ON CHANGES TAKING PLACE IN SEA WATER DURING STORAGE

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(Text-fig. 1)

Whipple reported in 1901 that when tap water was filled into glass bottles, the number of bacteria fell during the first 3–6 hr. by 10–25 %, and later increased by many hundred per cent, with a reduction in number of species. This rise in bacterial numbers was several times greater in small than in large bottles, and was reduced or even nullified if the water was kept agitated. A similar rise in bacterial numbers takes place when sea water is stored in glass vessels. Waksman & Carey (1935) found that multiplication took place in Seitz and in colloid-filtered water which had been inoculated with raw water, and from the oxygen used calculated that the rapid growth of bacteria breaks down about one-third of the organic matter in solution. Zobell & Anderson (1936) and Lloyd (1937), found a much greater increase in numbers of bacteria when the water was stored in small than in larger bottles, or in bottles where the water-glass surface area had been increased by filling the bottle with glass beads or rods. By observing the numbers at close intervals of time, Miss Lloyd found that the increase followed the course of a population curve; the peaks were determined by the volume of the container, but were not affected by the surface area of the water exposed to the air. Zobell & Anderson found that the peaks, or maximum number of bacteria found in the water, showed a rough direct proportion to the volume/surface area of the bottles, the maxima in small vessels being about twice the maxima in vessels ten times as large. They also observed that when the surface area was increased by a shallow layer of glass beads or silica grains, the resulting increase in numbers of bacteria was less than that calculated from the volume/area ratio. They concluded that not only this ratio but also the proximity of the main body of water to the glass surface played a part. The greatest increases were found in water between sand grains where populations of some twelve million bacteria per c.c. developed in water which maintained no more than a few hundred bacteria per c.c. in the sea.

Whipple had found that the marked difference between maximum bacterial population, which arose when tap water was stored in small and in large bottles, was much reduced if a small quantity of peptone (5 mg./l.) was added to the water. Zobell & Anderson noted that if nitrite and 10 mg./l. of peptone were added to sea water, there was a greater and more rapid loss of nitrite

in smaller than in larger vessels, but no such difference when 100 mg./l. of peptone was added. It appears that the volume effect only occurs when bacteria develop in water containing food substances at very great dilution. This conclusion was also reached by Heukelekian & Heller (1940) who found no growth of the bacterium *Escherichia coli* in solutions containing 0·5 and 2·5 mg. of glucose and peptone, but growth did occur if glass beads were added. With 25 mg./l. growth did take place, and with concentrations greater than this the effect of adding glass beads faded out.

As Whipple had found for tap water, Zobell and Anderson found a reduction in number of species when sea water was stored. Some twenty-five to thirty-five species were generally found immediately after the water had been collected, falling to nine or ten species by the time bacterial numbers had reached a maximum and to no more than four or five species after the maximum population had declined. After this decline the population remained relatively high for a long period, the numbers fluctuating from a few thousand to over a hundred thousand per c.c.—a sample of sea water which had been stored at 2–6° C. for 4 years was found to contain 209,000 bacteria per c.c.

In Zobell and Anderson's investigation a series of experiments was made dealing with the effect of oxygen on the proliferation of bacteria in stored sea waters. No material effect was observed unless the water was less than 50 % saturated with air. Waksman & Carey, on the other hand, observed greater growth in fully aerated than in partially aerated water.

The oxygen content of sea water stored at 16° C. in glass-stoppered bottles of different capacities after 20 days, and the maximal bacterial population reached (after 3–5 days) in similar bottles. The water initially contained 3·46 c.c. O_2 per litre and 231 bacteria per c.c.

Volume of sea water (c.c.)	10	100	1000	10,000
O_2 per litre	2·59	2·90	3·68	4·17
Bacteria per c.c.	1,475,000	1,080,000	673,000	382,000

Zobell, 1936. *Proc. Soc. Exp. Biol.*, Vol. xxxv, p. 271.

Both Zobell & Anderson (1936) and Waksman & Renn (1936) observed that in full and stoppered bottles the consumption of oxygen continued undiminished for some time after the bacteria in the water had reached maximum numbers and while the population was falling. The former investigators have shown that great numbers of bacteria develop on the glass surfaces; one experiment indicated that within 24 hr. more than twice as many were attached to the surface of the glass as were in the water. This accounts for the continued consumption of oxygen after the number of bacteria in suspension have declined.

The proliferation of bacteria when water is enclosed in glass vessels and the effect of their size is attributed by Zobell & Anderson to the water-glass surfaces:

(i) Providing a resting place for periphytic bacteria, many marine species having periphytic tendencies and at least some being obligate periphytes. In this connexion it is pertinent that saprophytic bacteria are attached to sus-

pended particles in the sea (Lloyd, 1930) and that bacteria are most numerous where plankton is most abundant, as observed by Waksman, Reuzer *et al.* (1933) who consider that 'bacteria exist only to a very limited extent in the free water of the sea, but are largely attached to the plankton organisms'.

(ii) Concentrating organic substances from very dilute solution on the surfaces owing to adsorption or other physical attraction.

(iii) Causing the diffusion of bacterial enzymes away from the cell, where it is attached to a solid surface, to be retarded; it has been generally observed that attachment to particles exerts a favourable influence on their enzymatic activity.

The second suggestion—that organic matter in solution is adsorbed on solid surfaces—is of particular interest. The authors state that the accumulation of a film of organic matter on glass slides soon after being submerged in sea water can be demonstrated by differential stains as well as by microchemical technique. Stark *et al.* (1938) also state that an accumulation of organic matter can be detected on glass slides which have been immersed for several hours in lake water. Their method of detection was based on the oxidation of a sulphuric acid-dichromate mixture. In neither of these communications is the exact technique described. The writer has been unable to obtain definite results on these lines, but a number of observations have been made which point to adsorption taking place.

It was noticed that when offshore water was kept in glass tubes a gelatinous ring of bacteria slowly developed at the meniscus, becoming apparent after five months (Harvey, 1925). This suggested that if their growth was due to local concentration of organic matter by adsorption, the latter took place to a greater extent at the meniscus, where potent physical forces come into operation, than on other parts of the glass surface. Experiments were therefore made to test this possibility.

If a clean glass or silica tube of about 1 mm. bore is dipped into sea water, the water rises in the tube and the meniscus takes up a position at a definite height above the surface, this height corresponding to a surface tension of approximately 72 dynes/cm. When the tube is either lowered or raised, the meniscus returns to this same height; it moves freely both up and down the tube, provided the glass is clean and moist. However, if a trace of various organic substances is dissolved in the water, such as 5 mg./l. of peptone or casein, it is otherwise. The water rises in the tube to approximately the same height as before, but if allowed to remain undisturbed for a few minutes and the tube is then lowered, the meniscus is lowered and is flattened owing to the lesser pull by the column of water below; finally the meniscus breaks free and rises again to the same height as before. If the water in the tube is covered with ether or benzene and allowed to stand, on lowering the tube the water-ether meniscus is lowered and becomes flatter, while the ether-air meniscus keeps its shape. It appears that a ring of organic matter is adsorbed on the glass where the meniscus has stood and this prevents water moving

readily up the tube past the ring, but does not hinder the movement of water down the tube past the ring. Using more concentrated solutions, a series of rings were formed where the meniscus had stood in the tube for a few minutes, each ring acting as a valve allowing water to pass freely downwards but hindering its passage in the reverse direction. The contact angle of water advancing past the ring is considerable, while the angle when the water retreats remains at or near zero.

At somewhat higher concentrations, such as 50 mg. of sodium caseinate per litre, the solution rose in a clean tube to almost the same height as sea water without this addition, but when allowed to stand the column of liquid gradually fell. It appears that the water is repelled by the ring of adsorbed protein; a similar gradual fall took place when the column of liquid in the tube was covered with benzene.

The addition of 0·2 mg./l. of sodium caseinate, or less, could be detected by filling a short column into a clean capillary tube and allowing this to rest horizontally for a few minutes, after which a tilt of several degrees was required to start the column of liquid moving, whereas similar tubes with similar columns of filtered sea water required a tilt of *circa* one-twentieth of a degree. Using this method of detecting adsorbable substances in a liquid, the following experiment was made. A shrimp was ground with water, filtered, and the filtrate added to filtered sea water in the proportion of extract from 0·2 g. wet weight of shrimp per litre of water. A quantity of glass-wool, previously cleaned with concentrated sulphuric acid, washed and dried, was added to a part of this liquid. After standing for half an hour, a column about 1 cm. long was drawn into a clean glass capillary and moved to and fro in order to wet the glass surface. This capillary, and also one filled in the same way with a similar column of the liquid which had not been in contact with the glass-wool, were adjusted on a horizontal plate, so that the columns of liquid lay in the central parts of the tubes which were placed alongside each other. The plate was then slowly tilted and the angle of tilt at which each column started to move was noted. The plate was again levelled and a fresh pair of readings obtained:

Liquid ex glass-wool required	2° 36′	0° 39′	1° 18′	1° 3½′ tilt
Liquid not treated required	5° 12′	3° 24′	3° 24′	>3½°

On repetition with a fresh pair of capillaries:

Liquid ex glass-wool required	0° 26′	1° 18′	0° 39′	1° 44′ tilt
Liquid not treated required	2° 10′	5° 12′	5° 12′	7°

Two hours later, that is, after the liquid had stood 2½ hr. in contact with the glass-wool, fresh tubes were prepared and gave results as follows:

Liquid ex glass-wool required	0° 13′	0° 13′	0° 26′	0° 26′	0° 13′ tilt
Liquid not treated required	0° 52′	2° 10′	2° 23′	0° 39′	0° 52′

This shows a marked falling off in the capability of the liquid which had stood in a glass vessel, and of that which had been in contact with glass-wool, to form a ring. A similar effect has been found in other experiments.

These experiments are interpreted as indicating that organic matter is adsorbed on a glass-water surface, the most rapid adsorption taking place where there is a glass-water-air or a glass-water-benzene or ether interface forming a meniscus. There is no indication of how much of the added organic substance is so adsorbed; it is not known to what particular substances the ring effect is due, but experiment suggests that it is not due to traces of oily impurities.

An attempt was made to evaluate the quantity of organic matter which was adsorbed from very dilute solution, such as 10 mg. of peptone per l., on a large surface exposed by glass-wool, about 50 sq. cm./c.c. of liquid. The most delicate method found for estimating the change, due to contact with glass-wool, was the quantity of dilute alkaline permanganate oxidized in 24 hr. at 30° C. by the liquid, the excess of permanganate being estimated by titrating the iodine set free on adding potassium iodide and a buffered mixture of sulphuric and boric acid. By acidifying with such a mixture, the trace of chlorine set free on acidifying permanganate in sea water was certainly reduced, if not eliminated; consistent triplicate titrations were obtained. The results of these experiments showed that only a small proportion of the oxidizable organic matter was adsorbed, possibly no more than the experimental error. The values obtained in the different experiments ranged from 0 to 7 % less in the liquid which had been in contact with glass-wool than in the control. Only a minute fraction of the added organic matter would be required to give a monomolecular layer; on the other hand, layers many molecules thick are known to build up on solid surfaces (Blodgett, 1935). Whether the presence of a monomolecular layer of nutrient on a surface would provide a sufficient local concentration of food to allow bacteria to grow, assuming it was rapidly renewed after being used, is not obvious.

It is an outstanding question why offshore sea water, which contains sufficient nutriment for the production of several million bacteria per c.c., and will in fact rapidly produce this population when in contact with clean sand grains, normally supports a population of no more than 10–200 bacteria per c.c. In addition to lack of solid surfaces, protozoa and other animals keep the bacterial fauna eaten down; this has been stressed by both Zobell and by Waksman & Hotchkiss (1937). The former investigator (1936) has also concluded that natural sea water contains a bacteriophage, or heat-labile substances inimicable to the growth of bacteria; added bacteria grew more rapidly in autoclaved than in Berkefeld filtered sea water.

Changes brought about by bacteria developing in stored sea water are the setting free of ammonia, phosphate and carbon dioxide, the interconversion of ammonia, nitrite and nitrate, and the utilization of oxygen.

The ammonia may either increase or decrease (Keys *et al.* 1935). When plankton is added to the water and it is stored in the dark, there is an increase in ammonia which later decreases as nitrite is formed; later nitrite decreases with an increase in nitrate (Von Brand *et al.* 1937). In a great number of

cases no change has been found in the nitrate and nitrite content of water from the open sea during storage, even if enriched with ammonia, unless it was obtained close to the bottom or bottom deposit had been added to it (Harvey, 1926; Cooper, 1937). It appears that nitrite-forming and nitrite-oxidizing bacteria are not usually present in offshore water, free from plankton, unless collected close to the bottom.

The phosphate in solution increases slowly during storage, the increase being sometimes preceded by a decrease.

These changes are brought about by bacteria; however, there is evidence that both the setting free of phosphate and changes in the nitrogen cycle may take place to some extent without their aid. Kreps (1934) found that changes in ammonium and nitrate took place in inshore water which had

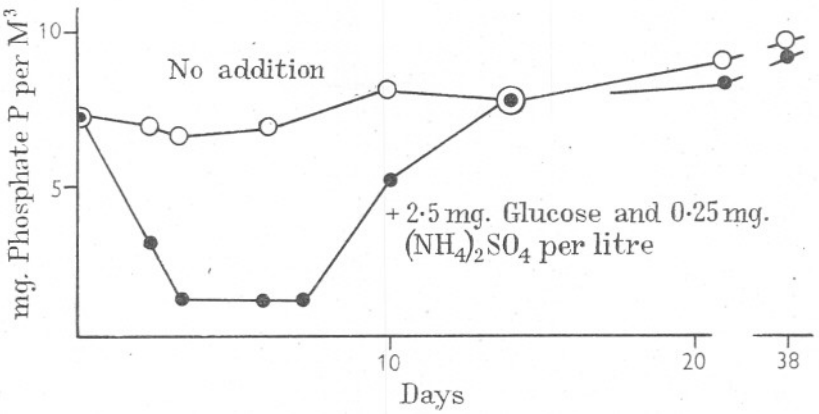

Fig. 1. Changes in phosphate concentration during storage in a sample of sea water, filtered through Whatman No. 3 paper. The lower curve shows the effect of increased bacterial growth due to the addition of phosphorus-free nutrient.

passed a Seitz filter or been poisoned with mercuric chloride, and suggested that sea water, particularly water near the bottom where organic matter was decomposing, contains enzymes which cause these changes. Keys *et al.* have also noted changes in ammonium content of water which had been sterilized with mercuric chloride, while Newcombe & Brust (1940) have noted that saturating water with chloroform reduces but does not stop phosphate being set free during storage.

Several investigations concerning the bacterial decomposition of organic matter added to sea water throw light on changes taking place during storage. Waksman & Carey (1935) have come to the following conclusions. Bacterial growth which occurs in clear water from the open sea consumes a similar quantity of oxygen as that brought about by the addition of $2\frac{1}{2}$ mg. of glucose per litre, and there are sufficient nitrogen compounds available for double the growth of bacteria which normally occurs when such water is stored. They

found sufficient available phosphorus or nearly sufficient in the waters they used for the requirements of the bacteria; adding phosphate had no effect or only slight effect upon their growth. On the other hand, Keys *et al.* have noted instances where the addition of phosphate has increased the oxygen consumption of stored waters. Renn (1937) found that when glucose was added to sea water the resultant growth of bacteria assimilated significant quantities of phosphate, and that this was soon regenerated following their death. This accounts for the decrease in phosphate which sometimes precedes an increase when sea water is stored. The writer has made similar experiments, adding glucose and an ammonium salt in order to provide ample organic matter and nitrogen for bacterial growth, and in some experiments enriching the water with phosphate. The results showed rapid and complete, or almost complete, regeneration of the phosphorus which the bacteria had utilized—that is, dephosphorulation of their body substances during autolytic breakdown. Previous experiments had shown that bacteria in a sample of sea water rapidly set free phosphate from nucleic acid and from casein in solution, but not from glycerophosphate, suggesting that the phosphatase enzymes of the bacteria in these samples of water could only deal with certain types of organic compounds.

The breakdown of organic phosphorus compounds and setting free of orthophosphate is of particular interest. Russell (1935, 1936) has shown a close correlation between the maximum concentration of phosphate which has been found in the water of the English Channel off Plymouth during winter and the subsequent abundance of young fish. Indeed, the fluctuations in maximum phosphate and in abundance of animal life have now followed each other rather closely for a number of years in this area. Redfield *et al.* (1937) have succeeded in showing by chemical analysis that there is a considerable accumulation of organic phosphorus in solution in the water of the Gulf of Maine during the summer, nearly all breaking down to phosphate during the ensuing winter. However, in an experiment by the writer when summer sea water was filtered and stored, no such quantity of phosphate was set free during succeeding months as would be expected to take place if the water had remained in the sea with a bacterial population at least several hundred times less. Some observations by Cooper (1935) point in the same direction; two out of four samples of sea water which had only been freed from larger plankton organisms showed little change in phosphate content when stored for several months, yet, where animal plankton had been added to this water, not only was all the phosphorus added in this form regenerated as phosphate but a considerable quantity in excess. He concluded that this excess was set free from dissolved organic compounds present in the sea water. It seems reasonable to surmise that some species of bacteria grow on the relatively rich food provided by the added animal plankton, which do not proliferate when plankton-free sea water is stored, and that these are able to dephosphorulate a greater variety of organic compounds.

The observations which have been discussed suggest a problem which

awaits, and would repay, solution. Russell's correlation has shown the value of a knowledge of the winter phosphate maximum of the water occupying an area, when studying the fluctuations in its total fauna from year to year; the same probably holds when studying differences between two bodies of water, due account being taken of such physical differences as the extent of vertical mixing. This winter maximum reflects the biological history and future of the water mass. The concentration of a conservative constituent such as chloride (salinity) reflects its physical past. To obtain the winter maximum requires the use of a ship over the period when the maximum is likely to occur, and exact colorimetric comparisons within a day or two of the samples being collected. The analysis of total organic and inorganic phosphorus, as made successfully by Redfield *et al.*, would require a team of workers to deal with the large number of samples necessary for a survey. Hence it would be particularly useful to reproduce in vitro the changes from organic phosphorus into ortho-phosphate which take place in the sea, presumably due, or mostly due, to the agency of bacteria, and which become apparent during autumn and winter when phosphate is not used as quickly as it is formed.

Summary

The rapid growth of bacteria when sea water is stored in glass vessels, and the possibility that this is brought about by the concentration of food by adsorption on the solid surface, are discussed. Some evidence is presented bearing upon such adsorption.

The regeneration of phosphate from dissolved organic phosphorus compounds in the sea and in stored water is considered.

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