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LOW-NUTRIENT R2A MEDIUM IN MONITORING MICROBIOLOGICAL QUALITY OF DRINKING WATER

The possibility of using low-nutrient R2A medium for determining the total count of aerobic mesophilic bacteria was investigated. Sampling of water from particular points of water treatment and distribution at Kruševac drinking water treatment plant was conducted. The samples were inoculated simultaneously on Plate Count Agar (PCA) and R2A media, and incubated at 37 °C and at room temperature. The bacterial count was determined after 48, 72, 120 and 168 h. The statistical analysis of the results showed significantly higher bacterial count on R2A medium compared to PCA. Moreover, a significantly higher bacterial count developed at room temperature compared to the temperature of 37 °C. R2A medium recorded 3.6% of unsafe samples in the distribution system after the 7-day incubation at room temperature. On the basis of the obtained results, an optimum method for determining the total count of aerobic mesophilic bacteria for all investigated waters has been defined. The process of incubation is predictable and it can be described by a mathematical model in the form of a polynomial of the second or the third power.

Key words: low-nutrient medium; R2A; aerobic mesophilic bacteria; drinking water.

Microbiological safety of drinking water is one of the most important demands for drinking water safety. There is no unique microbiological parameter on the basis of which the quality of drinking water can be practically determined. However, the total count of aerobic mesophilic bacteria is used as a basic indicator [1]. No significant correlation between microbiological safety of drinking water and the count of aerobic mesophilic bacteria has been established so far, as far as general human population is concerned. Nevertheless, opportunistic pathogens also belong to this group of bacteria. Those are bacteria that can cause numerous infections and be fatal at immuno-compromised people [2-4]. Some of these have very low infective doses and represent the most frequently recorded cause of unsanitary drinking water [5].

For aforementioned reasons, the application of a detection method which will indicate the condition of this parameter as authentically as possible is necessary. Considering that this parameter is monitored continually and in the long run, it is essential that the applied method is economical and comparatively simple to perform. Such are culturing methods which include the inoculation of a specific medium by a water sample and the incubation of the same media under specific conditions. Low-nutrient media, such as R2A, has been favoured lately [6-8]. Room temperature and the extended length of incubation (5 to 7 days) have also been favoured along with the low-nutrient media [9,10]. Today, two media are used for that purpose practically, both of them being high-nutrient: Nutrient agar and Plate Count Agar (PCA), with 48-hour incubation at 37 °C [11].

The objective of this study was to investigate the possibilities of the application of R2A medium in monitoring water quality during all phases of the water treatment and distribution, from its source to a consumer's tap, and to define the optimum conditions of incubation of water samples.

EXPERIMENTAL

Study area

The water supply source of the town of Kruševac is Ćelije reservoir, which was formed about 30 years ago by damming the river Rasina. The depth of the reservoir at the water-scoop is 30 m to 35 m. The

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reservoir water is transported to the drinking water supply plant from the depth of 20 m and through a 3 km pipeline by gravity. It undergoes five phases of treatment: prechlorination with low doses of chlorine, coagulation and sedimentation, ozonation, rapid sand filtration and final chlorination with approximately 1 mg/L of elementary chlorine. Final water is transported to consumers by the distribution system.

Basic physicochemical and microbiological characteristics of water at investigated points at water supply are given in Table 1.

Sampling

In the course of one-year investigation, sampling of water was conducted weekly at the following points of the treatment process:

- raw water entering the treatment process;
- prechlorination tank, after the exposure to the low concentrations of chlorine – chlorinated water;
- settler, after coagulation and sedimentation;
- Ozonator, after ozonation;
- Final water tank, after filtration and final disinfection.

The total of 250 samples were collected in this way: 50 samples from each point. The total of 250 samples were also collected from 16 representative points of the distribution system. The samples were taken into the sterile glass bottles of 250 mL volume. Before the sterilization, 0.15 mL of 5% solution of Na-thiosulphate was put into the bottles for taking water that contains chlorine in order to remove the chlorine. Immediatly after the sampling, the samples were transported in portable refrigerators at the temperature of 4 °C to a laboratory for further processing.

The preparation of media

Plate Count Agar (PCA) (Merck, code 1.05463.0500) was prepared by suspending 22.5 g of the powder in 1 L of purified water. The final pH of the solution was adjusted until it achieved the value of 7.0±0.2.

Reasoner's 2A Agar (R2A) (Merck, code 1.00416.0500) was prepared by suspending 15.2 g of the powder in 1 L of purified water. The final pH of the solution was adjusted until it achieved the value of 7.2±0.2.

Both solutions were sterilized in autoclave at 121 °C for 15 min. When the temperature dropped to 47 °C, the solutions were poured into sterile tubes.

Culturing

In order to determine the total count of aerobic mesophilic bacteria, each sample was inoculated on two media: PCA and R2A [6,7]. The inoculation was conducted with the pour plate technique by pouring molten and cooled-off (to approximately 47 °C) medium over the inoculum. The quantity of inoculum amounted to 1 mL of undiluted sample for all waters, except for the raw water, which was 10 times diluted.

Three agar plates were inoculated in each of two sets. One set of plates was incubated at 37 °C, and the other at the room temperature (20 to 22 °C). The bacterial count was recorded after 48, 72, 120 and 168 h. The mean bacterial count from all the three plates was recorded and expressed as a number of colony forming units CFU/mL. The obtained mean count was converted to 1 mL of a sample for the raw water.

Statistical analyses

The obtained results were statistically examined by the analysis of variance (ANOVA), with statistically significant level of $p < 0.05$ [12].

Furthermore, the regression and correlation analyses were performed for experimental data, using software CoRETV (Choice of Regression Equation between Two Variables) [13]. Using this software, the polynomials of the second and the fourth power, which describe the obtained results of the kinetics of growth of bacterial count in raw water, were chosen.

Table 1. Mean annual values of some physicochemical and microbiological parameters of water in certain phases of treatment and distribution

Parameter	Sampling sites					
	Raw water	Chlorinated water	Settler	Ozonator	Final water	Distribution network
Turbidity (NTU)	7.56	6.60	1.36	1.39	0.21	0.31
pH	7.77	7.69	7.31	7.31	7.29	7.28
$KMnO_4$ consumption, mg/L	9.96	9.00	6.54	6.05	4.60	4.85
Residual chlorine, mg/L	-	0.09	0.03	-	0.99	0.52
Total coliforms, counts/100 mL	57	0	0	0	0	0
Faecal coliforms, counts/100 mL	46	0	0	0	0	0
Faecal streptococci, counts/100 mL	14	0	0	0	0	0
Sulphite-reducing *clostridia*, counts/100 mL	94	9	1	0	0	0

RESULTS AND DISCUSSION

The mean annual counts of aerobic mesophilic bacteria, obtained by the application of different media and conditions of incubation at particular points in water distribution, are shown in Figures 1 to 6.

The results of investigation of the kinetics of growth of bacterial count on PCA and R2A medium at the temperature of 37 °C and the room temperature in the raw water are shown by means of a histogram in Figure 1.

A clear difference in bacterial colony count between the media is observed at the room temperature in favour of R2A medium. That difference is statistically significant at all incubation lengths, except after 48 h. Lower temperature decelerates the process of cell reproduction, so that a longer incubation is necessary for the forming of visible colonies [14]. After 48 h, a significant growth of bacterial count up to 72 h is recorded only on R2A medium. A low concentration of yeast extract, casein hydrolisate, peptone and glucose in this medium enables the growth of wide range of bacteria. In such conditions, fast-growing bacteria do not inhibit the growth of slow-growing ones, which occurs at high-nutrient media, such as PCA [15]. The results of the same samples, obtained after the incubation at 37 °C, indicate lower bacterial count on both media compared to the count obtained at the room temperature. Thus, bacterial count at the room temperature, after 168 h incubation on R2A medium is 10 times higher than at 37 °C (Figure 1). Since the environmental temperature of the source water ranges between 7 and 20 °C, when the bacteria are transferred to the temperature of 37 °C, the thermal shock kills a great number of them or, at least, inhibits their reproduction, so that colonies on agar are not formed [14]. The bacterial count on R2A medium is higher at this temperature, too. However, it is statistically significant only after 120 and 168 h. It means that R2A medium enables the recovery of thermally stressed bacteria and the increase in their count after five days of incubation.

After the first phase of the raw water treatment (prechlorination), the bacterial count in the water is drastically decreased. The results of the investigation of the kinetics of growth of bacterial count on PCA and R2A media at 37 °C and room temperature in chlorinated water are shown in Figure 2. The highest count is recorded at the room temperature on R2A medium after 168 h. Combined with lower temperature and extended incubation, that medium is particularly suitable for the recovery of chlorine-tolerant bacteria [15].

The results of the investigation of the kinetics of growth of bacterial count on PCA and R2A medium at 37 °C and room temperatures in water after sedimentation and ozonation are shown by means of a histogram in Figures 3 and 4. Both media clearly indicate considerably lower bacterial count after sedimentation and ozonation processes. Bacterial count in the settler is significantly higher on R2A medium compared to PCA only after 168 h (7 days) of incubation at the room temperature, which indicates that bacteria, weakened by physical and chemical treatments need longer period, lower temperature and low-nutrient medium for forming colonies. The same reason accounts for significant growth of bacterial count on R2A medium, from days 2 to 7 of the incubation at 37 °C and from days 2 to 5 of the incubation at 20 °C.

After exposure to high concentration of chlorine, bacterial count is very low in finished water, averagely

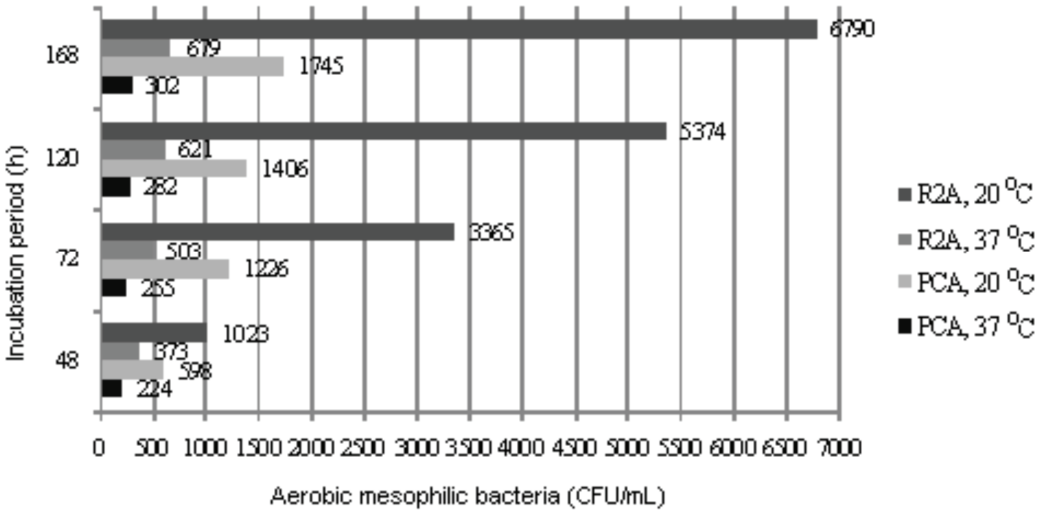

Figure 1. Mean annual count of aerobic mesophilic bacteria in raw water on examined media with different incubation conditions.

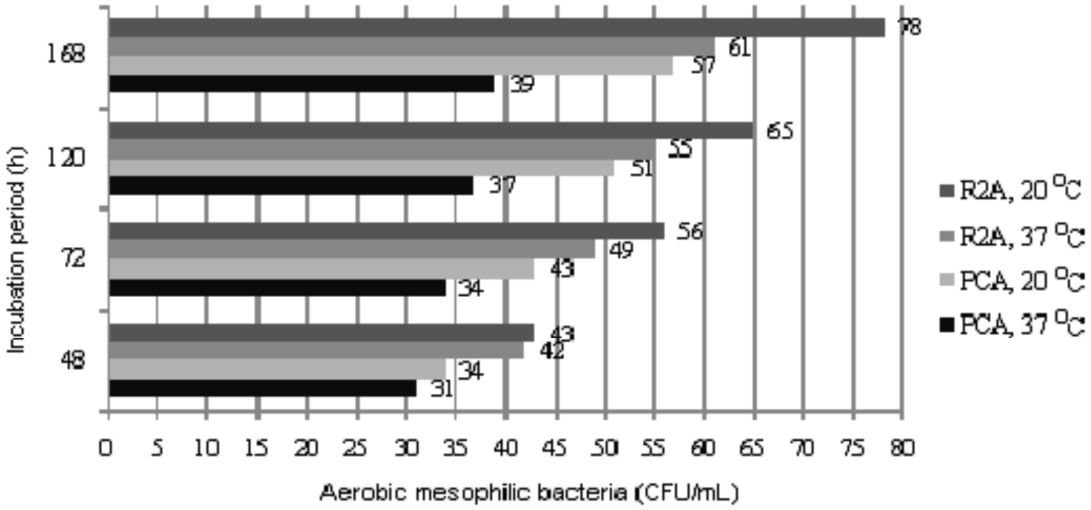

Figure 2. Mean annual count of aerobic mesophilic bacteria in chlorinated water on examined media with different incubation conditions.

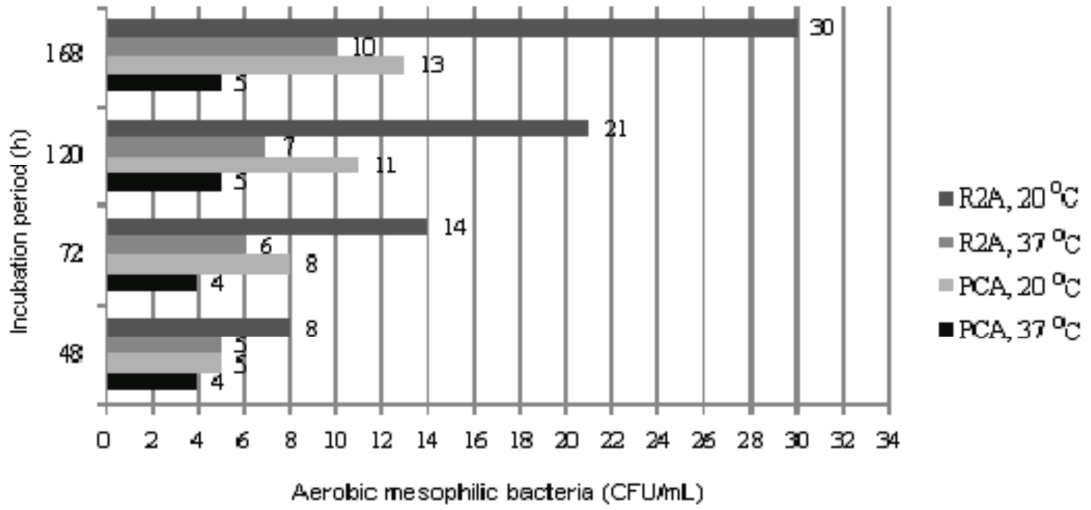

Figure 3. Mean annual count of aerobic mesophilic bacteria in settler on examined media with different incubation conditions.

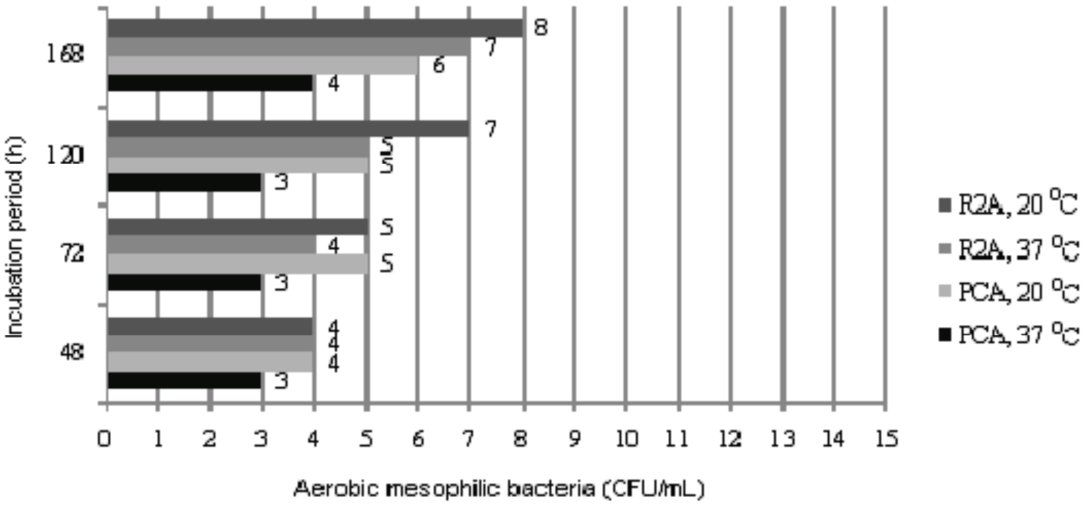

Figure 4. Mean annual count of aerobic mesophilic bacteria in ozonator on examined media with different incubation conditions.

< 1, on both media and at all incubation conditions, so that existing differences are not significant (Figure 5).

Although very low bacterial count is detected in the distribution system, some differences can be observed between investigated culturing methods (Figure 6). As with other waters, the most authentic view of bacteriological status is obtained on R2A medium after 7-day incubation at the room temperature.

The results of the investigation of water samples from a distribution system mostly indicate the sensitivity of R2A medium, on the basis of the number of samples in which bacteria are detected at all. Consequently, R2A medium detects the presence of bacteria in greater number of samples than PCA in all incubation conditions, except at room temperature after 48 h. The absence of difference after 48 h at room temperature indicates that bacteria need more time to develop visible colonies on low-nutrient media. Thus, R2A detects bacterial presence in 18.8% of samples more than PCA, after 168 h. It means that drinking water is nutritionally deficient environment, so that the medium with low concentration of the nutrient substance, such as R2A, is more suitable for bacterial growth [6].

Drinking water in a distribution system is subject to legal regulations for values of certain physicochemical and microbiological parameters [11]. The count of aerobic mesophilic bacteria is in legal limits, if incubation conditions of the samples regulated by the Standard Scale are observed. However, taking into consideration more realistic count of aerobic mesophilic bacteria (R2A, 168 h, 22 °C), the situation is different, and by this parameter, there are 3.6% unsafe samples.

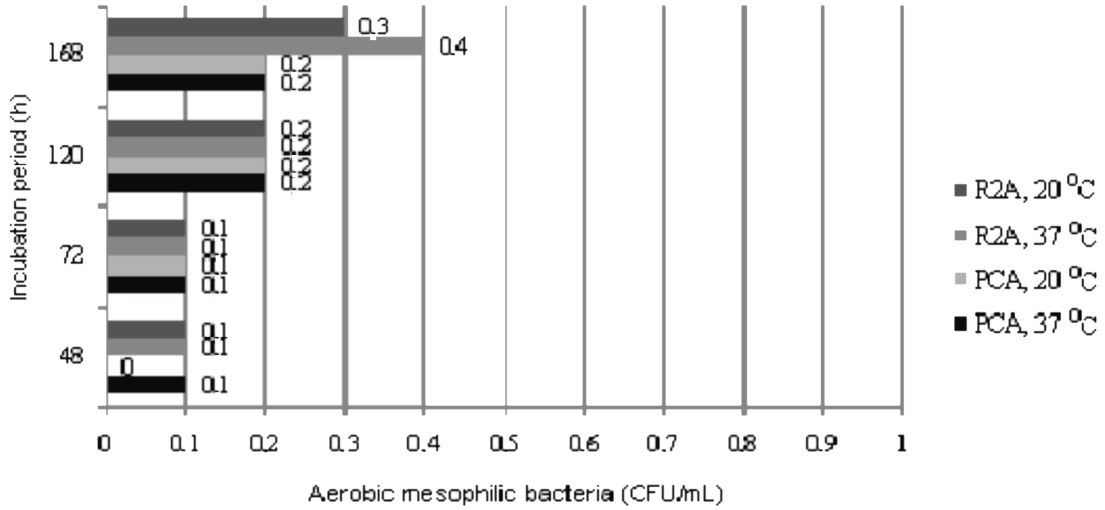

Figure 5. Mean annual count of aerobic mesophilic bacteria in finished water on examined media with different incubation conditions.

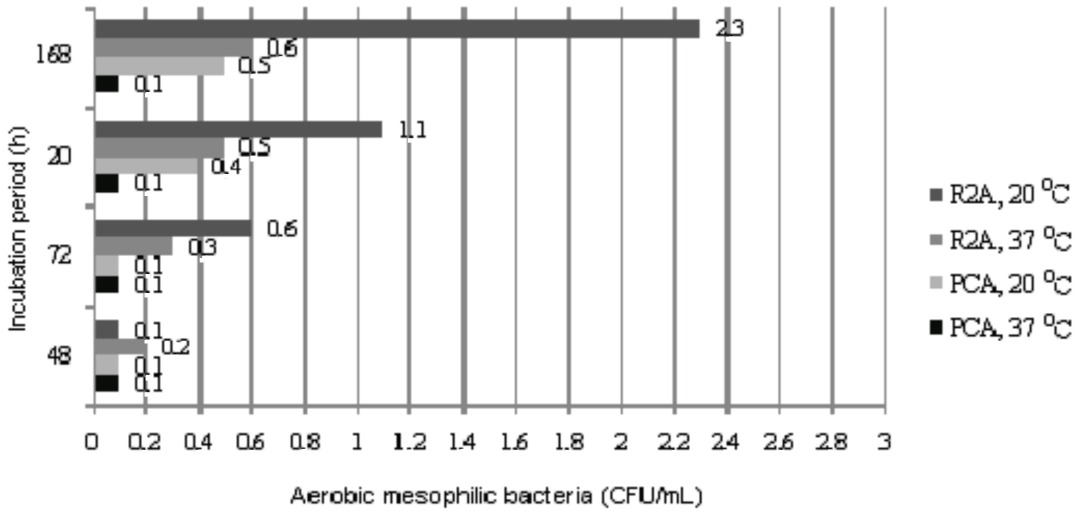

Figure 6. Mean annual count of aerobic mesophilic bacteria in distribution system on examined media with different incubation conditions.

On the basis of the obtained results, a culturing method which gives the most authentic results can be formulated. That method includes the inoculation of samples on R2A medium and their incubation of 5-7 days at room temperature. The formulated method, as all culturing methods, has its flaws. Firstly, culturing methods show significantly lower bacterial counts in water compared to microscopy, flow cytometry or ATP analysis [16-18]. Also, the results are obtained in only several days. However, culturing methods are more suitable for routine, everyday monitoring of drinking water, because they are more accessible, easier to perform and more economical. Moreover, set standards for drinking water take only results obtained by culturing methods into consideration.

Regression analysis

According to postulates of regression and correlation analysis, only experimental data for raw water should be subjected to this mathematical procedure. Using aforementioned software, CoRETV, the results of kinetics of bacterial culturing on R2A medium (at temperatures of 20 and 37 °C) and on PCA (at 20 and 37 °C) were tested. Polynomials of the second or the third power, shown in Table 2, describe the experimental results with high level of accordance (R > 0.97). These polynomials provide the count of colony forming units as a function of time (τ / h).

Slightly more complex dependence of the growth of bacterial count is detected on R2A medium at 20 °C, where the speed is somewhat less in the initial period up to 48 h. After 48 h, the speed of growth of bacterial count is considerably greater. At the initial 48 h of incubation, mainly colonies of fast-growing bacteria are detected on agar. Slow-growing bacteria demand extended incubation so that their colonies become visible. Hence, a significant increase in their count occurs only with the incubation longer than 48 h, which allows us to describe this experiment with satisfactory accuracy by polynomial of the third power.

Table 2. Mathematical models of kinetics of growth of aerobic mesophilic bacteria in raw water in different culturing conditions

Culturing conditions	Model	R
R2A, 20 °C	(CFU/mL) = 47.31 + 9.4474τ + 0.606τ^2 - 0.0026τ^3	0.9951
R2A, 37 °C	(CFU/mL) = 72.91 + 7.33τ - 0.023τ^2	0.9981
PCA, 20 °C	(CFU/mL) = 36.41 + 14.31τ - 0.026τ^2	0.9989
PCA, 37 °C	(CFU/mL) = 72.99 + 3.25τ - 0.012τ^2	0.9786

CONCLUSIONS

In the raw water samples, statistically significant difference in the count of aerobic mesophilic bacteria is observed between examined media, in favour of R2A medium, at both applied incubation temperatures, particularly over extended incubation periods. Furthermore, significantly higher bacterial count on both media is recorded at the room temperature compared to the temperature of 37 °C.

During certain phases of the treatment process, significant differences between the media, also in favour of R2A medium, are recorded only after the 7-day incubation and only at room temperature. Consequently, this temperature appears to be more appropriate even for the incubation of the very pure waters.

Although R2A medium was originally designed for ultra pure waters, these investigations prove the possibility of unification of a method for determining the total count of aerobic mesophilic bacteria at all points of water distribution, from the water source to a consumer's tap. The optimum method would involve the inoculation of water samples on R2A medium and their incubation at the room temperature in duration of 5 to 7 days for more polluted waters (raw water), or at least 7 days for pure waters (phases in the treatment process and a distribution system).

The process of incubation is predictable and it can be described by a mathematical model in the form of a polynomial of the second or third power. Therefore, these models could serve for prediction of the potential count of bacterial colonies in all incubation conditions applied in this study.

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NAUČNI RAD

NISKONUTRITIVNI R2A MEDIJUM U MONITORINGU MIKROBIOLOŠKOG KVALITETA VODE ZA PIĆE

U ovom radu istražena je mogućnost korišćenja niskonutritivnog R2A medijuma za određivanje brojnosti aerobnih mezofilnih bakterija. Vršeno je uzorkovanje vode iz pojedinih tačaka prerade i distribucije Fabrike vode za piće, Kruševac. Uzorci su paralelno zasejavani na Plate Count Agar (PCA) i na R2A medijum, i inkubirani na temperaturi 37 °C i na sobnoj temperaturi. Broj bakterija je određivan nakon 48, 72, 120 i 168 h. Statistička analiza rezultata pokazala je značajno veću brojnost bakterija na R2A podlozi u odnosu na PCA. Takođe, značajno veći broj bakterija razvio se na sobnoj temperaturi u odnosu na temperaturu od 37 °C. Rezultati dobijeni na R2A medijumu nakon 7 dana inkubacije na sobnoj temperaturi pokazali su neispravnost vode iz distributivne mreže u 3,6% uzoraka. Na osnovu dobijenih rezultata definisana je optimalna metoda za određivanje broja aerobnih mezofilnih bakterija za sve tipove ispitivanih voda. Proces inkubacije je predvidljiv i moguće ga je opisati matematičkim modelima oblika polinoma drugog, odnosno trećeg stepena.

Ključne reči: niskonutritivni medijum; R2A; aerobne mezofilne bakterije; voda za piće.