

## COMPARATIVE NUTRIENT AGAR STUDIES ON THE QUANTITATIVE SURVEY OF SAPROPHYTIC WATER MICROORGANISMS

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One of the most important endeavours of present-day research in water microbiology is to elucidate the dynamics of saprophytic and total microbial associations in waters of various trophism (OCEVSKI, 1966; DAUKSTA, 1967; OVERBECK, 1968 a, b; RHEINHEIMER, 1968; POTAYENKO, 1968; DAUBNER, 1969; DEUFE, 1969; FONDEN, 1969; ROMANENKO, 1969, and others).

The majority of water microbiologists employs RAZUMOV's direct method (1932) in order to make quantitative surveys of total microbial flora living in waters and in sediments, consequently, the obtained results are well comparable. To standardize the counting of saprophytes would also be desirable (BABENZIEN, 1962). The nutrient agar used in hygienic practice is quite inadequate for counting the autochthon, slowly growing water inhabiting bacteria (STRZELCZYK et al. 1967) thus, there is an ardent quest to find new nutrient media and the line of suggested and applied nutrient agars has not yet come to an end (HESSE and NIEDNER, 1898; FRED et al. 1924; STARK and MCCOY, 1938; OPPENHEIMER and ZOBELL, 1952; FERRER et al. 1963; STRZELCZYK et al. 1967; MELCHIORRI-SANTOLINI and CAFARELLI, 1967; FONDEN, 1967, 1968) which makes comparative study extremely difficult.

It is important to choose the right composition of the nutrient agar in the process of plate-pouring and spreading when determining the number of saprophytes, however, besides this, a large number of other factors may also influence the number of bacteria appearing on the agar-slide (CARLUCCI and PRAMER, 1957; JONES and JANNASH, 1959; BUCK and CLEVERDON, 1960; GUNKEL et al. 1960; FONDEN, 1967; CLARK, 1967; STRZELCZYK et al. 1968).

To take into consideration all the above presented facts we thought important to try and apply some new nutrient agar in order to count the saprophytic microorganisms in connection with the new and detailed microbiological investigation of Lake Balaton. To this effect we have carried out a comparative research on the basis of 12 nutrient agars, relying partly on literary data and partly on our own resources; our samples were taken from Lake Balaton, from the Inner Lake of Tihany, from Velence Lake, and from River Danube and cultured accordingly.

### Material and method

The samples from Lake Balaton have been taken at some 500 metres from the shoreline in front of our Research Institute, the other sample taking localities, the Inner Lake of Tihany and River Danube, were in the littoral

TABLE 1  
 The composition

		Nutrient agar I.a	Nutrient agar I.b	Complex agar II.	TAYLOR's agar III.	Sodium caseinate agar IV.
Distilled water,	ml	—	1000	1000	1000	1000
Lake	"	—	—	—	—	—
Tap	"	1000	—	—	—	—
Agar	g	15	15	15	15	15
K <sub>2</sub> HPO <sub>4</sub>	g	—	—	0.5	0.2	0.2
Na <sub>2</sub> HPO <sub>4</sub>	g	—	2.0	—	—	—
KNO <sub>3</sub>	g	—	—	0.2	—	—
NaNO <sub>3</sub>	g	—	—	—	—	—
MgSO <sub>4</sub> · 7H <sub>2</sub> O	g	—	—	0.2	0.05	0.2
(NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub>	g	—	—	—	—	—
FeSO <sub>4</sub> · 7H <sub>2</sub> O	g	—	—	—	—	trace
FeCl <sub>3</sub>	g	—	—	trace	trace	—
NaCl	g	—	3.0	—	—	—
Glucose	g	—	—	0.5	—	1
Glycerin	ml	—	—	—	1	—
Soluble starch	g	—	—	—	0.5	—
Bacto pepton	g	5	10	—	0.5	—
Bacto beef extract	g	3	1.5	—	—	—
Bacto yeast extract	g	—	—	0.05	—	—
Sodium caseinate	g	—	—	0.1	0.5	1.0
Mud extract	ml	—	—	—	—	—
Powder alga	g	—	—	—	—	—

Note: X.a — Lake water agar of Balaton; X.b — Lake water agar of Lake Belső

zone (Table 3). While the mud-samples of Velence Lake originate from the yellow-coloured oxidized micro-zone and from the black-coloured reduced zone. The watersamples have been taken by the help of a Francev sampler (KUZNETSOV, 1952) into a sterile, 250 ml flask from a depth of 50 cm. The mud-samples have been secured by using an Ekman-Birgs drege and from the so obtained mud-blocks by the help of instruments cauterized in alcohol flame from inside we took our samples. The samples have been elaborated in the following hour of collecting from sites Lake Balaton and Inner Lake while samples originating from River Danube on the next day. The mud from Velence Lake has been stored in a refrigerator for a longer period of time.

The total microbial plankton quantity has been determined by RAZUMOV's direct method (1932).

The slides were sealed from 10–10 ml of 42 °C nutrient agar, whose composition is shown in Table 1. Our pulverized alga-nutrient medium have been obtained from the laboratory mass-culture of *Scenedesmus obtusiusculus* CHOD. The desiccated alga fragments were ground in a ball and tube mill. To the mud-extract agar 800 g mud from Lake Balaton was boiled in 2 litre Balaton water, then following sedimentation it was passed through filter paper. Considering the low number of bacteria in Lake Balaton, 0.1 and 1 ml lake water without dilution were inoculated, while in case of the Inner Lake of Tihany and River Danube the degree of dilution was 10 and 100-times, respectively. In the case of mud-samples coming from Velence Lake we made dilutions of 10-, 100- and 1000-times. In the case of sealings we had 2 controls

## of the mediums

JENSEN's agar V.	OPPENHEIMER's agar VI.	Fe-peptone agar VII.	Powder alga agar VIII.	Mud extract agar IX.	Lake water agar		KRASHENNIKOVA's agar XI.
					X.a	X.b	
1000	—	—	—	—	—	—	400
—	1000	—	1000	—	1000	1000	500
—	—	1000	—	—	—	—	100
15	15	15	15	15	15	15	15
0.5	0.1	—	—	—	—	—	0.01
—	—	—	—	—	—	—	—
—	—	—	—	—	—	—	—
0.2	—	—	—	—	—	—	—
—	—	0.1	—	—	—	—	0.005
—	trace	0.1	—	—	—	—	—
trace	—	—	—	—	—	—	—
—	—	—	—	—	—	—	0.05
1	—	0.1	—	—	—	—	—
—	—	—	—	—	—	—	—
—	—	—	—	—	—	—	—
—	5	5	—	—	—	—	—
—	—	—	—	—	—	—	—
0.2	1.0	—	—	—	—	—	—
—	—	—	—	1000	—	—	—
—	—	—	5	—	—	—	—

without inoculum and 3—5 parallels. The slides were incubated at 25 °C and as the colonies appeared they were counted under 10-times of magnification on the 1st, 2nd, 3rd, 6th, 12th, 14th, 32nd and 36th day. The results obtained have always been referred to 1 ml and 1 g of the original sample.

### Results and discussion

It was striking to observe that the bacterioplankton quantity of Lake Balaton and that of the Inner Lake of Tihany shows significant difference (*Fig. 1*). The total microbial plankton quantity (nearly  $12 \cdot 10^6/\text{ml}$ ) of the Inner Lake of Tihany when compared to the data found in literature (ROMANENKO, 1969) shows a pronounced degree of eutrophism. Likewise is the number of saprophytes examined on every nutrient agar greater, more than one order of magnitude, than the values obtained for samples taken from Lake Balaton.

The comparison of the examined nutrient agars (*Fig. 1*) unambiguously prove that in hygienic practice as well as in water microbiology the media used are inadequate for bacterial cultures, and only a low number of colonies develop. Sealings of various samples originating from different waters, and taken at different times, the proportion of bacteria developing on various culture media varies in every case, but we never obtained favourable culture values on nutrient agar.

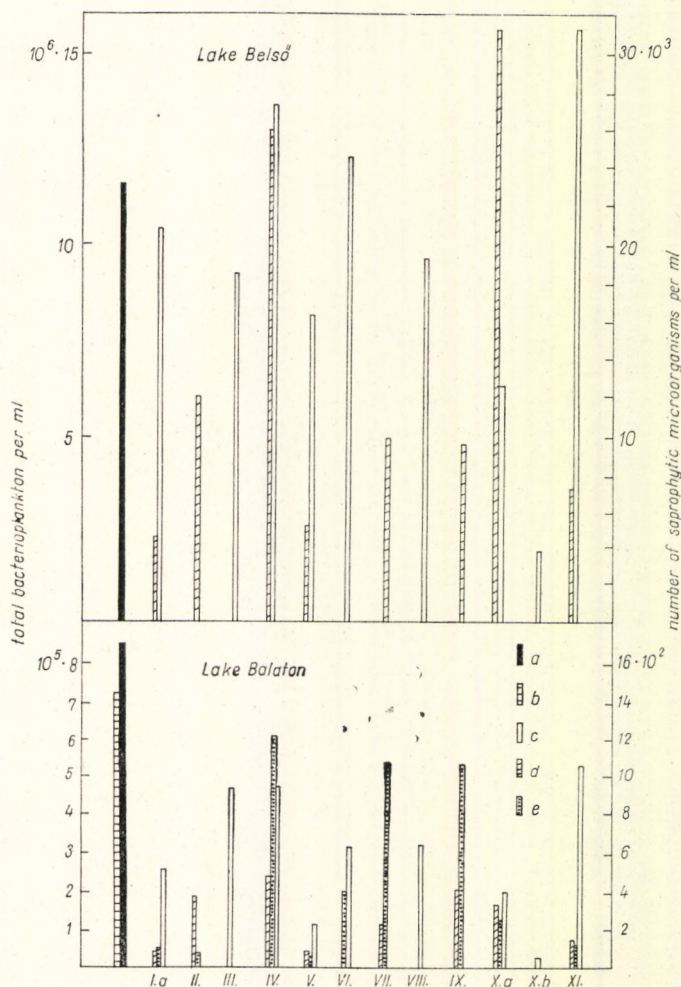


Fig. 1. Appearing colonies on the examined nutrient mediums and the total quantity of the microbial plankton in plate pouring carried out in different times from Lake Balaton and from the Inner Lake of Tihany. Total microbial plankton: (a) 19th VIII, 1968; (b) 8th IX, 1968. Plate pourings: (c) 6th VIII, 1969; (d) 19th VIII, 1968; (e) 8th IX, 1968

From among the culture media rich in organic materials the one containing sodium-caseinate appeared to be the most effective, for the largest number of colonies developed in this type of culture media. The OPPENHEIMER medium and the iron-peptone agar are also better for bacterial cultures than the nutrient agar, although, the proportions vary in the case of individual sealings. We examined three culture media rich in organic material and yielding high values for a period of one week by daily sealings (Table 2) using an inoculum originating from the water of Lake Balaton.

The sodium-caseinate medium in this particular case also has yielded the highest value. The modified iron-peptone agar proved to be only half as efficient as regards the quantity of bacteria, than the former. The OPPENHEIMER medium has yielded the lowest values in every case of examination.

TABLE 2

Media	Time of plate pouring						
	1968 IX. 19	20	21	23	25	26	27
	number of individuals/ml						
IV	1013	1200	2730	1950	2088	—	2000
VI	325	253	810	690	510	450	520
VII	594	560	1320	710	1600	110	—

In examining the water samples taken from River Danube in order to indicate the pollution we have shown the oxygen consumption of samples measured with  $\text{KMnO}_4$  (Table 3).

It is interesting to note that in one instance in the most polluted water sample on the sodium-caseinate agar we obtained a low value. On a small decrease of pollution the sodium-caseinate agar again yielded the highest values and the samples taken from the comparatively pure water of River Danube in both cases the biggest number of bacterium colonies developed on the sodium-caseinate agar.

TABLE 3

Media	Time of plate pouring and Origin of samples			
	22. X. 1969 Megyer	22. X. 1969 Nagymaros right shore	4. XI. 1969 Branch of River Danube at Soroksár Quay at Ferencváros	5. XI. 1969 Petőfi bridge leftshore
	number of individuals/ml			
I/a	—	—	5 310	2 870
I/b	12 350	10 850	8 960	20 220
VI	19 050	35 660	30 190	24 800
IV	41 280	29 370	37 000	14 180
Oxygen consumption $\text{O}_2$ mg/l	6.7	7.7	12.1	13.8

A significant difference has been observed between these two nutrient agars, too. Nutrient agar containing various salts has yielded higher values.

The highest number of bacterium colonies developed again on the sodium-caseinate agar from samples originating from the oxydized microzone and the reduced zone of Velence Lake (Table 4).

Both on the OPPENHEIMER agar and on the sodium-caseinate agar the samples taken from the oxidized microzone yielded more than twice as many bacteria than from samples originating from the reduced zone. At the same

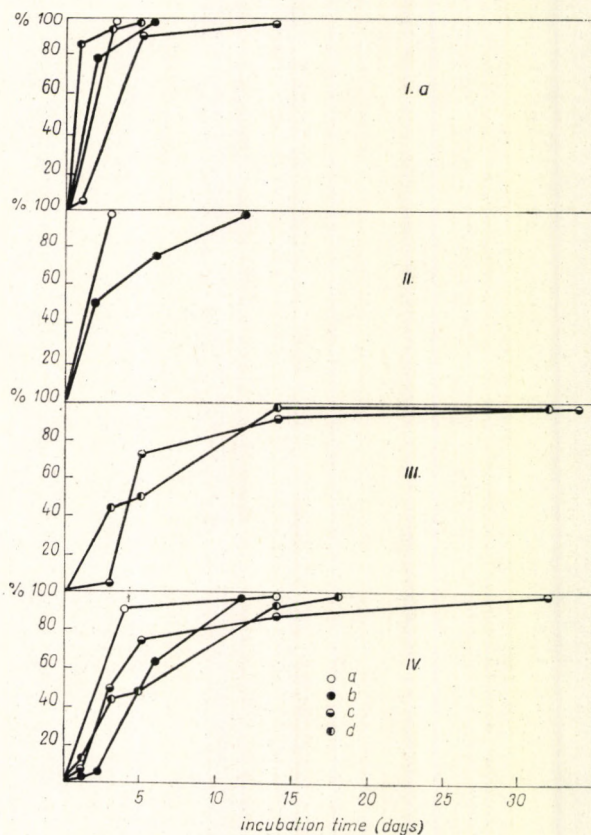
TABLE 4

Media	Origin of samples	
	Oxydized microzone, yellow mud	Reduced zone, black mud with a seell of $\text{H}_2\text{S}$
	number of individuals/ml	
I/b	3 684 000	3 477 000
VI	8 156 000	3 408 000
IV	13 210 000	5 240 000

time we have not found such a significant difference on nutrient agar, thus, when we work with nutrient agar only in the examined mud zones, we find the quantity of saprophytes well-nigh the same.

From all the above it issues that from the already employed media containing much organic materials the sodium caseinate agar appears to be the most favourable for counting saprophytic microorganisms especially for samples taken from Lake Balaton, and for other types of waters as well. In the case of strongly polluted waters it is well advisable to use nutrient agars containing a variety of salts.

In Poland, STRZELCZYK and his collaborators carried out investigations in 1967 on Lake Jeziorak and found that the sodium-caseinate agar did not give as high a value as the iron-peptone agar, for the latter yielded the highest values. Notwithstanding, in Hungary and elsewhere the sodium-caseinate agar has gained a wide acceptance for the examined waters (FRED. et al. 1925; FRED and WAKSMAN, 1928; STARK and MCCOY, 1938; TAYLOR, 1940; POTTER and BAKER, 1956, 1961; COLLINS and WILLOUGHBY, 1962; WILLOUGHBY and COLLINS, 1966).



*Fig. 2.* Nutrient media marked I.a, II, III, IV showing the dynamics of appearing colonies in plate pouring carried out in different times from Lake Balaton and from the Inner Lake of Tihany. Lake Balaton: (a) 19th VIII, 1968; (b) 8th IX, 1968; (c) 6th VIII, 1969. Inner Lake of Tihany: (d) 6th VIII, 1969

The other advantage of the sodium-caseinate agar yielding higher number of bacteria than the bacterium colonies appearing on the slide are small, they are clearly delineated from one another, as compared to those cultured on nutrient agar whose colonies are large, spreading fast and becoming liquefied, consequently, they render counting difficult. The chromogenic bacteria also develop on the sodium-caseinate agar in a higher percentage (STRZELCZYK et al. 1967).

The medium containing lake water and algal powder yielded a better result when inoculated with the water of Lake Balaton than the same procedure carried out on nutrient agar. Somewhat smaller values were obtained when inoculated with the water of the Inner Lake of Tihany. Here, the colonies were small and easy to count. Surrounding a number of colonies appearing 4–6 days after inoculation, on a dark green agar slide, striking light zones may be perceivable. By counting the colonies having lighter zones we might get some information to the quantity of microorganisms probably taking part in the decomposition of pigment materials.

The dynamics of the development of the final number of bacterial colonies on media enriched with organic materials (Figs 2 and 3) is rather

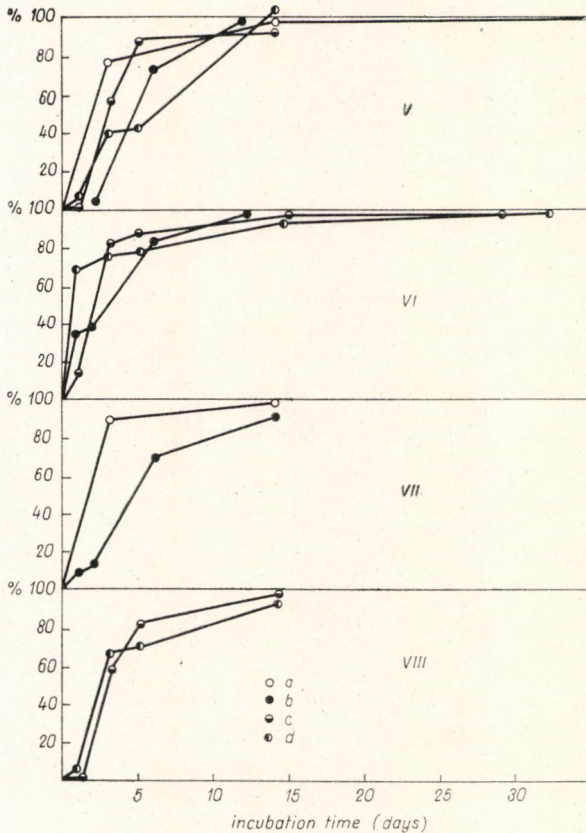


Fig. 3. Nutrient media marked V, VI, VII, VIII showing the dynamics of appearing colonies in plate pouring carried out in different times from Lake Balaton and from the Inner Lake of Tihany. For symbols see Fig. 2

variable. The fastest rate of growth has been observed on nutrient agar. On the fourth day 85% of the final number of bacterial colonies appear. On the other media, the rate of growth is slower, and by the 14th day 95–100% of the final number of bacterial colonies appears.

The other group of examined media not enriched with organic materials, but consisting of natural substrate or containing salts is shown in *Table 1*. On these culture media far many colonies developed than on the nutrient agar rich in organic materials, and quite frequently they yielded the highest values among all the culture substrates examined (*Fig. 1*). It was quite surprising that when Balaton water was inoculated on the lake-agar deriving from the supertrophic water of the Inner Lake of Tihany we obtained 10-times less number of bacterial colonies, while inoculated with the water of the Inner Lake of Tihany this number was only 4 when using lake-agar deriving from the water of Lake Balaton. The cause of this phenomenon may be sought for in the high algal content of the Inner Lake of Tihany whose colour is permanently of a green hue. In preparing culture substrates, as the result of repeated boiling, a large quantity of organic material as well as materials inhibiting the growth of bacteria become dissolved. An extravagant increase in organic materials, as has already been seen, may inhibit the growth of a part of the bacterial flora inhabiting the water.

The development of the final number of colonies on the lake water and mud-extract agars containing merely natural substrate as well as on oligo-carbophilic medium, is slower than on substrates enriched with organic materials (*Fig. 4*).

The majority of colonies appearing on the agar-slides containing natural substrate develops on the borderline of the agar and glass. This peculiar phenomenon may be due to surface effects (ZOBELL, 1943). The colonies are frequently small, indistinct, spotlike, difficult to perceive and thus counting is hindered. The development of colonies is very slow, and besides the saprophytic colonies other colonies also appear on the agar slide. For this particular cause the agarized nutrient media containing natural substrate are used only for special investigations. For example, the nutritive material supply of a lake may be calculated with their help quite easily, as well as the developmental rate of the natural microflora, etc. The MPN procedure was combined with a membrane filtering by MELCHIORRI-SANTOLINI and CAFARELLI (1967), where the lake waters was used for nutritive solution, receiving a higher number for bacteria than in nutritive solution enriched with organic materials.

When comparing the properties of nutritive media, on the one hand, those which has been enriched and examined by us, and on the other hand, those containing the natural substrate taking into consideration the properties of bacteria regarded to be autochton and zymogen as established by WINOGRADSKY (1932), the following conclusion is well justified: that zymogen bacteria are able to develop on nutrient media enriched with organic materials, while media containing only natural substrate is good for autochton bacteria only. GIBSON (1957) says that the WINOGRADSKY's classification of soil bacteria is also valid for water inhabiting microorganisms.

We should, of course, bear in mind that only a supposed parallelism exists, for WINOGRADSKY's classification is rather general, as the above presented results conclusively prove that on various culture media, how divers representatives of natural microbial associations may develop. The difference



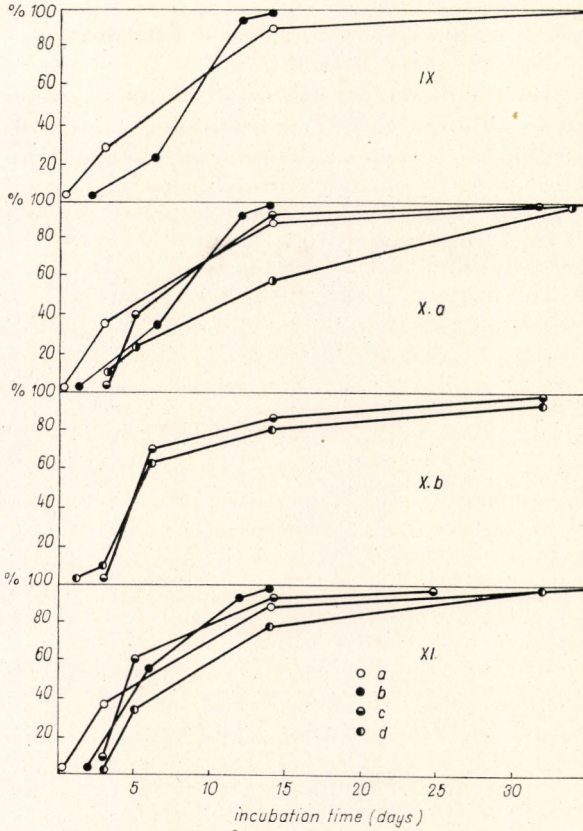


Fig. 4. Nutrient media marked IX, X.a, X.b, XI showing the dynamics of appearing colonies in plate pouring carried out in different times from Lake Balaton and from the Inner Lake of Tihany. For symbols see Fig. 2

between the result obtained by plate pouring and direct counting is mainly due to a high degree of selective capacity of the various nutrient media than to the high number of dead organisms counted on the membrane filter, for the quantity of dead organisms, measured by any type of method, compared to the total number hardly reaches a mere 20% (RAZUMOV, 1962).

### Summary

1. Significant differences have been observed between culture media used by us for counting saprophytic microorganisms.

On the basis of results obtained by comparative analysis, for the waters in Hungary the sodium-caseinate agar proved to be the most suitable for counting saprophytic microorganisms. This medium yielded the highest number for bacteria, the colonies were small, did not liquefy and the chromogenic bacteria occur in them in greater numbers.

2. It was interesting to note that on nutrient media containing only natural substrates and salts a large number of bacteria were able to develop. The slow rate of growth of the colonies and the difficulty in their counting make them suitable, primarily, for "special" investigations.

3. The authors in studying the microflora of waters by direct and indirect methods found that significant differences exist between the results of the two methods, which, according to them, are mainly due to the selecting property of the employed nutrient media as against the supposed high percentage of dead organisms counted on the membrane filter.

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## ÖSSZEHASONLÍTÓ TÁPTALAJ-TANULMÁNYOK

### A SZAPROFITA VIZI MIKROORGANIZMUSOK MENNYISÉGI FELMÉRÉSÉRE

*Oláh János és Vásárhelyi Réka*

#### Összefoglalás

1. A szaprofita mikroorganizmusok számlálására általunk felhasznált táptalajokon nagymértékben eltérő eredményeket tapasztaltunk. Az összehasonlító vizsgálataink eredményei alapján a nátrium kazeinátos táptalaj bizonyult a legalkalmasabbnak a szaprofita mikroorganizmusok számlálására.

A legmagasabb baktériumszámot adta, a telepek aprók, nem összefolyók és közöttük a kromogén baktériumok is nagy számban szerepelnek.

2. A csak természetes szubsztrátumokat és sókat tartalmazó táptalajokon megelégedően sok baktérium képes fejlődni. A kolóniák lassú növekedése és számlálásuk nehézsége miatt azonban elsősorban „speciális” vizsgálatok elvégzésére alkalmasak.

3. A szerzők a vizek mikroflórájának direkt és indirekt módszerekkel történő tanulmányozása során a két módszer eredményei közötti nagy különbségeket — szemben a membrán filteren számolt elhalt sejtek feltételezett magas százalékkal — elsősorban az alkalmazott táptalajok szelektáló hatásának tulajdonítják.

СРАВНИТЕЛЬНОЕ ИЗУЧЕНИЕ ПИТАТЕЛЬНЫХ СРЕД ДЛЯ КОЛИЧЕСТВЕННОГО  
ИЗУЧЕНИЯ САПРОФИТНЫХ ВОДНЫХ МИКРООРГАНИЗМОВ

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1. В развитии сапрофитных микроорганизмов на используемых нами различных питательных средах наблюдались различия. На основе сравнительных исследований самой лучшей питательной средой, для подсчёта микроорганизмов, оказалась питательная среда содержащая казеинат натрия. Эта питательная среда дала самое большое число бактерий. Маленькие колонии не сливаются и между ними в большом числе находятся хромогенные бактерии.

2. На питательных средах содержащих только натуральные субстраты и соли развивается удивительно много бактерий. Из за медленного роста колоний и трудного подсчёта они пригодны в основном для специальных исследований.

3. Авторы обнаружили при изучении водной микрофлоры прямыми и косвенными методами значительные различия между результатами двух методов. Эти различия трактуются как результат того, что использованные среды обладают селективными свойствами.