RELATION BETWEEN FOOD CONCENTRATION AND SURFACE FOR BACTERIAL GROWTH[1]

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Received for publication April 19, 1940

Storage of waters, underground, surface, sea and sewage, removed from their natural environment results in an initial multiplication of bacteria. The initial increase is reflected in the total plate counts as well as in the numbers of coliform organisms. The increase cannot be attributed to the change in temperature, as incubation of the waters at the same temperatures as in their natural environment does not prevent it. This phenomenon is the more puzzling because the waters in their natural environment do not undergo similar increases.

Zobell and Anderson (1936) obtained greater increases of bacteria in sea water stored in small volumes. This was attributed to the contact of the water with the proportionate larger solid surface area in small receptacles. The solid surfaces provide a resting place for periphytes as well as concentrating the nutrients in a film thereupon. The bacteria may multiply upon the solid surface without necessarily being firmly attached to it.

Stark, Stadler and McCoy (1938) found that in support of Zobell's results, measurable amounts of organic matter accumulated over a period of hours on the surface of chemically clean glass slides suspended in sterile lake water, indicating that the accumulation of organic matter is independent of and precedes bacterial growth. Butterfield (1933) on the other hand, found that size of container does not make any difference in the increase

[1] Journal Series Paper, New Jersey Agricultural Experiment Station, New Brunswick, New Jersey Division Water and Sewage Research.

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of bacteria in stored river water. That volume-surface ratio does not influence bacterial growth curves obtained in culture media was indicated by Rahn (1932), but all the waters which manifest an initial increase in bacteria are poor in nutritive value for bacterial growth. Even sewage is poor in nutrients in relation to culture media. Zobell and Anderson (1936) state that the effect of surface area on bacterial multiplication is manifest only in dilute solutions. When 100 mgm. of peptone and 1 mgm. of KNO_2 were added per liter of sea water, there was no difference between small and large receptacles in the rate of nitrite reduction.

There is little information available in regard to limiting concentration of food materials on the growth of different bacteria. Butterfield (1929) found that 0.5 mgm. each of glucose and peptone per liter, though not optimum for the growth of *Aerobacter aerogenes* was by no means a limiting concentration as the organisms multiplied after an extended lag period. Friedlein (1928) reported that *Escherichia coli* did not grow in a sodium lactate medium at concentrations lower than 0.1 per cent (1000 p.p.m.) and in a glucose medium in concentrations below 0.016 per cent (160 p.p.m.). Bacterial growth was determined by turbidity measurements and it is a question whether the failure to obtain increases in turbidity at these low food concentrations can be taken as a positive proof of the absence of growth.

Experiments reported below were therefore undertaken to secure the following information:

(1) The limiting concentration of nutrients for the growth of *Escherichia coli.*

(2) The effect of increasing the solids surface on the limiting concentration of food for the growth of *Escherchia coli.*

(3) The concentration of food materials at which the addition of surfaces fails to exert an influence on the growth of *Escherichia coli.*

(4) Comparison of the type of surface on the growth of natural water flora.

(5) Effect of biologically active surface on the growth of bacteria in water.

METHODS

Glucose and peptone were selected as the nutrients. A series of concentrations containing from 100 p.p.m. to 0.5 p.p.m. of glucose and of peptone were made. The menstruum was 50 p.p.m. phosphate buffer water. Twenty milliliter portions of the different concentrations of the media were placed in 250 ml. Erlenmeyer flasks. To one series of flasks 50 grams of 4 mm. glass beads were added in conjunction with the medium while the other series served as control without glass beads. The glass beads, as well as all glassware, were thoroughly treated with cleaning solution and washed. A sufficient number of flasks of each concentration of the medium was made so that after sampling, the flask was discarded. The medium was sterilized and inoculated with a suspension of a 24-hour culture of *Escherichia coli.* The organism was harvested from agar slopes, washed free of nutrients by centrifuging two times, and suspended in phosphate buffer. One milliliter portions of the inoculum were added to each flask and incubated at 20°C. At intervals, a set of flasks representing different concentrations of nutrients with and without the beads was shaken vigorously for 1 minute and the number of organisms determined by agar plates incubated at 37°C. for 48 hours.

RESULTS

A set of typical results is given in figure 1. The parallel determinations from duplicate flasks showed a satisfactory agreement. The results show that at 0.5 p.p.m. glucose and peptone concentration the *Escherichia coli* failed to grow in 72 hours in the absence of beads. With 2.5 p.p.m. food concentration the growth was only slight. In the presence of beads on the other hand there was considerable growth even at 0.5 p.p.m. glucose and peptone concentration. The growth level attained with glass beads between 0.5 and 12.5 p.p.m. concentration was practically the same. The numbers of *Escherichia coli* were higher in the presence of beads over the corresponding concentrations without beads up to 25 p.p.m. Beyond this concentra-

tion the numbers of organisms were practically the same whether beads were present or not.

Although *Escherichia coli* can grow both under aerobic and anaerobic conditions, the objection might be raised that the ob-

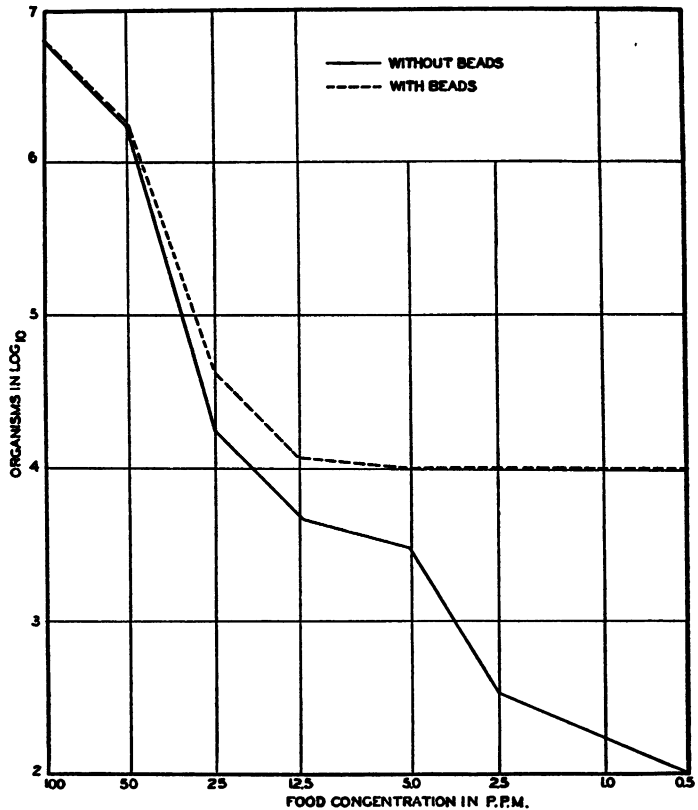

FIG. 1. NUMBERS OF ESCHERICHIA COLI (IN LOG_{10} PER ML.) IN VARIOUS CONCENTRATIONS OF GLUCOSE AND PEPTONE DURING 72 HOURS' INCUBATION WITH AND WITHOUT GLASS BEADS
(Initial number 170 per ml.)

served results were affected in the higher concentrations by the absence of dissolved oxygen. That this was not the case is indicated from the results of the following experiment. The same medium used previously was distributed into 1 liter flasks in 300 ml. quantities. The concentrations of glucose and peptone

varied from 0.5 p.p.m. to 100 p.p.m. The media were inoculated with a 24-hour culture of *Escherichia coli* and incubated for a period of 48 hours. At the end of this period the medium was siphoned carefully into 150 ml. glass stoppered bottles and dissolved oxygen determined according to the Rideal-Stewart modification of the Winkler method. The results are given in table 1. Dissolved oxygen was present in all the concentrations of glucose and peptone except in the 100 p.p.m. In these large flasks there was a surface-volume ratio of 15:1, whereas the surface-volume ratio in the 250 ml. flasks with 20 ml. portions the medium

TABLE 1

*Dissolved oxygen content of different concentrations of glucose peptone broth inoculated with Escherichia coli**

CONCENTRATIONS	DISSOLVED OXYGEN
p.p.m.	*p.p.m.*
100.0	0
50.0	4.0
25.0	7.8
12.5	8.0
5.0	8.0
2.5	8.0
0.5	8.0

* After 48-hour incubation of 300 ml. portions in 1 liter Erlenmeyer flasks.

was 2:1. It is therefore reasonable to assume that dissolved oxygen was present in the smaller flasks containing 20 ml. of the medium even at 100 p.p.m. of glucose and peptone. Hence the results reported in the first experiment were not influenced by the differences in dissolved oxygen content at various food concentrations.

The effect of different types of surface on bacterial numbers was then compared. The menstruum was unpolluted surface water with its natural population. The water was placed in beakers and (1) kept under quiescent conditions, (2) aerated, (3) agitated continuously by means of paddles, and (4) kept in contact with sand. Thoroughly cleaned and washed fine Ottawa sand was used. The volume of water added was just sufficient

to wet the sand. In order to obtain representative samples known quantities of sterile water were added to the sand, and vigorously shaken, after which the sand was discarded. Bacterial numbers were determined by nutrient agar plates incubated at 20°C. for 48 hours. The results are given in figure 2. The bacteria increased in the control up to 24 hours and thereafter

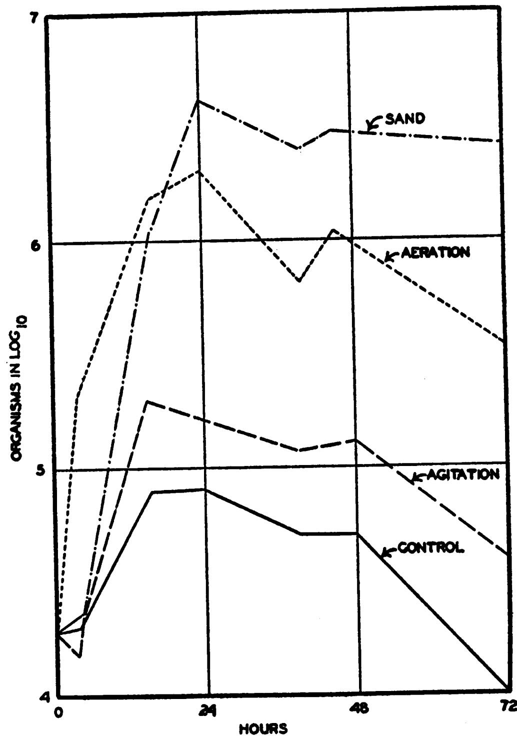

FIG. 2. NUMBERS OF BACTERIA (AGAR PLATE COUNTS) IN SURFACE WATER (*a*) KEPT UNDER QUIESCENT CONDITIONS, (*b*) IN THE PRESENCE OF SAND, (*c*) AGITATED, AND (*d*) AERATED

decreased gradually up to 72 hours when there were fewer organisms remaining than initially. Agitation caused an increase of organisms to a higher level than were present in the control. With aeration and with sand the numbers were increased to even higher levels. The numbers dropped after 72 hours aeration but the number of organisms remaining at this time was greater than the initial number. With sand, the numbers did not show an

appreciable decrease even after 72 hours. The aerated water did not show an initial lag period.

The effect of gentle stirring of sewage by means of submerged paddles on bacterial numbers is illustrated in table 2. It is unlikely that reaeration was materially increased by the stirring to influence the results. The numbers in the unstirred sewage did not increase while in the stirred sample there was a 2.5-fold increase.

TABLE 2

Effect of stirring of sewage on bacterial numbers

Bacteria in millions per ml.

TIME	NOT STIRRED	STIRRED
hours		
0	8.2	8.2
4	8.0	7.4
24	4.3	21.2

TABLE 3

Effect of addition of stones covered with biologically active slime on the numbers of bacteria in water

Bacteria in thousands per ml.

TIME	NON-POLLUTED WATER		POLLUTED WATER	
	Control	Stones added	Control	Stones added
hours				
0	22	30	316	303
9	30	27	1,100	795
26	800	230	6,850	3,900
72	230	23	416	37
96	37	110	30	64

The effect of solid surfaces covered with biological slime on bacterial numbers of the supernatant water was investigated. Stones from the bed of an unpolluted stream were collected and placed in battery jars one layer deep. The jars were then filled with polluted and unpolluted stream water. Controls consisted of the same waters without the stones. The results are given in table 3. Storage of both the polluted and unpolluted water resulted in increases in bacterial numbers, those in the polluted

water being higher. When these waters were stored in the presence of slime-covered stones, the increases in bacterial numbers were not as great.

DISCUSSION

Under the experimental conditions the limiting concentration of glucose and peptone for the growth of *Escherichia coli* was about 2.5 p.p.m. of each of the ingredients. This result was obtained by inoculating with very small quantities of thoroughly washed cells. It is conceivable that with larger inoculations the limiting concentration might be even lower. The limiting food concentration is not a fixed value but is dependent on the amount of surface in contact with the medium. By increasing the amount of surface per unit volume of the medium, as was done experimentally by the introduction of clean glass beads, the limiting concentration is moved downward and appreciable growth is obtained even at 0.5 p.p.m. concentration. Up to 25 p.p.m. glucose and peptone concentration, increasing the surface-volume ratio by the introduction of the glass beads resulted in increase in the growth of *Escherichia coli.* Beyond this value the addition of glass beads had little effect on the numbers. Furthermore, increasing the concentration of glucose and peptone from 0.5 to 25 p.p.m. had little influence on the ultimate numbers of *Escherichia coli* in the presence of glass beads. Increasing the concentration of food above 25 p.p.m. resulted in higher bacterial numbers, both with and without glass beads. It appears that food concentration was the limiting factor up to 25 p.p.m. glucose and peptone and that within this range the addition of surface enabled the organisms to produce greater growth presumably due to the surface concentration of the food and the bacteria (Stark, Stadler and McCoy, 1938; Zobell and Anderson, 1936). At 50 and 100 p.p.m. the addition of surfaces did not result in greater increases in bacterial numbers.

Escherichia coli is not a true periphyte. Therefore, the concentration of these organisms on solid surfaces is induced only by the dearth of food. Furthermore, the attachment is superficial, because shaking readily detaches them. A 24-hour growth of

this organism in the presence of glass beads, after shaking for 1 minute, yielded 41 million organisms per milliliter. When the menstruum was poured off and the glass beads were shaken with fresh sterile water (9 ml.) an additional 2 million bacteria were obtained per milliliter. On repeating the process a second and third time 520,000 and 500,000 organisms per milliliter were obtained, respectively. This would indicate that the attachment of the organisms on the surface is superficial and that under quiescent conditions there may be a continuous interchange of organisms between the menstruum and the surface. Therefore, the numbers obtained in the presence of glass beads do not represent the total numbers of organisms present but only those that could be detached by shaking.

It should also be pointed out that the minimum food concentration for bacterial growth will vary with the type of organisms. In general, the larger the organism the higher will be the limiting food concentration (Butterfield, 1929).

It is our contention that the increase in the bacterial numbers on aeration and agitation is to be attributed to surface phenomenon similar to those which govern the action of sand or glass beads. That the increase is not due to dispersion of the organisms originally present in the water is indicated by the fact that agitation for the first five hours did not result in an increase. Dissolved oxygen, as such, did not exert an influence on the results because the water, being unpolluted, had a low oxygen-consuming capacity and was thoroughly reaerated prior to use. The dissolved oxygen value at the end of the period was 7.4 p.p.m. in the control, 8.0 p.p.m. in the aerated sample and 7.8 p.p.m. in the agitated sample. It is improbable that the changes in the CO_2 tension brought about by the treatment could have affected the bacterial numbers. The differences in the bacterial numbers attained seem to be in the order of the increases in the surface. The lowest increase over the control was obtained with agitation and the highest with sand and aeration.

Sand filtration of liquid wastes such as sewage and water to obtain highly purified effluents is an old established practice. Bacteriologically this method seems to be ideally suited for the

removal and oxidation of soluble impurities from water in very low concentrations. With such low concentrations of organic material as are present in water and sewage, the only possible rapid biological action seems to be in the presence of solid surfaces enabling the surface concentration of food and bacteria. These solid surfaces soon become coated with a highly specialized slime wherein biological action takes place at a high rate. Surfaces free from slime make possible the oxidation of low concentrations of organic matter but when these surfaces become coated with slime the rate of oxidation is greatly accelerated.

A similar phenomenon is encountered in the activated sludge process of sewage treatment. In this process bacteria capable of growing in colonial form (*Zooglea ramigera*) are the predominant forms (Butterfield, 1935). The surfaces to which these organisms attach themselves may be those of inert inorganic or organic particles. The organisms grow in masses embedded in capsular gel and form large flocs. Sewage, compared with culture media represents a very dilute substrate, and contains both dissolved and dispersed material. The dispersed matter is concentrated on the surfaces of the floc and acted upon at a very high rate by the mass of organisms present in the floc. There is, however, no evidence of surface concentration of the dissolved materials, which are probably directly assimilated by the organisms during the circulation of the floc through the liquid. Mixing is therefore essential, in addition to an adequate supply of oxygen to maintain aerobic conditions. Both mixing and maintenance of aerobic conditions may be accomplished either by mechanical agitation or by aeration. Aeration and agitation have been shown to increase the number of bacteria in surface waters by virtue of increasing the internal surface of the liquid and consequent contact between the organisms and their food material.

The increase of bacteria in water removed from its natural environment and stored in the laboratory has been attributed to increase in the surface-volume relationship (Stark, Stadler and McCoy, 1938). However, when a surface water is removed from its natural environment, there is brought about in addition, a change in the relationship between the water and the stream bed.

The stream bed is covered with active biological slime which may remove bacteria from the water and prevent an increase in their numbers. On the other hand, in waters stored in the laboratory this factor is not present and the increased surface results in greater numbers. The addition of stones covered with slime to water reduced the increase in bacterial numbers over the control without the stones. The amount of active surface film in relation to the volume of water was greatly increased under laboratory conditions as compared with natural conditions. At the same time, however, the amount of food material introduced by the slime was also increased. It is probable that considerable diffusion of soluble, and some dispersion of insoluble, particles took place when the stones covered with film were placed in the water, resulting in an increase in the numbers of bacteria, while the active surfaces would tend to decrease the numbers of bacteria in water by adsorption. The net effect of the addition of slime covered stones therefore is the resultant of these two factors, an increase in the numbers but of a lower magnitude than without the slime-covered stones. A differentiation should be made between the action of inert surfaces as such, which would tend in highly dilute substrates to increase the numbers of bacteria, and active surfaces of slime which would exert an opposite effect. Under natural conditions inert surfaces are seldom encountered without an active slime.

SUMMARY AND CONCLUSIONS

For the purpose of determining the effect of food concentration and surface on the growth of bacteria, washed cultures of *Escherichia coli* were inoculated into glucose and peptone medium of concentrations varying from 100 to 0.5 p.p.m. in flasks of 250 ml. capacity containing 20 ml. of the medium. To one series 50 grams of 4 mm. clean glass beads were added. The numbers of organisms were counted by the plate method after different periods of incubation.

Under the conditions of the experiment the growth of *Escherichia coli* did not take place in glucose and peptone concentrations of 0.5 and 2.5 p.p.m. The addition of glass beads to the medium

at these concentrations permitted a considerable growth of these organisms. The effect of glass beads was noticeable up to 25 p.p.m. concentration of glucose and peptone. Beyond this concentration the numbers of *Escherichia coli* with and without beads were practically the same.

The population of stream water is similarly affected by the addition of clean sand. Aeration and agitation also result in higher counts of the flora of the water. Stirring of sewage likewise results in an increase of bacterial numbers.

The addition of stones covered with biologically active slime to surface waters does not prevent the increase in the numbers of bacteria but considerably reduces the magnitude of the increase.

Surfaces enable bacteria to develop in substrates otherwise too dilute for growth. Development takes place either as bacterial slime or colonial growth attached to the surfaces. Once a biologically active slime is established on surfaces, the rate of biological reaction is greatly accelerated.

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