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THE APPLICATION OF THE REPLICA TECHNIQUE IN INVESTIGATIONS OF PHYSIOLOGICAL GROUPS OF BACTERIA IN SURFACE WATERS

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Introduction

Microbial populations, according to their composition, characterise various types of surface waters. Especially in polluted waters, the relations between single physiological groups of bacteria can reflect changes taking place under the influence of polluting waste materials (Daubner 1972, Rheinheimer 1975, Mitchell 1972, Tržilova 1976, Ritter 1977). The 2nd International symposium of hydromicrobiologists in Bratislava (1975) emphasized the importance of further investigations of the role of various groups of bacteria which take part in the circling of the matter and energy in waters; it was suggested that new methods should be introduced into practice.

In the paper, some results obtained by our modification of the replica technique method (Lederberg and Lederberg 1952) are presented as a new approach to ecological investigations of physiological groups of bacteria in surface waters.

Material and Methods

The presence of saprophytic bacteria on MPA, proteolytic bacteria, phosphomineralizing, phosphomobilizing and amylolytic bacteria was investigated in November 1978 in the section of the river Sava from Ščitarjevo to Galdovo covering about 75 km.

The samples of water were taken every two hours through the period od 24 hours, in the localities of Ščitarjevo, Dubrovčak, Tišina and Galdovo, and a mixed sample was taken for bacteriological investigations. Dilutions of samples from 10^{-2} to 10^{-5} were prepared before inoculation, and each plate of MPA (meat peptone agar) was inoculated with 0,2 ml of the

material. Incubation was 48 h at 20° — 22° C. The plates with 20—40 developed colonies of bacteria were selected, and their print was taken by the method of replica technique (Lederberg and Lederberg 1952), with the help of a round surface (Petri dish) covered with a fine velvet (Fig. 1). The velvet with the bacteria was pressed down on the surface of special media to prove the examined physiological groups.

The colonies of bacteria developed in identical arrangement on all the plates after incubation, so that the number of bacteria of single groups, as well as their relations inside microbial populations on MPA (Fig. 2), could be determined.

Besides that, the possibility of application of replica technique in determining the influence of ecological factors on the bacterial activity was examined too. So, the influence of temperature on the proteolytic activity of aerobic saprophytic bacteria from the Sava water was investigated. After replication, the plates were placed into incubators at various temperatures (16° C, 22° C, 26° C, 30° C and 37° C), and the proteolytic colonies were counted after 24 hous.

Simultaneously with bacteriological examinations, some hydrochemical parameters, such as dissolved O_2, $KMnO_4$ consumption and BOD_5, were defined as well.

Te number of proteolytic bacteria was determined on the basis of the zones of hydrolysis on MPA with 1,5% gelatine, after addition of $HgCl_2$. Incubation 24 h, temperature 25° C.

The phosphomineralizing bacteria were determined on the Menkina agar with lecithyne. Incubation 5 days, temperature 25° C.

The phosphomobilizing bacteria were determined on the medium with $Ca_3(PO_4)_2$, and the amylolytic bacteria on the starch-agar after addition of iodine solution. Incubation 72 h, temperature 25° C.

All these media are described in a book by Daubner (1972). Hydrochemical parameters were determined after Standard Methods (1976).

Results and Discussion

From the results of chemical and bacteriological analyses (Table 1, Fig. 3) it can be concluded that the Sava at Dubrovčak is considerably organically loaded; it is the consequence of mixing of the river water with waste waters from the city of Zagreb, which flow out after Ščitarjevo (Šoštarić and Stilinović 1979). With the help of replica technique it was proved that an increase in the number of saprophytic bacteria at Dubrovčak was followed by an increase in the values of other bacterial groups too, which is a sure sign of loading of the river with municipal waters (Daubner 1972, Rheinheimer 1975, Melberga and Vavere 1974, Phelps 1944, Rodina 1972, Grigorjeva 1975).

Downstream from Dubrovčak, the number of bacteria of all groups decreased, as a result of the processes of self purification; and at Galdovo, the bacteriological picture was like that of Ščitarjevo before the inflow of waste waters from Zagreb. Especially high were the values of phosphomobilizing and amylolytic bacteria at the places of the greatest pollution, while in the less polluted parts their number was considerably reduced. The finding of the greater number of phosphomobilizing bacteria in the more polluted parts of the river is in accordance with the results of Mosevič and Danilevič (1955) who found that the number of these bacteria in water depended considerably upon the quantity of organic matter.

Table 1. Results of measurements of some hydrochemical parameters in the river Sava from Ščitarjevo to Galdovo in November 1978.

Tabela 1. Rezultati mjerenja nekih hidrokemijskih parametara u rijeci Savi od Ščitarjeva do Galdova u studenom 1978.

Sampling place Mjesto	Dissolved oxygen mg/l Otopljeni kisik mg/l	$KMnO_4$ consumption mg/l — 10 min $KMnO_4$ potrošak mg/l — 10 min	BOD_5 mg/l BPK_5 mg/l
Ščitarjevo	8.0	30.2	5.8
Dubrovčak	6.1	86.4	10.2
Tišina	7.8	42.8	6.9
Sisak—Galdovo	7.2	38,9	6.1

Fig. 4. presents the relations between the examined physiological groups of bacteria and the number of saprophytic bacteria on MPA, expressed in percentages.

At the time of examination a sudden decrease of the percentage of proteolyic and phosphomineralizing bacteria could be observed in the highly polluted parts of the river (Dubrovčak), while at the same time the percentage of phosphomobilizing and amylolytic bacteria increased. In these investigations, the greatest part in the population of saprophytic bacteria on MPA consisted of inorganic phosphate mobilizing bacteria in the more polluted parts of the river, and of proteolytic bacteria which hydrolyzed gelatine in the less polluted parts. As the results of relations between saprophytic bacteria on MPA and physiological groups of bacteria, obtained by replica technique, are not comparable with the existing literature, we mention that similar results were obtained in our investigations of some others surface waters too (Stilinović 1978, 1979).

Fig. 5 shows the results of examinations of the influence of incubation temperature upon the proteolytic activity of bacterial strains from the river Sava, using the method of replica technique. The results obtained after 24 hours of incubation at various temperatures show clearly that the highest number of proteolytic bacteria developed at 22° C and 26° C. In addition to the fact that the data on the activity of proteolytic bacteria in relation to the temperature, were obtained fast, it was possible to establish the temperature valence of each individual strain separately.

Further examinations of the qualitative composition of the populations of saprophytic bacteria on MPA, by the method of replica technique, should include also other physiological groups of bacteria in surface waters (fat-hydrolyzing, ammonifying, denitrifying, mineral-oils, urea, phenol decomposing bacteria, etc.). On the basis of the results obtained it will be possible to ascertain the values of the method for estimating the state and nature of pollution, as well as the processes of selfpurification in surface waters. The method of replica technique can also be used in various ecological investigations in the field of hydromicrobiology. A special advantage of the method of replica plating in hydromicrobiology is in the minimal use of laboratory material and in time saving and it is possible to work out a relatively great number of samples at the same time.

Summary

The method of replica technique is a new way of determining the presence of physiological groups of bacteria in surface waters. By using the method of replica technique it was established that the percentage of proteolytic and phosphomineralizing bacteria decreased, in relation to the number of saprophytic bacteria on MPA, at the places of the greatest pollution of the river Sava, while at the same time the percentage of amylolytic and especially of phosphomobilizing bacteria increased.

The same method was used in examining the influence of temperature on the proteolytic activity of the bacterial strains from river Sava, and it was established that the highest number of bacteria on MPA hydrolyzed gelatine between 22° C and 26° C.

A special advantage of the replica technique method in hydromicrobiology is in the minimal use of laboratory material and in time saving, and it is possible to work out a relatively great number of samples at the same time. The reported method is very simple and feasible in all laboratories.

Fig. 1. Petri dishes wrapped with velvet for replica technique method.

Sl. 1. Petrijeve zdjelice omotane baršumom za metodu replika tehnike.

Fig. 2. The colonies of proteolytic, phosphomobilizing and amylolytic bacteria on nutritious media, obtained by the method of replica technique.

Sl. 2. Kolonije proteolitičkih bakterija, fosfomobilizatora i amilolitičkih bakterija na hranjivim podlogama dobivene metodom replika tehnike.

Fig. 3. The proportion of some physiological groups of bacteria in the number of saprophytic bacteria on MPA, isolated from water of the Sava downstream from Zagreb in November 1978.

Sl. 3. Zastupljenost nekih fizioloških grupa bakterija unutar broja saprofitnih bakterija na MPA izoliranih iz vode Save u studenom 1978.

Fig. 4. The proportion of some physiological groups of bacteria in the number of saprophytic bacteria on MPA, isolated from river Sava downstream from Zagreb expressed in percentage

Sl. 4. Zastupljenost nekih fizioloških grupa bakterija unutar broja saprofitnih bakterija na MPA izoliranih iz Save nizvodno od Zagreba, izražena u postocima.

Fig. 5. Results of examinations of the influence of temperature upon the proteolytic activity of bacterial strains using the method of replica technique.

Sl. 5. Rezultati ispitivanja utjecaja temperature na proteolitičku aktivnost bakterijskih sojeva primjenom metode replika tehnike.

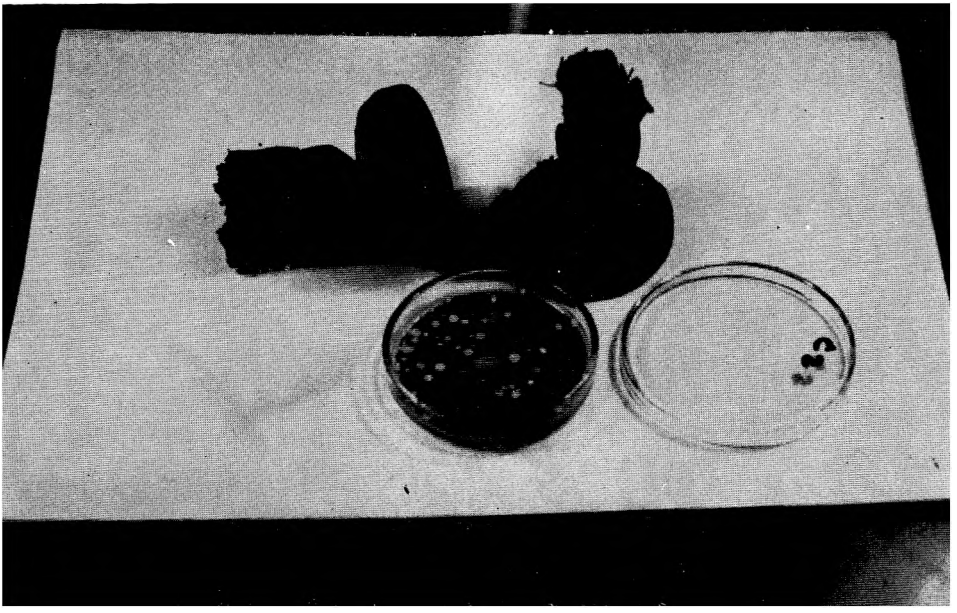


Fig. 1. — Sl. 1.

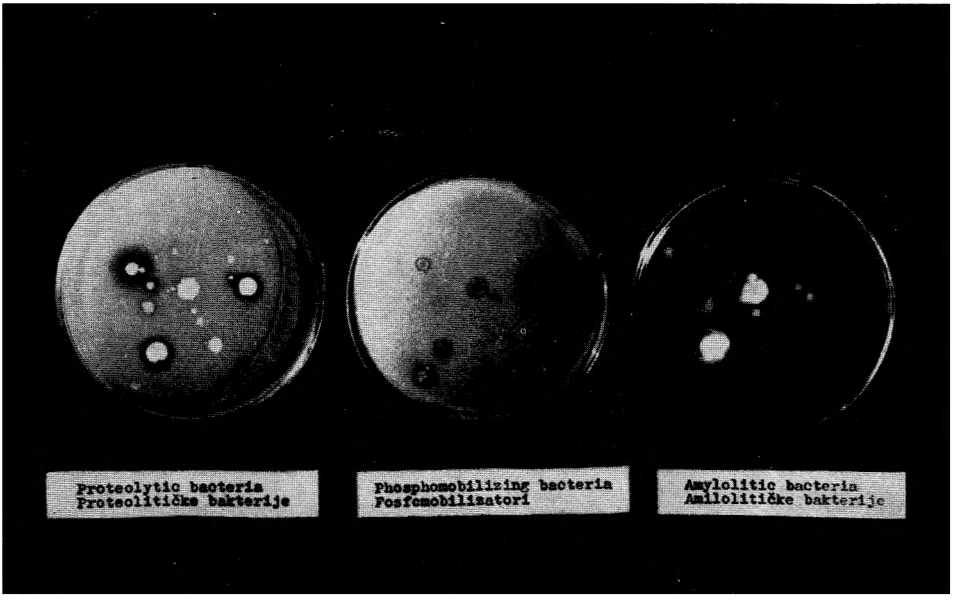
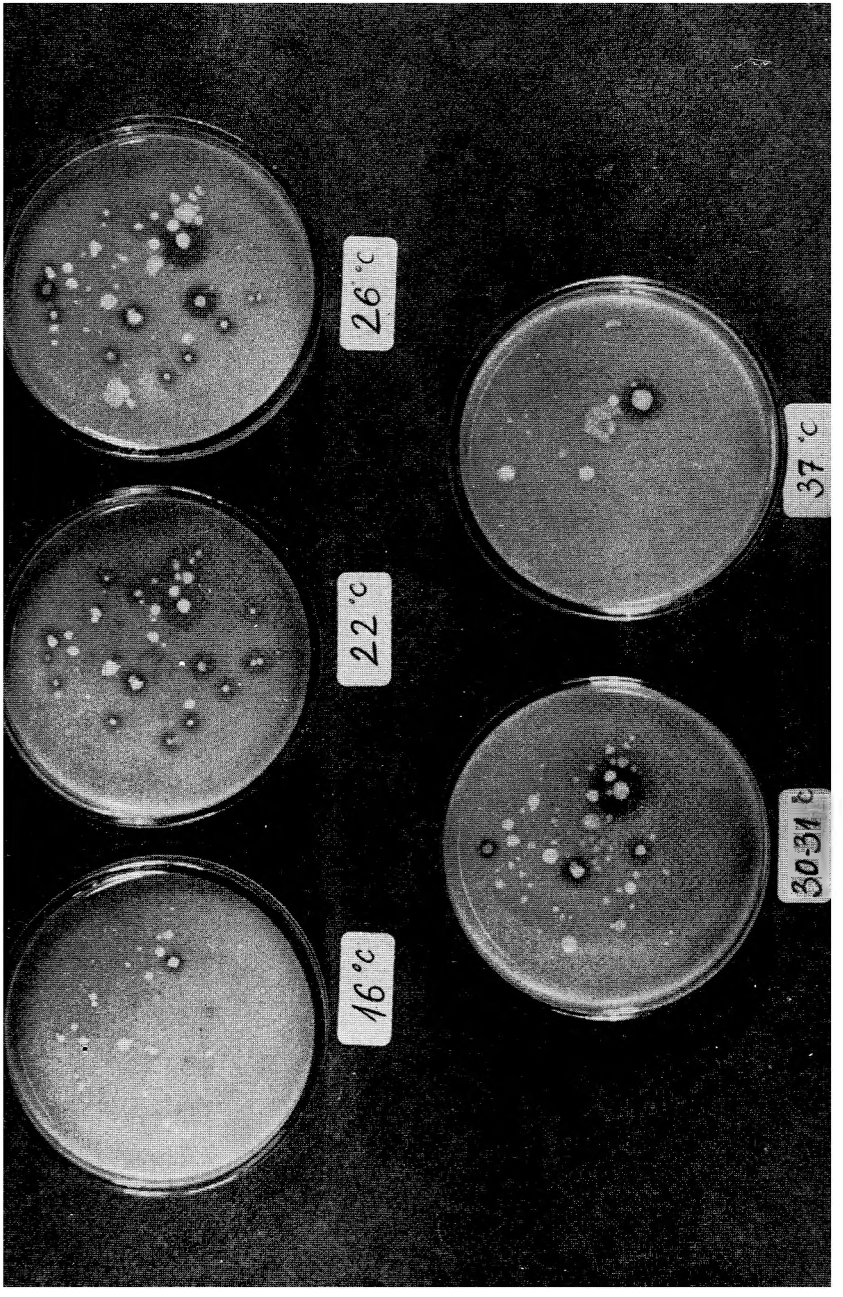


Fig. 2. — Sl. 2.



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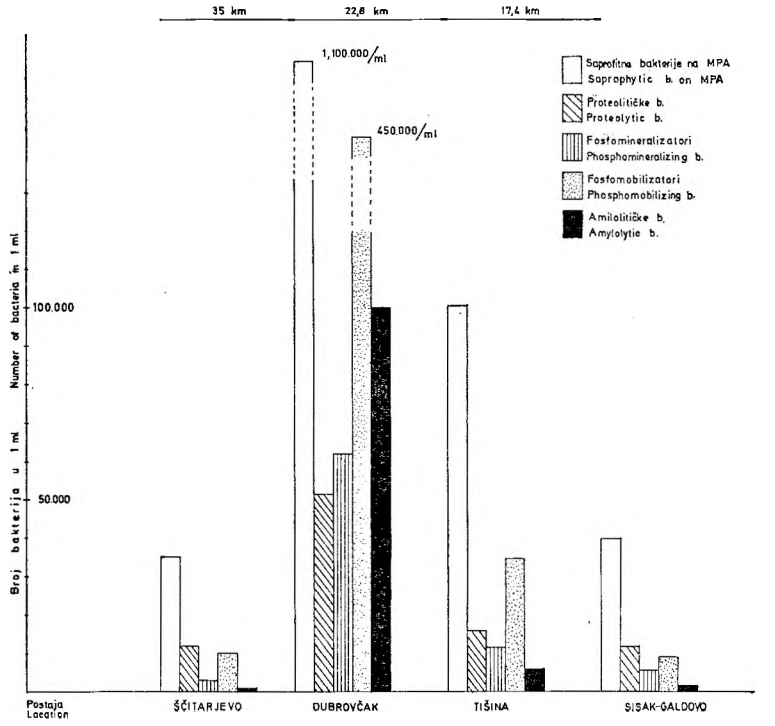

Fig. 3. — Sl. 3.

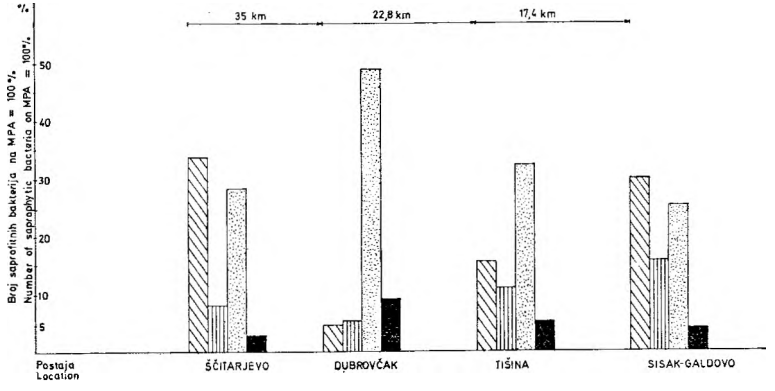

Fig. 4. — Sl. 4.

References

Daubner, I., 1972: Mikrobiologie des Wassers. Akademie Verl. Berlin.

Grigorjeva, L. V., 1975: Sanitarnaja bakteriologija i virusologija vodoemov. Medicina. Moskva.

Lederberg, J., E. M. Lederberg, 1952: Replica plating and indirect selection of bacterial mutants. J. Bact., *63*, 399—406.

Melberga, A. G. i *A. K. Vavere*, 1974: Mikroorganizmi kak faktor samoočišćenija ustjevogo rajona reki Daugava. Faktori samočišćenija ustjevogo rajona reki Daugava. Zinantne, Riga.

Mitchell, R., 1972: Water pollution microbiology. Wiley-Interscience, New York, London, Sydney, Toronto.

Mosevič, M. i *M. Danilevič*, 1955: Rol biohimičeskih procesov v krugovorote fosfora v vodoemah. Izv. Vses. naučno-isled. In-ta ozernogo i rečnogo ribnogo hozjajstva, *34*, 149—156.

Phelps, E. B., 1944: Stream Sanitation. J. Wiley and Sons, Inc. New York.

Rheinheimer, G., 1975: Mikrobiologie der Gewässer. Verb. Gustav Fischer Ver. Jena.

Ritter, R., 1977: Ökologische und physiologische Bakteriengruppen: Ergebnisse hydrobakteriologischer Untersuchungen. II Inter. hydromikrob. Symposium Smolenice. VEDA Ver. der Slowakischen Akad. der Wissenschaften, Bratislava, 113—136.

Rodina, A. G., 1972: Methods in Aquatic Microbiology. Univ. Park Press, Baltimore, Butterworths, London.

Stilinović, B., 1978: Istraživanja zastupljenosti nekih fizioloških grupa bakterija u rijeci Savi od Krškog do Podsuseda. (u tisku).

Stilinović, B., 1979: Zastupljenost nekih fizioloških grupa bakterija u površinskim vodama različite kvalitete. (u. tisku).

Standard Methods for the Examination of Water and Wastewater, 12, 1967. American Public Health Association, New York.

Šoštarić, N. i *B. Stilinović*, 1979: Kompleksna analiza kvalitete rijeke Save prije i poslije utoka otpadnih voda Zagreba Ekologija, *14*, 2, 117—125.

Tržilova, B., 1976: Die Ergebnisse der Bestimmung einiger physiologischer Gruppen von Mikroorganismen in Oberflächengewässern mit neuen Methoden. Biologija (Bratislava) *31*, 3, 179—185.

SAŽETAK

PRIMJENA REPLIKA TEHNIKE U ISTRAŽIVANJIMA FIZIOLOŠKIH GRUPA BAKTERIJA U POVRŠINSKIM VODAMA

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U ovom su radu kao nov pristup istraživanjima ekologije fizioloških grupa bakterija u površinskim vodama, iznijeti neki rezultati dobiveni vlastitom modifikacijom replika tehnike (Lederberg and Lederberg 1952). Ispitivana je zastupljenost proteolitičkih bakterija, fosfomineralizatora, fosfomobilizatora i amilolitičkih bakterija unutar populacija saprofitnih bakterija izoliranih na MPA (mesno-peptonski agar) iz savske vode nizvodno od Zagreba (Ščitarjevo — Galdovo) u mjesecu studenom 1978.

Najveći broj bakterija svih grupa nađen je kod Dubrovčaka, što je posljedica zagađenosti Save otpadnim vodama Zagreba nizvodno od Ščitarjeva (fig. 2), na što upućuju i neki hidrokemijski parametri (tab. 1). Nizvodno od Dubrovčaka opada broj saprofita i bakterija ispitivanih grupa, što je znak autopurifikacije rijeke, pa kod Galdova dobivamo u vrijeme istraživanja bakteriološku sliku sličnu onoj kod Ščitarjeva prije ulijevanja kolektora grada. Metodom replika tehnike moglo se utvrditi da se obzirom na opterećenost rijeke dolazi do promjena odnosa među pojedinim fiziološkim grupama s obzirom na broj saprofitnih bakterija. Tako u manje opterećenim dijelovima rijeke najveći udio u populacijama saprofita na MPA imaju proteolitički sojevi i nešto manje fosfomobilizatori, dok su u jače zagađenim apsolutno dominantni samo fosfomobilizatori (fig. 3).

Metodom replika tehnike mogu se u hidromikrobiologiji relativno brzo dobiti podaci o utjecaju raznovrsnih ekoloških faktora na aktivnost bakterija. Ispitivanjem utjecaja temperature na proteolitičku aktivnost sojeva iz savske vode moglo se u roku od 24 sata konstatirati da najveći broj saprofitnih bakterija na MPA sa želatinom pokazuje hidrolitičku aktivnost od 22° C do 26° C.

Primjenom replika tehnike u istraživanjima fizioloških grupa bakterija postiže se minimalna potrošnja laboratorijskog posuđa i hranjivih podloga, jer je potrebno dobiti samo kolonije saprofitnih bakterija u povoljnom razređenju na MPA (radi daljeg repliciranja), a to je i inače rutinska pretraga voda u svakom mikrobiološkom laboratoriju. Također se postiže velika ušteda u vremenu, što omogućuje ispitivanje većeg broja uzoraka odjedanput. Posebno je važno to da se opisana metoda može zbog svoje jednostavnosti i lagane tehničke izvedbe primijeniti u svakom hidromikrobiološkom laboratoriju.

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