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ACTIVITY OF BACTERIA IN THE LIBERATION OF PHOSPHATE FROM THE SEA SEDIMENTS IN BOTTOM WATER

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In examining the influence of some factors in the production and mineralization of organic matter in sea-water, Buljan (1935) has found that it is possible to attain the deconcentration of phosphate gathered in thin sea-bottom sediments and lead them into a solution, if there are for this purpose suitable morphometric and hydrographic conditions. — The experiments undertaken by the afore mentioned observer have shown that besides the H-ion concentration, also the aerification of water is a factor affecting the quantity of phosphate liberated in water, i.e. that in semi-aerobic conditions is dissolved about 29,6% of phosphate and in strictly anerobic conditions about 53,8% of phosphate reckoning according to the quantities which are liberated in aerobic conditions.

Our experiments aimed to ascertain (1) whether, and under what conditions sea-water itself dissolves and preserves in the solution a determined quantity of phosphate from mud, and (2) whether, and to what extent, the bacterial flora of the mud and sea-bottom water partake in the process of phosphate diffusion in water.

Acknowledgment

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Material and Method of work

In our experiments we used the mud from the gulf of Kaštela. — The mud was collected by means of a Petersen's corer, from a depth of 30 m. and its characteristics were the following: granulometric

elaboration of the mud according to which the particles of sediments are grouped in four categories showed to possess:

89,53% particles smaller than 0,01 millimeters
8,97% particles from 0,01 to 0,05 millimeters
0,96% particles from 0,05 to 0,10 millimeters
0,72% particles from 0,10 to 2,00 millimeters.

Consequently with regard to the quantity of sediment in the single fractions and according to the colour, therefore according to the texture, the mud which we employed in our experiments answers to dark grey clay. The dampness of the mud before sterilization was 49,3%, the specific weight was 2,811 gr. and pH was 8,18. The contents of mineral matter determined by heating to incandescence amounted to 78,56% and organic matter to about 21,42%.

Immediately before the use the mud is well homogenized, weighed and directly put into well cleaned oxygen flasks of 300, 500 and 1000 cm^3. The sterilization of the mud was performed in an autoclave (120° C. during 30 minutes, for each experiment, twice in an interval of 24 hours).

The sea-water used in the experiment was also taken from the gulf of Kaštela at a distance of about 1000 m. from the shore. The water for the experiments with the single pure bacterial cultures was filtrated through filterpaper before the sterilization in the autoclave (120° C. for 30 minutes for each experiment, twice in an interval of 24 hours) or was filtered through the Berkefeld filter (N) for experiments with mixed bacterial cultures.

For the examination of the activity of the single bacteria we took 12 pure cultures which we isolated from the mud by means of inoculation on the nutrient broth under aerobic and anaerobic conditions (Tab. 1). Some of the bacterial characteristics have been determined in the usual way, according to the »Manuel of Methods for pure culture study« (1950). In order to examine the activity of the mixed bacterial flora we used 48 hours bacterial cultures from different selective broths (Cviić 1955), which we inoculated with the suspension of the same mud. The counting of the bacteria was done by the method of the dish on the nutrient broth of the usual composition (Cviić 1953). This method affords only a survey on the fluctuations of the number of heterotrophic bacteria in the experiment and these data served as a control of the fluctuations of the bacterial populations in the experiment, and not as its absolute number.

Tab. 1. SOME CHARACTERISTICS OF THE BACTERIA EMPLOYED IN THE EXPERIMENTS.

Strain No.	Shape and appearance of the colony — pigmentation	Shape of cell	Size of cell in	Relation towards oxygen	Gram stain	Creation of spores
215	Irregular shape, wrinkled, bright opaque, yellowish-brown	rods	1, 4—2, 5×0,5, 0,7	facultatively aerobic	+	+
217	Round, bulging, bright, opaque yellow		1,0—1,3	„	+	+
258	Round, flat, turbid-rough opaque, without pigment	rods straight or twisted	6,5—8, 5×1,0—1,2	„	+	+
295	Round, raised, bright, opaque without pigment	rods straight or coiled	1.5—2, 8×0,6—0,8	„	+	+
301	Round, flat opaque, bright green	rods	2,1—3, 5×0,9—0,1	„	—	+
302	Irregular, raised, bright opaque, without pigments	rods	1,4—3, 0,×0,4—0,7	„	—	+
310	Round, raised bright, transparent, without pigments	cocci	0,9—1,4	„	+	+
311	Round, split flat, bright opaque, without pigment	rods	3,5—4, 2×0,9—1,3	„	—	+
312	Round, undulated at the edge, flat, rough, turbid, opaque without pigment	rods	1,9—3 0×0,4—0,6	„	—	+
313	Round, bright opaque, without pigment	rods	1,0—2, 3×0,6—0,8	„	—	+
314	Round, flat, bright, opaque without pigment	rods straight and coiled	4,2—6, 4×0,8—1,2	„	—	+
315	Round, flat, bright, opaque without pigment	rods	1,3—4, 2×0,7—0,9	„	—	+

The free phosphate in sea-water was determined by means of Deniges' colorimetric method modified by Cooper (1938). Oxygen was determined by means of the modified Winkler's method, H-ion concentration by means of electrical pH meter (Radiometer — Copenhagen).

Experiment and results

I. CHEMICAL SOLUTION OF PHOSPHATE FROM THE MUD

The preliminary experiments had already shown that sterile mud, well stirred with a determined quantity of sterile sea-water augments the contents of free phosphate in water. In 10 oxygen flasks, volume of about 300 cm^3 we put 10 gr. of mud, we sterilized it and filled up to the top with sterile sea-water which had 2,5 mgr/m^3 $P\text{-}PO_4$. The mud and water were well mixed and let to stay from 24 till 48 hours, in order that the mud might deposit again. The analysis of the water showed that the latter now contained 25,0 and in some flasks even 30,0 mgr/m^3 free $P\text{-}PO_4$. It means that the quantities of free phosphate in water was augmented by solution for 22,5 to 27,5 mgr/m^3, namely on average for about 24,5 mgr/m^3.

In the further experiments we took 10 flasks of about 500 cm^3 volume and dropped into each of them 20 gr. of mud. After a similar procedure as in the previous experiment, we ascertained that the quantity of free phosphate in water was augmented on average for

Tab. 2. INFLUENCE OF THE WATER VOLUME AND QUANTITY OF MUD ON THE CHEMICAL SOLUTION OF FREE PHOSPHATE FROM MUD.

Experiment No.	300 cm^3 water			
	1000 cm^3 water from 10 gr. mud dissolved $P\text{-}O_4$ mgr/m^3	from 20 gr. mud dissolved $P\text{-}O_4$ mgr/m^3	from 10 gr. mud dissolved $P\text{-}O_4$ mgr/m^3	from 20 gr. mud dissolved $P\text{-}O_4$ mgr/m^3
1	34,0	50,5	28,0	43,0
2	35,5	52,0	26,0	41,5
3	33,5	51,0	28,5	42,0
4	32,0	52,5	25,5	42,5
5	33,0	50,0	26,5	41,0
Average	33,6	51,0	26,9	42,0
on 100 cm^3 water, dissolved	11,2	17,0	2,6	4,2

about 40,8 mgr/m³ P-PO₄ and the increase varied from 36,0 to 54,0 mgr/m³.

The repetition of the aforesaid experiments showed that the varitions of the quantity of phosphate dissolved in water are fairly remarkable and we attributed them to the difference in volume of the flasks and particularly to the difference in the kind of mud which we had taken for these experiments from various regions. Namely, ZoBell (1939) had already expressed the opinion that the texture of sediments with the contents of organic matter has an influence in the bacterial quantity, more than some other factors. Also from the results Buljan (Op. cit.) — who in his experiments used mud from various parts of the Adriatic, — we can see that the differences in the quantities of dissolved phosphate were fairly remarkable, since, for instance, in strictly anaerobic conditions were dissolved 442, 482, 366, 541 and 962% phosphate. This is the reason why in all our further experiments we used always the same mud from the gulf of Kaštela, of which we have given the characteristics in our previous pages.

In order to establish whether and in what measure the volume and quantity of mud affects the chemical solution of free phosphate, we carried on parallely experiments with flasks of 300 cm³ and of 1000 cm³. We used the same, well homogenized mud and the same sea-water, containing 2,5 mgr/m³ free phosphate. The mixing of the mud with water was carried on after 24 hours and on Tab. 2 we give the results obtained.

From Tab. 2 we can see that the sea-water in 10 gr. of mud dissolved on average 33,6 mgr/m³ free phosphate if the volume of water was 300 cm³, and 26,9 mgr/m³ if the volume of water was 1000 cm³. From 20 gr. mud was dissolved 51,0 mgr/m³ phosphate in 300 cm³ of water, and 42,0 mgr/m³ in 1000 cm³ of water. If we take 1000 cm³ of water as a unit of volume, then it appears that from 10 gr. mud on 100 cm³ in the smaller flask was dissolved 11,2 mgr/m³ phosphate, and in a flask about three times larger only 2,6 mgr/m³. From 20 gr. of mud was dissolved in the flask of smaller volume 17,0 mgr/m³ phosphate per 100 cm³ of water, and in the flask of a bigger volume only 4,2 mgr/m³. Accordingly the counting of free phosphate solution per unit of volume shows that there is dependency between the volume of disponible water and the quantity of dissolved phosphate, as well as the quantity of raw mud and the volume of water.

The results obtained show then that the quantity of chemically dissolved free phosphate and the volume of water are in an inverted proportion, i.e. with the same quantity of disponible mud in the unit of water volume, dissolves the more free phosphate as the less is the total volume of water. On the other hand it was shown that between the quantity of dissolved free phosphate and the quantity of mud there is a direct proportional relation, i.e. with the same quantity of water more free phosphate is dissolved if the quantity of mud is bigger and viceversa.

The question arose whether the dissolved free phasphate remain in the solution or the mud absorbs them again and how far does the quantity of oxygen in water influence this, i.e. aerobic and anaerobic conditions.

An exeriment was carried on in which equal quantities of sterile mud (20 gr.) and sterilized sea water (P—PO4 — 4,0 mgr/m^3), 0_2—0,76 ml/l. ph — 7,42) were put into three series of each five oxygen flasks of about 500 cm^3. The first five flasks (a) were not filled with water up to the top, but between the stopper and the water surface was left an air space, which had to be used as a reservoir of oxygen. The second series of five flasks (b) were filled with water up to the stopper to prevent the diffusion of oxygen from the air. The third five flasks (c) were filled up to the stopper and into the water, as a means of oxygen reduction was put 0,1 gr/lit. $NaHSO_3$, which had taken all the oxygen from the mud and water. The flasks were incubated in a water bath at a temperature of 24°C. Every 2—3 days a flask of each series was taken out and by means of a slender tube was pipetted out a quantity of water sufficient for the analysis. The analysis of the quantity of phosphate was performed and as a control of the experiment was performed the analysis of the quantity of bacteria, of oxygen and of H-ion concentration. The inoculation of water on the agar Petri's dishes showed that the flasks all the time of the experiment had remained sterile.

On Fig. 1 we show the results of the analyses of free phosphate in water. It was proved that the quantitiy of phosphate during 13 days fluctuates slightly under aerobic, the so-called semi-aerobic and strictly anaerobic conditions. At the beginning there was in all three cases a

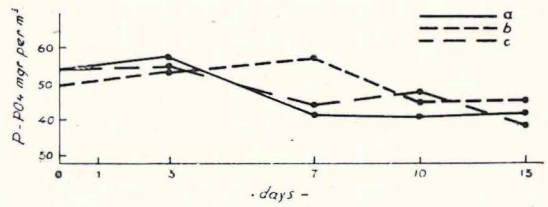


Fig. 1. Chemical solution of phosphate from the mud under aerobic (a) semi-aerobic (b) and strictly anaerobic (c) conditions.

slight increase of phosphate which may be ascribed to a possible subsequent solution and towards the end of the experiment the quantity of phosphate in all three cases was slightly decreasing, — which would mean that there was a certain sedimentation or renewed absorption of phosphate in the mud. It was proved, therefore, that chemically dissolved free phosphate remained substantially in the solution for the thirteen days during which the experiment lasted.

In the experiment the contents of oxygen and H-ion concentration fluctuated very slightly, i.e. remained substantially the same till the end of the experiment. The quantity of oxygen varied from 1,42 to 1,73 ml/l. in the flasks »a« and »b« and in the flasks »c« it was O ml/l., pH varied from 7,41 to 7,67.

In the further succession of experiments we had to get the answer to the second question, namely whether and in what measure the bacterial flora of the mud and sea-water partake in the process of diffusion of free phosphate from the mud into the water.

II. BIOGENETIC LIBERATION OF PHOSPHATE FROM NATURAL MUD INTO NATURAL SEA-WATER

In the first experiment we wished to ascertain what amount of free phosphate would be diffused from natural non-sterilized mud in which not only bacterial flora had remained untouched, but also other mud organisms in natural non-sterilized water, and whether there is in this diffusion any difference between aerobic, semi-aerobic and strictly anaerobic conditions.

We took three series of oxygen flasks of about 500 cm^3, we sterilized them and in each of them we weighed 10 gr of well homogenized natural mud. We poured then sea-water (P—PO^4 — 3,0 mgr/m^3, 02 — 5,57 ml/lit, pH — 8,09) in three series of 4 flasks each. Series »A«: in the flasks was poured $^3/_4$ volume of water, so that between the stopper and the water surface remained an air space, and the flasks were stopped up with a cotton plug. Series »B«: the flasks were filled with water up to the top and well closed with a glass stopper which was paraffined. Series »C«: the flasks were filled to the top with sea-water into which we had poured 0,1 gr/lit $NaNSO_3$ and well closed with a paraffined glass stopper. The water and mud in the flasks were well shaken and incubated in a water bath at a temperature of 24°C. Every 2,4 etc. days from each series was taken a flask, some clear water was pipetted from it and then the analysis was performed: the number of bacteria per cm^3, the quantity of free phosphate, the quantity of oxygen and H-ion concentration. The results of these analyses are shown on Tab. 3 and the diffusion of free phosphate in this experiment is represented by means of curves on Fig. 2.

Tab. 3. BIOGENETIC LIBERATION OF FREE PHOSPHATE FROM NATURAL MUD IN WATER UNDER AEROBIC (A), SEMI-AEROBIC (B) AND STRICTLY ANAEROBIC (C) CONDITIONS, QUANTITY OF BACTERIA, PHOSPHATE AND H-ion IN THE SINGLE STAGES OF THE EXPERIMENT.

Experiment	After days	Bacteria per cm^3 × 10	P-PO_4 mgr/m^3	5,22 ml/m^3	pH
A	2	130	26,0	5,22	7,83
A	4	160	48,0	3,32	8,13
A	8	11	71,0	3,50	7,25
A	12	12	95,5	4,20	7,08
B	2	140	28,5	1,83	7,87
B	4	175	42,5	0,30	8,05
B	8	25	77,0	0	7,68
B	12	27	103,0	0	7,52
C	2	41	79,0	0	7,25
C	4	83	104,0	0	7,18
C	8	16	106,5	0	7,30
C	12	8	114,5	0	7,32

The liberation of free phosphate into water (Fig. 2) did not flow in all three flasks in an equal measure. Already, after 48 h. occurred a differentiation which remained, or was augmented, till the end of the experiment. In the experiments made under aerobic and semi-anaerobic conditions the differences in the rise of phosphate through the duration of the experiments were not remarkable, but the quantity of liberated phosphate was remarkably bigger in semi-anaerobic conditions. Under strictly anaerobic conditions the rise of phosphate in water was by far bigger than in the first two cases, and the biggest difference was after four days, when it amounted to 56,0, respectively to 62,0 mgr/m^3. Towards the end of the experiment, namely after 12 days, the differences were lessened and the quantity of phosphate in water was augmented

in comparison with the beginning by 02,5 mgr/m³ under aerobic conditions, by 100,0 mgr/m³ under semi-anaerobic conditions and by 111,5 mgr/m³ under strictly anaerobic conditions.

The analyses of the disponible oxygen (Tab. 3) show that there was in the series »A« during the experiment enough of it for a full activity of aerobic and facultatively aerobic bacteria. In the series »B« the quantity of oxygen went abruptly dropping and was quite consumed between the fourth and eighth day, after which the phosphate could be liberated only by the activity of facultatively aerobic or strictly

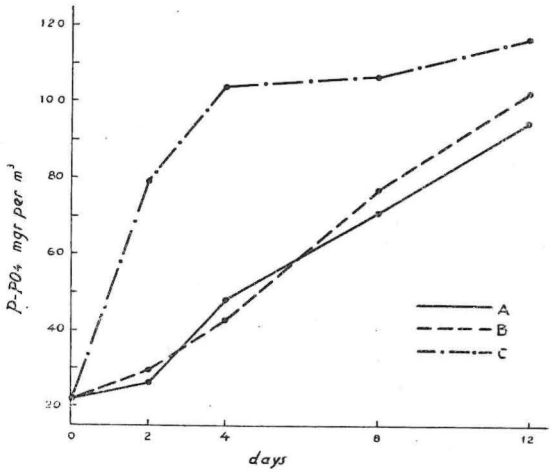


Fig. 2. Biogenetical liberation of phosphate from natural non-sterilized sea mud in sea water; »A« under aerobic, »B« under semi-aerobic, »C« under strictly anaerobic conditions.

anaerobic bacteria. The H-ion concentrations was in the course of the experiments in all three series substantially in a gradual oscillation towards the neutral, but with a general tendency to a rise of H-ion concentration, respectively a dropping of pH.

The number of heterotrophic bacteria (Tab. 3) suddenly augmented in the first four days and afterwards it dropped in all three cases. More detectable differences in the quantity of these bacteria appeared in strictly anaerobic conditions »C« where the average number of bacteria per mc³, was remarkably lesser than in the other two cases (»A« and »B«). Very likely it is a question here of the action of Na-sulfite which in the employed concentration, it is true, does not act toxicantly on bacteria, as showed by the preliminary experiments with the pure bacterial cul-

tures, but which in a determined number of bacteria retards their growth. The data obtained about the number of bacteria represent only an incomplete picture of the number of aerobic and facultatively anaerobic heterotrophic bacteria, and by no means their actual number active in the experiment. These data can, therefore, serve only as a rough and ready control of the motion of a part of the bacterial populations in the experiment. We can rightly presume that the number of bacteria is by far larger with regard to the numerous physiological groups of bacteria registered in the mud by various observers, (ZoBell 1942 and 1943, Kriss 1954) and which could not be registered by our method.

The results obtained in the previous experiments obviously show that a fairly large quantity of free phosphate was liberated biogenetically into water in the course of the experiment (the analysis of the water showed that chemically was dissolved about 21,5 mgr/m^3 P-PO$_4$ on 500 cm^3 of water), that the dissolved phosphate during 12 days not only remained in the solution, but was constantly rising and that the biggest growth of phosphate was in the strictly anaerobic conditions. Consequently our data correspond to the results obtained by Buljan (Op. cit.) However the question arose if in this diffusion of free phosphate besides bacteria participated also some other group of organisms from the mud or from the added sea-water. It is known, in fact, that some protozoa and other animals as well as some sea moulds and fungi can live in anaerobic conditions (ZoBell 1945) and we have to presume that these also, as many other organisms in mud, may have a direct or indirect part in the diffusion of phosphate into water. We endeavoured to clarify this question in our following experiment.

III. BIOGENETIC LIBERATION OF PHOSPHATE FROM STERILE MUD IN SEA WATER INOCULATED WITH SUSPENSION OF MUD BACTERIA

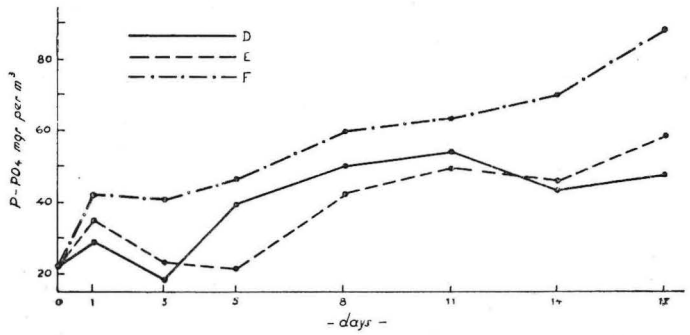
We weighed 10 gr. of the same well homogenized mud into three series of 7 oxygen flasks (D, E and F) we sterilized twice in autoclave and filled with twice sterilized sea-water (P—PO$_4$ — 3,5 mgr/m^3, O$_2$ — 3,21 ml/lit., pH — 8,14). In the flasks of the series »D« we left on the top an air space and we closed them with a cotton plug, the flasks series »E« were filled to the top and paraffined the glass stopper; into the flask series »F« we poured sterilized sea-water till the top, to which we added NaHSO$_3$ (0,1 gr/lit.) and after inoculation we paraffined the glass stopper. All the flasks were inoculated with 0,5 ml. of bacteria sunspension, which we arranged as follows: the mud suspension was inoculated in six different selective

broths; after 24 hours we put into a sterilized pumkin 3 ml. of culture from each broth, we stirred well together and then inoculated the flasks with this mixture. The cultures in the flasks after stirring were incubated in a water bath at a temperature of 24°C. Three separate flasks with mud and water were left non inoculated-sterile and after 24 hours we ascertained that in them were chemically dissolved on average 18,5 mgr/m³ free phosphate in each flask. The results of the water analysis in the cultures, carried on at determined intervals of time, are shown on Tab. 4 and Fig. 3.

Tab. 4. BIOGENETIC LIBERATION OF FREE PHOSPHATES FROM MUD IN WATER PERFORMED IN MIXED BACTERIAL CULTURES UNDER AEROBIC (D), SEMI-AEROBIC (E) AND STRICTLY ANAEROBIC (F) CONDITIONS, AND VARIATION OF THE QUANTITY OF BACTERIA, OXYGEN AND H-ion CONCENTRATION IN THESE CULTURES.

Experiment	After days	Bacteria per cm^3 $\times 10^3$	P-PO_4 mgr/m^3	O_2 ml/lit	pH
D	1	500	29,0	1,87	8,12
D	3	670	15,5	0,98	7,78
D	5	590	40,0	1,65	7,68
D	8	400	50,5	1,92	7,70
D	11	350	54,0	1,32	7,58
D	14	390	43,0	1,20	7,60
D	17	180	48,5	2,10	7,45
F	1	560	35,0	2,15	7,97
F	3	710	22,0	1,16	7,87
F	5	700	20,5	0,56	7,73
F	8	370	41,0	0,19	7,72
F	11	270	49,5	0,10	7,55
F	14	400	44,0	0,06	7,52
F	17	300	59,0	0	7,40
F	1	60	41,0	0	8,09
F	3	86	40,5	0	7,85
F	5	106	46,0	0	8,05
F	8	104	59,0	0 H_2S	8,00
F	11	150	63,0	0 „	7,89
F	14	120	69,5	0 „	7,73
F	17	190	89,0	0 „	7,65

In this experiment it was also shown that the highest rise of free phosphate, from the beginning to the end of the experiment was in the bacterial cultures cultivated under strictly anaerobic conditions (Fig. 3) and lesser was the rise under aerobic and semi-aerobic conditions. The differences in the quantities of liberated phosphate in these three cases are explicit, but not so big as in the previous experiment (Fig. 2). Besides in this experiment there were remarkable oscillations in the quantitiy of phosphate in the single intervals of time, whereas in the previous experiment none was observed. Particularly restless are the curves of the phosphate rise in the cultures cultivated under aerobic and semi-aerobic conditions, where the quantities of diffused phosphate in their reciprocal relation rose and dropped several times in the course of the experiment. This manifestation is very likely caused because by the sterilization of the mud, some physical and biologic-ecologic conditions were altered, whereby the natural course of biogenetic and abiogenetic processes was deranged, respectively was deranged the natural balance in the medium. About the reciprocal antagonistic action of bacteria in a mixed bacterial culture of sea-water, up to now we have enough particulars W a k s m a n (1937 and 1941) ascertained lately that in the struggle for food the bacterial species to which the conditions of the environment suit better, drive back the other bacterial species. L e w i s (1929) proved that *Pseudomonas fluorescens* produce in the culture toxic matter which hinders the growth of other bacteria and moulds. Z o B e l l and A n d e r s o n (1936) T a y l o r (1940) and C h a r l t o n (1955) and many other authors have also ascertained that a direct and indirect antagonistic action exists between the single bacterial species in the mixed bacterial cultures. adducing various



F i g. 3. Biogenetical liberation of phosphate from sterilized sea mud and sea water inoculated with bacterial suspension; »D« under aerobic, »B« under semi-aerobic »F« under strictly anaerobic conditions.

opinions about the causes of such manifestation. It is obvious that in our case too, since in the sterile mud and sea water were put various bacterial species from the mud suspension, these bacterial species, having come into a new, ecologically-unbalanced medium, executed reciprocally a remarkable antagonistic action, which, according to the conditions of the medium, respectively according to the processes themselves in such a medium, was at times more strongly and sometimes more weakly expressed and directed now in this and then in that direction.

The consumption of oxygen and the fluctuation of H-ion concentration (Tab. 4) were in this experiment substantially the same like the one in the previous experiment. Although the quantity of oxygen in water was rather different in the series D, E, F, it seems it did not affect the bacterial activity in the decomposition of organic matter, respectively in the liberation of phosphate from mud. In the series »F« where all the oxygen was already reduced at the beginning of the experiment, and after the eleventh day appeared H_2S in water, the liberations of phosphate flowed indisturbed and even bigger quantities were liberated than in aerobic and semi-aerobic conditions. This phenomenon may be connected to the results ZoBell and Anderson (1936), who ascertained that the contents of oxygen in sea water is a factor which, it is true, affects the bacterial activity, but had no importance for dense bacterial populations which appear in small volumes of sea water.

In the cultures of this experiment the fall of pH towards the end of the experiment was still more evident than in the previous one. For this phenomenon most probably is in part responsible the bacterial creation of desaminasis, for which Gale (1943) ascertained that it gives an impulse for the change of pH towards acid and that in addition to decarboxylasis it acts in the cultures as a mechanisms of neutralization by means of which the organism attains a degree of internal stabilization, if it finds itself in a medium which shifted from neutrality. It is inevitable, on the other hand, that the very biochemical processes themselves, which have taken place at the same time in the cultures in the course of the experiment, i.e. biogenetic transformation of organic material and abiogenetic reaction in the mud itself, — have influenced the H-ion concentration.

The quantity of heterotrophic bacteria in the single stages of the experiment (Tab. 4) was, also in this case, bigger in the cultures kept under aerobic and semi-aerobic conditions than in those under strictly

anaerobic conditions, only in the first and second cultures the maximum of bacterial development was attained after the third, respectively after the fifth day, and in strictly anaerobic conditions the maximum of bacterial development was attained only close to the end of the experiment. Since we had inoculated the flasks with mud suspension from selective broths in which could grow only aerobic and facultatively aerobic bacteria, it is plain that in our case only these were active in the processes of decomposition of organic matter, respectively of biogenetic liberation of phosphate. ZoBell (1946) mentions that of the bacterial colonies isolated from the seamud, over 90% were facultatively aerobic and therefore only a relatively small number of mud bacteria is strictly anaerobic. Consequently, according to their physiological action in our experiment there were by all means in the suspension employed various types of bacteria that attacked and decomposed the organic matter from the mud, liberating in doing so certain quantities of phosphate.

If we compare the data about the quantities of dissolved phosphate in the previous experiment (series »D, E, F«) with the quantities of dissolved phosphate in the experiment with non-sterilized mud and water (series »A, B and C«) we can affirm that at the close of the experiment there was in the first: A = 95,5 mgr/m^3, B = 103,0 mgr/m^3 and C = 144,5 mgr/m^3 of free phosphate and in the second experiment there was: D = 48,5 mgr/m^3, E = 59,0 mgr/m^3 and F = 89,0 mgr/m^3 free phosphate. Therefore in the experiment in which the natural equilibrium had not been deranged by sterilization, bacteria had regenerated about 40% more phosphate, than in the experiment in which the mud and water had been sterilized. By the experiments which followed we wished to verify this phenomenon under exactly controlled and equal conditions for both the cases.

IV. BIOGENETIC LIBERATION OF PHOSPHATE FROM NATURAL AND STERILE MUD UNDER STRICTLY ANAEROBIC CONDITIONS

In the oxygen flasks of about 500 ml. volume we weighed for each 10 gr. of well homogenized mud. The first series of five flasks (G) were sterilized twice and filled to the top with sterile sea water (P—PO_4 — 2,5 mgr/m^3, pH — 8,09) to which we added 0,1 gr./lit. Na-sulfite, we inoculated the flasks with 2 ml. of bacterial suspension from selective broths and then we paraffined the glass stoppers. In the second series of five oxygen flasks (H) we did not sterilize the mud, but filled the flasks to the top with sea water (P—PO_4 — 3,5 mgr/m^3 pH — 8,08) which we filtered through a Berkefeld filter (N) in order to remove from it all the organisms and to which we also added to reduce the oxygen 0,1 gr./lit. Na-sulfite. After

Tab. 5. BIOGENETIC LIBERATION OF FREE PHOSPHATE FROM MUD, UNDER STRICTLY ANAEROBIC CONDITIONS IN CULTURES INOCULATED WITH BACTERIAL SUSPENSION (G) AND IN CULTURES WITH NATURAL BACTERIAL FLORA FROM MUD (H) AND PARTICULARS ABOUT THE FLUCTUATION IN THE NUMBER OF BACTERIA AND H-ion CONCENTRATION.

Experiment	After days	Bacteria per cm^3 X 10^3	P-PO_4 mgr/m^3	O_2 ml/lit	pH
G	1	217	27,0	0	8,08
G	2	316	31,5	0	8,06
G	4	250	43,0	0	8,12
G	6	230	58,0	0	8,19
G	10	330	78,0	0	8,09
H	1	153	32,5	0	7,99
H	2	255	46,0	0	8,12
H	4	310	52,0	0	8,13
H	6	269	73,5	0	8,09
H	10	289	92,0	0	8,03

having paraffined also for these flasks the glass stoppers, both flasks and mud were well and equally mixed, all the flasks were incubated in water bath at a temperature of 24° C. In specially prepared sterile flasks the water chemically dissolved 21,5 mgr/m^3 free phosphate. The results of this experiment are shown on Tab. 5 and Fig. 4.

In this experiment it appeared again that bacteria in natural conditions of non-sterilized mud, dissolve bigger quantities of free phosphate from the mud than are dissolved by the bacterial suspension of the same mud cultivated 24 h. on selective broths. Since it may be supposed that with the suspensions must have come again in the culture the principal groups of bacteria that were also before in the mud, and were perhaps removed some other organisms, in such a case the main responsibility for this reduction in the quantitiy of dissolved phosphate should be ascribed to the mud. To this latter the sterilization had inevitably altered some physical, chemical and very likely also biochemical faculties, lessening and slackening the activity of the bacterial flora, particularly of some physiologic bacterial groups. In this way, by all means was afforded a bigger possibilty for the antagonistic action of

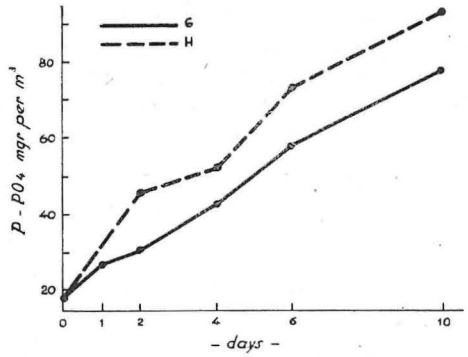


Fig. 4. Biogenetical liberation of phosphate from sterilized mud and sea water inoculated with bacterial suspension »G« and natural mud and sea water »H«. Both the experiments were carried on under strictly anaerobic conditions.

some bacterial groups, to which the conditions of the medium suited much better than to other bacterial groups, the activity of which was temporarily or permanently rejected. It will be however necessary to dedicate a particular attention to this phenomenon to which we simply endeavoured to give here a general explanation.

In order to get a picture about the activity of the single pure bacterial cultures in the biogenetic solution of free phosphate from the mud, we isolated from the mud 12 bacterial strains and with them carried on single experiments.

V. BIOGENETIC LIBERATION OF PHOSPHATE BY PURE BACTERIAL CULTURES

In the oxygen flasks about 300 ml. volume we weighed into each 10 gr. of well homogenized mud, the flasks and mud were twice sterilized and then filled to the top with twice sterilized sea-water. The quantity of P—PO_4 in the sterile sea water was in some flask series 3,0 mgr/m³ and in others 1,5 mgr/m³, the quantity of oxygen was 2,58 or 2,20 ml/lit. and in some flask series there was 9,09 pH and in others 8,15. The series of flasks were inoculated with three drops each of 48 h. pure broth culture and then flasks were closed with glass stoppers, the mud and water in them were well stirred and incubated in a water bath at a temperature of 24,0° C. In order to ascertain the quantity of phosphate dissolved by water itself, i.e. chemically, we left on purpose for each series a sterile flask and the analysis after 24 h. showed that there were chemically dissolved from, 25,0 to 30 mgr/m³ of free phosphate.

Tab. 6. PARTICULARS ABOUT THE BIOGENETICAL LIBERATION OF FREE PHOSPHATE FROM MUD IN WATER IN THE DEVELOPMENT OF 12 BACTERIAL CULTURES AND ABOUT THE VARIATION OF OXYGEN AND pH IN THE SAME CULTURES.

After days	Bacteria per $cm^3 \times 10^3$	P-PO_4 mgr/m^3	O_2 ml/lit	pH	Bacteria per $cm^3 \times 10^3$	P-PO_4 mgr/m^3	O_2 ml/lit	pH	Bacteria per $cm^3 \times 10^3$	P-PO_4 mgr/m^3	O_2 ml/lit	pH
	Strain 215				*Strain 217*				*Strain 258*			
1	48	28,0	2,37	8,22	1	33,0	2,27	8,23	1	29,5	2,50	8,09
3	97	40,0	1,94	8,16	2	16,0	2,08	8,18	3	32,0	2,37	8,10
5	120	81,0	1,44	8,01	5	54,5	1,60	8,99	4	66,0	2,16	7,99
7	130	70,5	1,18	8,03	90	74,5	0,35	7,90	12	87,5	1,89	7,96
10	120	84,5	0,90	7,88	80	86,5	0,20	7,83	15	103,5	1,80	7,91
13	100	112,0	0,46	7,85	73	98,0	0,14	7,84	10	109,0	0,98	7,89
	Strain 295				*Strain 301*				*Strain 302*			
1	3	29,5	2,26	8,22	62	27,0	1,80	8,00	56	23,0	1,35	7,94
3	6	35,5	2,07	8,15	31	49,5	1,07	7,81	37	43,0	0,97	7,70
5	21	48,0	1,63	7,91	41	57,0	0,99	8,06	42	66,0	0,93	8,03
7	53	72,0	1,13	7,88	29	72,0	1,25	8,05	74	77,0	0,67	8,05
10	48	91,5	1,00	7,75	16	94,0	0,74	8,03	67	103,5	0,60	8,02
13	25	122,0	0,74	7,74	19	84,0	0,05	7,03	59	112,0	0,36	7,75
	Strain 310				*Strain 311*				*Strain 312*			
1	73	25,0	1,87	7,93	15	25,0	2,70	7,74	14	23,5	2,50	7,75
3	81	51,0	1,29	7,91	14	43,0	2,51	8,21	16	40,0	2,42	8,08
5	54	72,0	1,32	8,02	32	68,5	2,13	7,99	16	64,0	2,30	7,87
7	28	89,5	1,12	8,04	29	92,0	1,97	8,05	15	87,0	1,71	7,88
10	30	99,0	0,75	8,01	11	86,5	1,82	8,06	29	74,5	1,41	7,85
13	25	104,5	0,37	7,88	40	72,0	1,45	7,95	52	88,0	1,12	7,92
	Strain 313				*Strain 314*				*Strain 315*			
1	13	22,0	2,82	7,74	40	19,0	2,41	7,69	12	21,0	2,82	7,79
3	13	35,0	2,58	8,00	51	37,0	1,92	8,08	13	36,0	2,52	8,04
5	16	72,0	2,35	7,94	63	78,5	1,62	7,85	11	63,5	2,28	7,89
7	27	94,5	1,90	7,93	42	92,5	1,46	7,88	12	86,0	1,86	7,84
10	41	91,5	1,53	7,90	54	97,0	1,42	7,91	28	77,5	1,59	7,85
13	48	96,0	0,52	7,88	58	101,0	0,67	7,82	34	93,0	1,14	7,79

The results of the experiment shown on Tab. 6 and on Fig. 5 point out that all the pure bacterial cultures from the mud dissolved considerable quantities of free phosphate. It is visible in the meantime that with all the cultures such dissolution was not of the same intensity, nor were the quantities dissolved after 13 days, — namely at the close of the experiment, — even approximately alike. — In the same way the

development of the bacterial population was very unbalanced in the individual pure cultures. In some cultures the quantity of dissolved phosphate in the course of the experiment was in constant rise (strains 258, 295, 302 and 314), with others during the experiment it dropped

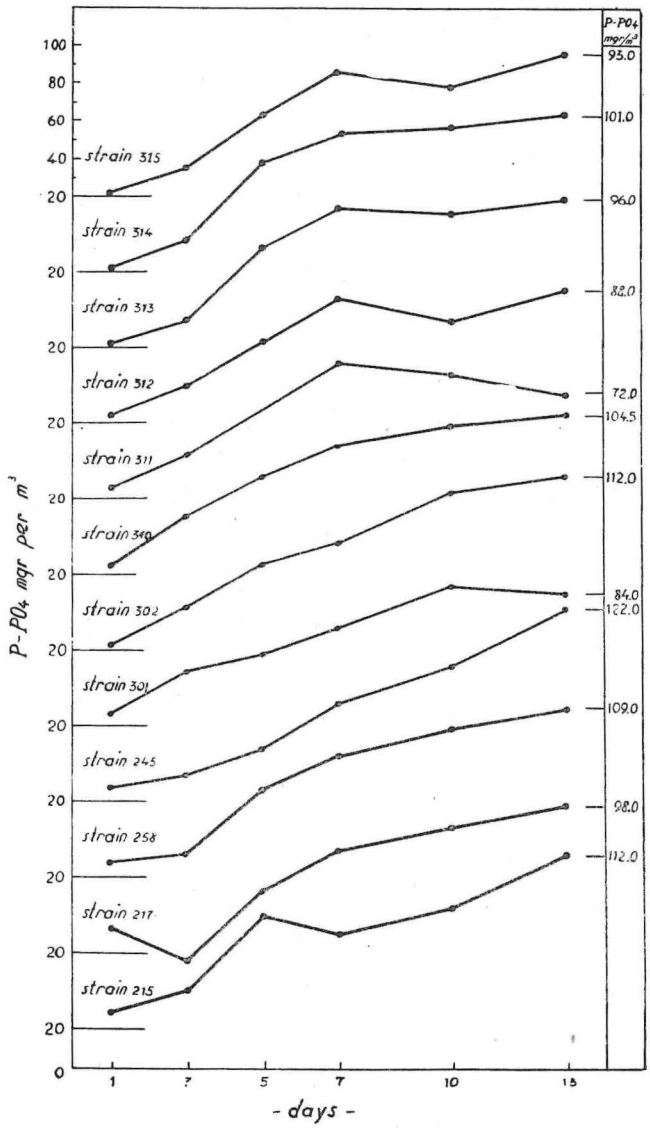


Fig. 5. Biogenetical liberation of phosphate from sterilized mud in sea water inoculated with pure bacterial cultures. All the experiments were carried on under semi-aerobic conditions.

and rose again (strains 215, 217, 312, 313 and 315) and with some towards the close of the experiment it was dropping (strains 301 and 311).

The quantity of dissolved free phosphate at the end of the experiment varied in the cultures from 72,0 to 122,0 mgr/m³ and the average for all the 12 cultures amounts to 99,2 mgr/m³. — If we deduct the quantity of chemically dissolved phosphate (27,5 mgr/m³) from the quantity of phosphate registered after 1, 3, 5, 7, 10 and 13 days, then we get the curve on Fig. 6 which shows the actual rise of the biogenically dissolved free phosphate within 13 days. After 24 h., i.e. after the first day there was a slight deficit of phosphate, for the bacteria in the cultures assimilated about 2,1 mgr/m³ of the chemically dissolved phosphate. Continuing the experiment till the seventh day, the quantity of dissolved phosphate rises abruptly and from the seventh to the thirteenth day it rises somewhat slower. From the daily average rise of biogenetically dissolved phosphate we reckoned that within 24 h. was dissolved, respectively regenerated, on average, about 10,3 mgr/m³ phosphate in every bacterial culture, which for 11 days amounts to about 113,3 mgr/m³.

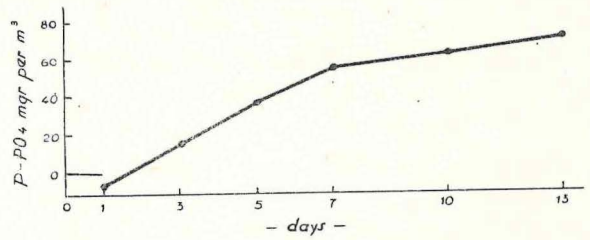


Fig. 6. The actual average quantity of biogenetically dissolved phosphate in 12 experiments with pure bacterial cultures, after deduction of chemically dissolved phosphate.

It is obvious that the aforesaid figures indicate approximately only those quantities of phosphate which are registered as free phosphate in water solution, whereas bacteria, by all means, dissolved considerably larger quantities, but the latter are not registered, since bacteria at once utilized them for the construction of their cells. We may however suppose that also these quantities of phosphate consumed by the bacterial cells are not insignificant, since the contents of phosphor in the bacterial cells is fairly high. According to the data collected from various observers Porter (1946) states that it amounts, on average from about 2,5% to

5,0% and with some bacterial species even till 8,91%. If we consider that in our cultures the 24 h. growth of bacterial biomase was of only 7,3 mgr/m³, namely as much as it is found in the Caspian Sea (Kriss 1954, Osnickaja 1954) then such a growth would spend for the construction of the cells from about 1,72 till 2,00 mgr/m³ of phosphate within 24 hours. Having regard to the fact that in our case it is a question of bacterial culture cultivated under laboratorial conditions we are bound to suppose that the bacterial biomase in our cultures was by all means much bigger. Accordingly we may reckon that this consumption of phosphate for the construction of the bacterial cells was in our cultures at least twice or three times bigger.

It is interesting that the maximum of bacterial development (Tab. 6) was observed in the first part or in the middle of the experiment, i.e. that the number of bacteria per cm³ was dropping by the close of the experiment, that is when the quantity of dissolved phosphate was substantially still rising. The same manifestation could also be observed in the previous experiments. Here probably we have to do with processes similar to those ascertained by Kreps (1934) who advanced the opinion that the absence of specific microorganisms must needs not mean also the cessation of the corresponding specific biochemical processes, since these may be continued also with organic catalyzators or enzymes. The author thinks that the bacterial enzymes are concentrated on the sea hottom and that they continue their function there long after the bacteria have disappeared. — ZoBell (1939) found that bacteria are distributed in the mud of the sea bottom to a depth of 60 cm. and that in deeper layers they break down. The microbic processes meanwhile are continued even in greater mud depths and also the afore mentioned author ascribes this phenomenom to the activity of bacterial enzymes which are collected in the sediments. Bacterial enzymes comprising reductases and oxydases are found in large concentrations in contact water (ZoBell 1946).

The fluctuation of oxygen in this experiment (Tabl. 6) was rather irregular, but in all the bacterial cultures the consumption of oxygen was constant, i.e. the quantities of disponible oxygen towards the close of the experiment lessened irregularly, but uninterruptedly. Although, then, the consumption of oxygen and the multiplication of bacteria were substantially paralel, they were not directly proportional. Waksman and Carey (1935) found that the quantity of oxygen utilized by a determined number of bacteria is not always quite uniform in their

cycle of life. To explain these irregularities, to the parallelism between bacteria and oxygen we must add that a part of oxygen is utilized also for the process of chemical oxydation in the mud, which also remained out of control in the experiment.

In any case in this experiment the quantity of disponible oxygen could be neither a prohibiting, nor a promoting factor for the oxidation of organic matter, respectively for the biogenetical liberation of free phosphate, since on one hand the quantities of disponible oxygen varied above 0,31 ml/lit. for which quantity ZoBell (1943) ascertained it represented the lower border necessary for the undisturbed oxidation of organic matter, and on the other hand all the pure bacterial cultures employed in the experiment were facultatively aerobic (Tabl. 1).

Discussion about the results

Waksman, Carey and Reuszer (1933) noticed that there are in the mud specific bacterial groups which can decompose residues of zooplanktons and fitoplanktons, liberating in doing so such quantities of nitrogen which enable the activity of these bacteria. The above mentioned observers furthermore conclude that the proteins were decomposed with the same speed in the mud as in sea water, but that the production of CO_2 and the decomposition of the remaining constituent elements of plankton was faster in the mud. — In decomposing zooplanktons, bacteria liberate parallely nitrogen and phosphorus and the relation of the liberated N:P is equal to 1,5:1 (Waksman, Hotchkiss, Carey and Hardman, 1938). The results of the experiment Seiwel and Sciwel (1938) showed that about half the phosphorus contained in freshly caught zooplanktons was decomposed within 24 h. after the breakdown of the zooplanktons. Similar results were obtained by Cooper (1935) who found that within 12 hours from one fourth to one third of the phosphorus contained in sea plankton is liberated by bacterial action in the form of phosphate. A sudden regeneraiton of phosphates in sea water may occur also during the bacterial decomposition of fitoplanktons. (Waksman et al. 1937).

Thus various observers have ascertained that in the mud of the sea bottom biochemical processes are performed faster than in sea water, that these processes enable and promote the bacterial activity in the general decomposition of disponible organic matter and that one of the

consequences of such decomposition is the quicker regeneration of phosphate. To these findings we have to add the results obtained by Renn (1937) who ascertained that, as soon as the development of bacterial populations in sea water enriched with phosphore and glucose, has passed its maximum, appears a faster regeneration of phosphate. The same author furthermore affirms that the bacterial cells in laboratorial conditions do not restrain phosphorus for more than some days, but they liberate it in water. According to this author the speed of regeneration depends on the quantity of glucose with which he enriched sea water.

The increase in the quantitiy of phosphate in sea water established in our experiments is therefore on one hand a consequence of the favourable biochemical conditions existing in the mud and contact water, and on the other hand, as it has been proved in our experiments, it is a consequence of the enrichment of sea water with phosphate which the latter chemically dissolves from the mud. — Both these conditions permit in determined conditions a speedy bacterial regeneration of phosphate, i.e. a bacterial activity in the passage of phosphate from the mud into sea water as one of the consequences of decomposition of organic matter existing in the mud. It was shown that in this activity there are remarkable differences between the single bacteria, since while one, in determined conditions, increases the quantity of phosphate in the water even till 122 mgr/m^3, the other, under the same conditions, increases it only to 72,0 mgr/m^3, (Tabl. 6). Whether these differences depend on a larger consumption of phosphate by the bacterial cells of some bacterial species, or they are a consequence of the general slackened activity of some bacterial species towards the regeneration of phosphate from the disponible organic matter, or they are the consequence of the action of some outer factors, which by their action retard or lessen the activity of some bacterial species in the regeneration of phosphate, or they are a consequence of the common action of all the aforesaid factors, for the time being it is difficult to assert.

Up to now we have not yet data about the fact whether there exists in the sea a specific bacterial flora performing the mineralization of organic phosphate, or in general the transformation of phosphorus links. It is known however, that a great many of lipolytic and preteolytic sea bacteria decompose organic matter with the liberation of phosphate (ZoBell, 1946).

The aerobic oxidation of sulphur in the absence of CO_2 was followed in Thiobacillus thioxidans by the disappearance of anorganic phosphorus, and when at the end of this process the cells aerobically fixed CO_2 in the basis returned anorganic phosphorus (Stephenson, 1949) Newburgh (1954) in the meantime in a slow fixation CO_2 *Thiobacillus thiooxidans* could not ascertain a liberation of anorganic phosphate. The bacterial reduction of phosphate to phosphine appears in the course of putrefaction of proteinic material. Budakov (1927) found an organism which can reduce phosphate to phosphite and further to hiposphophite and phosphine. Menkina (1950) found in the ground bacteria which mineralize some forms of organic phosphate (lecithin and nucleic acid). These and some other results (Wade, 1952, Mitchell and Moyle, 1953) show that there exists specific bacterial transformation of phospahte in the various media, and since this is one of the problems which in the question of the general metabolism of matter in the sea plays a very important role, particular attention should be consacrated to it.

SUMMARY

1) In the experiments we observed the fluctuation in the number of heterotrophic bacteria, free phosphate, the quantity of oxygen and H-ion concentration, and the obtained results are discussed.

2) It has been ascertained that under controlled laboratorial conditions, sea water in contact with sea mud, dissolves chemically from the latter determined quantities of free phosphate. The quantity of chemically dissolved phosphate from certain quantities of mud, are in the reversed proportion with the quantity of disponible mud. So from 10 gr. of mud are chemically dissolved in 300 cm^3 of water about 33,6 mgr/m^3. The dissolved quantities of phosphate remained in the solution for 13 days and at the close of the experiment they were in a slight decrease.

3) From the natural sea mud are liberated within 12 days in the conditions of the experiment, in natural sea water bigger quantities of free phosphate under strictly anaerobic (93,0 mgr/m^3) than under aerobic (69,0 mgr/m^3) or semi-aerobic conditions (81,5 mgr/m^3). The liberated quantities of phosphate were in constant rise during 12 days, as long as the experiment lasted.

4) The suspension of various bacterial species liberates after 17 days from sterile mud and sterile sea water bigger quantities of phosphate under strictly anaerobic (70,5 mgr/m^3) than under aerobic (30,0 mgr/m^3) or semi-aerobic conditions (40,5 mgr/m^3).

The total amount of liberated phosphate is less than the one liberated from the natural mund and sea water. Also under these conditions the dissolved phosphate was substantially in constant increase during the 17 days of the experiment.

5) Pure bacterial cultures liberate from the mud, under determined conditions on average about 10,4 mgr/m^3 of phosphate in 24 hours and the quantitiy of liberated phosphate in the single cultures varies greatly.

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AKTIVNOST BAKTERIJA PRI IZLUČIVANJU FOSFATA IZ MORSKIH SEDIMENATA U PRIDNENU VODU

Vlaho CVIIĆ

Institut za oceanografiju i ribarstvo, Split

Kratak sadržaj

1) U eksperimentima se pratilo kolebanje broja heterotrofnih bakterija, slobodnih fosfata, količina kisika i koncentraciju H-iona. Dobiveni rezultati su diskutirani.

2) Utvrđeno je, da — pod kontroliranim laboratorijskim uslovima — morska voda u dodiru s morskim muljem otapa iz ovoga kemijskim putem određene količine slobodnih fosfata. Količina kemijski otopljenih fosfata iz neke količine mulja u obrnutom je razmjeru s količinom vode, a u upravnom razmjeru s količinom raspoloživog mulja. Tako se iz 10 gr mulja, stavljenih u 300 cm^3 morske vode, kemijski otopi oko 33,6 mgr/m^3 fosfata, a u 1000 cm^3 vode 29,9 mgr/m^3. Otopljene količine fosfata ostale su u otopini 13 dana, a prema kraju eksperimenta bile su u slabom opadanju.

3) Iz prirodnog morskog mulja izlučuju se kroz 12 dana, u uslovima eksperimenta, u prirodnu morsku vodu veće količine slobodnih fosfata pod striktno anaerobnim (93,0 mgr/m^3), nego pod aerobnim (69,0 mgr/m^3) ili poluaerobnim uslovima (81.5 mgr/m^3). Izlučene količine fosfata bile su u stalnom porastu kroz 12 dana, koliko je trajao eksperiment.

4) Suspenzija raznih bakterijskih vrsta oslobađa, nakon 17 dana, iz sterilnog mulja i sterilne morske vode veću količine fosfata pod striktno-anaerobnim (70,5 mgr/m^3), nego pod aerobnim (30,0 mgr/m^3) ili poluaerobnim uslovima (40,) mgr/m^3). Ukupne količine oslobođenih fosfata su manje od onih oslobođenih iz prirodnog mulja i morske vode. I u ovim uslovima otopljeni fosfati bili su uglavnom u stalnom porastu kroz 17 dana trajanja eksperimenta.

5) Čiste bakterijske kulture oslobađaju, u određenim uslovima, iz mulja prosječno po 10,4 mgr/m^3 fosfata u 24 sata, a količina oslobođenih fosfata, u pojedinim kulturama, jako varira.

Tiskanje završeno 30. VII. 1956.

Tisak: Novinsko-izdavačko poduzeće »Slobodna Dalmacija« — Split