

ESTIMATION OF WATER POLLUTION BY A BIOLOGICAL REACTION.

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(With Plate II, 1 Figure in the Text, and 7 Charts.)

It is well known that certain motile Protozoa and bacteria tend to form aggregations near the surface film of water owing to a tropism to an optimum oxygen tension. The reaction to be described is based on this fact and concerns *Polytoma uvella* Ehr. and certain bacteria associated with it in culture. *Polytoma* is an encapsulated flagellate which commonly appears in macerations and is structurally closely allied to *Chlamydomonas* but is without chlorophyll and lives as a saprophyte. It has the habit of fixing itself temporarily by its flagellar end when in a suitable position. The associated bacteria are, firstly a *Spirillum* and, secondly, several species of motile bacteria of which one has been provisionally identified as *Bacillus terminalis* Migula.

The reaction is carried out in tubes of 4 mm. bore. One-tenth of a c.c. of the culture is placed in the tube by means of a pipette provided with a long glass bead which is shrunk on about a centimetre from the orifice, a device which provides against fouling the sides of the tube. In a few moments an aggregation of the aerotactic organisms can be seen as a well-defined layer lying a little below the meniscus, its depth varying with the activity of the culture. A column of water is then run gently on to the inoculum to a depth of 40 mm., the tubes being graduated at the two necessary points. An ordinary fine pipette is used and the teat is so manipulated that when rather more water than is required is drawn up the teat is fully distended. The tube is then held almost horizontally, surface tension keeping the inoculum in place, and the pipette is advanced with its tip pressed to the glass and a little water held in the angle thus formed. When the inoculum is reached the water in the angle flows on to it and forms a cushion at the slightest pressure on the teat. The tube can then be held upright and the rest of the water added boldly. Inoculum and water now lie distinct and for several days there is little mixing by diffusion. The aggregation, moving without any disintegration, climbs and lies like a white blanket at the junction of the fluids. It then proceeds to find its new stable zone in the added water and, provided the blanket keeps its continuity, this lies a few millimetres below the top of the column. The rate of progress up the tube bears a close relationship to the oxygen content of the added water.

The aggregation and its upward movement is the result of the action of

two forces. Firstly there is the action of gravity, for if the organisms were not negatively geotropic they could not find the surface quickly in water such as crude sewage which is practically devoid of oxygen in solution. This force is simply demonstrated. Two tubes are filled with a suspension of the culture in water deficient in oxygen, one being kept upright and the other inverted. The blanket forms densely in the upright tube about 4 mm. below the meniscus and includes most of the *Polytoma* in the suspension, but in the inverted tube most of the *Polytoma* pass to the closed tip forming there a loose cloudy aggregation; a feeble blanket forms at a greater distance from the meniscus than in the upright tube and may contain some *Polytoma* but is mostly bacterial. In most of the tests the ratio of the depths of the blankets is as 2 : 1, but this ratio is not constant and can be varied with the concentration of the suspension. The greater depth from the meniscus of the feeble blanket in the inverted tube is thus due to the fact that fewer organisms compose it and so less oxygen is consumed. If the tubes are now reversed the dense blanket fades in a few moments and the feeble one becomes intense and establishes itself nearer to the meniscus. Each feature seen before in the one is now copied in the other.

The second force is the tropism to an oxygen concentration less than that which is stable with the atmosphere, as Fox (1921) demonstrated with *Bodo*. In the slowly climbing blanket these forces almost balance one another. The organisms consuming oxygen in front of them, the negative geotropism forces them gently up till they reach that position where consumption of oxygen just equals the amount taken up by the water. Then the two forces are exactly balanced. This position in tap water lies 4–7 mm. below the meniscus, given of course a sufficiency of organisms to keep the blanket intact. If the wastage on the climb is such that a hole forms in the blanket then oxygen can pass through and the aggregation comes to rest or may begin to descend again.

When this reaction was encountered it was thought that it might have more than theoretical interest. The ordinary methods of estimating the quality of sewage effluents are of a chemical nature and admittedly empirical. They are carried out under highly artificial conditions and lump together inert oxidisable substances and organisms. They disregard the fact that there are in bacteria bed effluents, organisms which are of a beneficial nature and will continue to improve the water if it is kept exposed to air and light. A biological test which takes account of these factors should therefore be of use, and it is with this in view that the preliminary study of the reaction has been made.

The idea of employing aerotactic organisms as oxygen indicators is not novel. Engelmann (1881) employed motile bacilli to demonstrate photosynthesis. He suggested the similar use of certain Protozoa and foresaw a breadth of application for the method not yet realised. Fox (1921) used *Bodo* to demonstrate the respiratory areas in *Chironomus* larvae, and Thorpe (1930) adopted the same method for another insect larva. These studies were carried out in thin films under the microscope.

METHOD OF CULTURE.

The strain of *Polytoma* employed was obtained by aerating the humus from the final settlement tanks of the Leeds Corporation Sewage Works. An initial selection of the aerotactic organisms was made by means of the reaction described, the climbing aggregation in superimposed water being drawn off with a pipette for subculture. This contained *Polytoma* dominating, but also *Bodo*, *Oikomonas* and *Pleuronema* in some numbers. *Polytoma* was isolated from the other Protozoa by culture in hanging drops. In this connection it may be mentioned that *Polytoma* is not readily centrifuged down in unaerated sterile water, as it fixes itself to the tube sides with great power. In water saturated with oxygen it goes to the bottom of a tube of its own accord or may be centrifuged down with great ease. In this way it may be washed, but it has not yet been obtained free from bacteria and it is therefore not known whether it will thrive in their absence.

The medium is composed of equal parts of the humus from the final settlement tanks of the sewage works and bacteria bed effluent. The mixture is placed in tubes of 1 in. diameter and digested and partially sterilised at 80° C. for 2 hours. The humus then coagulates and can be shaken down, the supernatant fluid being left limpid and not readily miscible with water, a point essential to the proper working of the reaction. If the mixture is raised to boiling-point the humus flocculates and does not settle readily. The medium is inoculated with 1 c.c. from a previous culture and the tubes are kept unplugged at room temperature in beakers covered with glass. *Polytoma* multiplies rapidly till a density of a million or more to the c.c. is reached, the bacterial population being of the order of 800 million per c.c. The culture is best for use when about 5–8 days old, becoming sluggish later.

Behaviour of the aggregations.

The aggregation consists of a layer of the motile bacteria (*vide infra*), thickened and made more stable by the *Polytoma* which swim to and fro in the zone or adhere to the glass in its neighbourhood. *Polytoma* is so dense in the zone that under the low power of the microscope the impression is gained that it is the sole inhabitant. A good climbing blanket is about 0.5 mm. in thickness. The reaction is read partly by the rate of climb, the junction of the fluids being zero, and partly by the appearance of the blanket. Two or three tubes are set up for each water examined and the mean of the readings is taken. Standing tap water is included in every series as control.

As regards experimental error, readings taken after an hour's climb agree to 1 mm. in the vast majority. Among 380 pairs read after 3–6 hours' climb 50 per cent. are identical, 35 per cent. differ by 1 mm., 10 per cent. by 2 mm., 4 per cent. by 3 mm. and 1 per cent. by 4 mm. Readings of 456 pairs after 24 hours' climb gave 35 per cent. identical, 35 per cent. differing by 1 mm.,

17 per cent. by 2 mm., 6 per cent. by 3 mm., 3 per cent. by 4 mm. and 4 per cent. by more than 4 mm.

The chief experimental error arises in sampling the culture. The organisms being somewhat zoned in this it is difficult to draw off a number of exactly equivalent samples. If the amount required for a series is drawn off and mixed repeatedly as the tubes are inoculated an error arises through increasing aeration. The best method appears to be to draw off rather more than is required and run it into a shallow dish to form a layer 1–2 mm. deep. The small samples required can then be fairly drawn from this. About 10 min. should elapse between inoculating the tubes and adding the water to allow the aggregations to form. The series should then be dealt with in the order in which they have been inoculated.

Experimental series have been set up in tubes of bores varying from 2.9 to 8.2 mm. In the narrower ones there is difficulty in placing an equal amount of culture in each, and with the wider ones there is a greater tendency to upward diffusion of the inoculum. The size first selected of 4 mm. bore gave least experimental error.

Agreement in appearance of the aggregations between members of a pair is remarkable, but unfortunately does not lend itself to exact definition. The following conventions are adopted: *B*, the blanket retains much of its initial density; *b*, the blanket has lost at least half its initial density; *c* is a cloud-like aggregation; *bc* is intermediate between the last two: a circle round one of the last three symbols indicates that it is just visible; *M* is an aggregation in contact with the meniscus; *R* indicates heavy and *r* slight roping.

Influence of oxygen on rate of climb.

Oxygen in solution in water was estimated by the convenient Miller test as modified by J. T. Thompson (1927), the amount being recorded as parts by weight per 100,000 water. Tap water was deoxygenated by boiling and another sample was saturated with the gas. Intermediate conditions were obtained by mixing the samples. Chart I shows the nature of the climb in various concentrations thus obtained.

In standing tap water the blanket moves upwards steadily without losing much in density and reaches the stable zone, about 4 mm. below the meniscus, in a few days. In tap water deficient in oxygen it moves at first more rapidly with some fading but slows down as the water re-absorbs oxygen. With excess of oxygen it retires in proportion into the depths of the inoculum and may appear to be lying on the bottom as though the organisms were stunned.

In highly polluted water such as crude sewage the blanket breaks up and the individuals reach the meniscus in less than an hour and rest there for a time with ropes falling. The whole column of water may appear to be boiling gently with the activity of these ropes. In other cases the gathering at the meniscus may be more gentle, each *Polytoma* taking its place on the glass as

it arrives till a more or less symmetrical formation develops like an inverted crown. The method of gathering is related to the strength of the sewage. Later the aggregation retires a little from the meniscus and again in the depth adopted there is relationship to strength.

Between the extremes of steady climb and hurry to the top all variations have been observed and correlated with the amount of oxygen in solution as shown by the Thompson-Miller test. Especially there is an important phase in which the blanket breaks up and for some hours no trace of aggregation is to be found. The organisms re-gather within 24 hours in the upper half of the tube and the distance of the zones from the meniscus in a series of sewage dilutions of from 5 to 50 per cent. is correlated with the oxygen in solution in

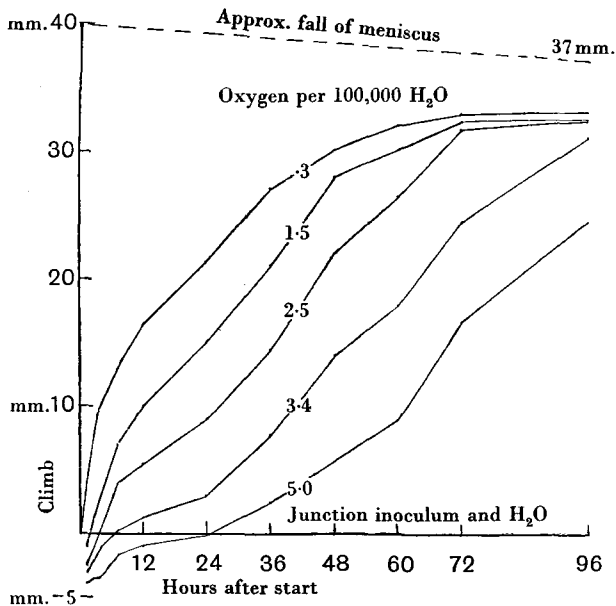


Chart I. Illustrating the influence of oxygen in solution in tap water on the rate of climb of *Polytoma* blanket. Each curve represents the average climb in three tubes.

the dilutions after standing 24 hours. It seems probable that from this a factorial expression of the strength of a sewage can be devised.

Chart II is obtained by a direct count of *Polytoma* in a series of sewage dilutions after 24 hours' climb. Each point represents the actual number counted in a 2 mm. field along a vertical line. It indicates the dense aggregations obtained in tap water and highly polluted water (20 per cent. sewage) and the more scattered aggregations formed in intermediate conditions, in which, however, the graded condition is very clear.

When under the influence of an excess or deficiency of oxygen *Polytoma* develops a different appearance to that in normal culture. The organism contains much starch which is ordinarily scattered in the cell but may be more aggregated before or behind the central nucleus. If the organisms are examined

from the meniscus of crude sewage it is found that the starch is concentrated behind the nucleus in most individuals. In those at the bottom of water with excess of oxygen the starch in nearly all is concentrated at the anterior flagellar

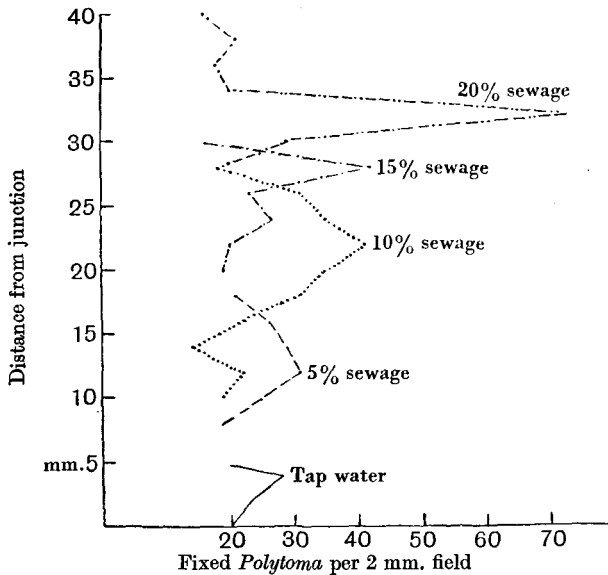


Chart II. Illustrating the distribution of *Polytoma* individuals after 24 hours' climb in tap water and water contaminated with increasing amounts of sewage. The lower parts of the curves are omitted for simplicity. The peak of the tap water curve is low because a larger proportion of *Polytoma* are swimming in the zone.

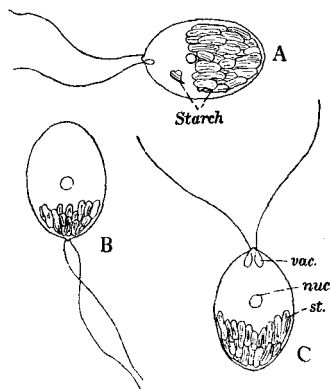
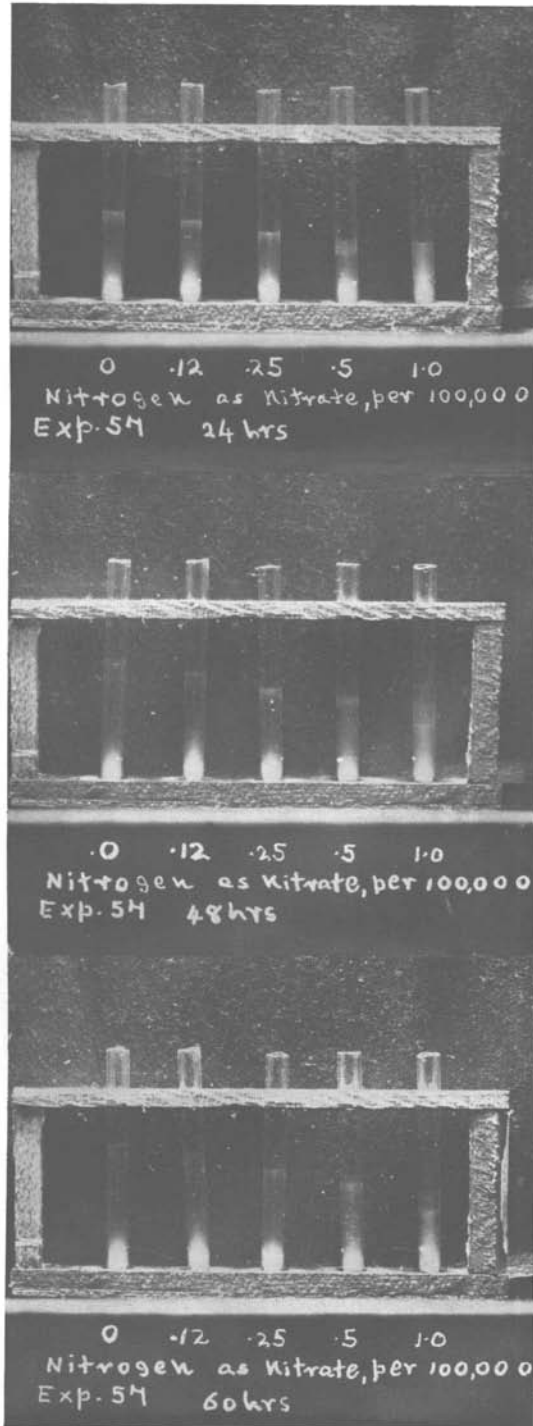


Fig. 1. *Polytoma wella* in dilute iodine solution, showing variable distribution of starch. A, from culture, starch somewhat scattered; B, from water with excess of oxygen, in most individuals starch collects anteriorly; C, from deoxygenated water, tendency of starch to collect posteriorly. (Semi-diagrammatic, $\times 600$.)

end (see Fig. 1). By transferring the organisms from one extreme to another the starch can be made to move backwards or forwards. It is to be supposed that this starch movement changes the centre of gravity of the organisms and



Showing the appearance of the *Polytoma* blanket and the lag produced in its climb by the presence of small amounts of nitrate.

so orientates them towards the surface or downwards as the case may be. If this is so it explains the ease with which they keep to a definite zone even when not attached to the glass. This point will be dealt with in detail elsewhere.

Influence of nitrate and nitrite on rate of climb.

When the reaction is tested with the effluents from the bacteria beds it is found that with those of high quality, although the oxygen in solution is perhaps 10–20 per cent. less than that of standing tap water, the climb of the blanket is slower. Secondly, in nearly every sample of bed effluent tested, after the blanket has climbed some distance, a second and more feeble aggregation separates from the inoculum and follows the first at a distance

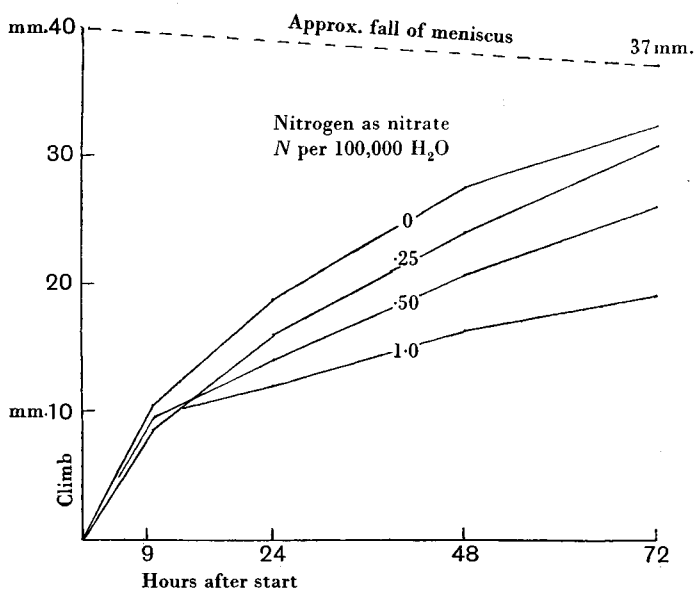


Chart III. Illustrating the lag in climb of the *Polytoma* blanket when nitrate is added to tap water. Each curve represents the average climb in three tubes.

up the tube. In these details the tubes of a pair mirror one another to the utmost detail.

These effects may be produced in tap water by adding nitrates or nitrites as the potassium salts in such quantities as occur in the effluents. Chart III and Plate II illustrate one of the experiments in which nitrogen as nitrate was added in varying amounts to tap water and a proportionate lag was produced in the climb just as with increased oxygen there is a proportionate lag. There is an important difference however, since with increased oxygen the lag is most marked at the beginning but with added nitrate there is usually no lag at first. It develops about the ninth to the twelfth hour and intensifies up to a point as time goes on. Seven similar series to the one illustrated have been set up and in six cases the lag was delayed but in the seventh the nitrate

lag showed itself from the beginning. Three similar series have been set up with nitrogen added as nitrite and in each the lag developed at the beginning of the reaction.

As regards the secondary blankets produced by nitrates there is some variation with the activity of the culture. In an active culture all the *Polytoma* leave the inoculum with the first aggregation, the second one being at first bacterial but collecting *Polytoma* as it climbs from the wastage of the first blanket. In a very sluggish culture *Polytoma* is concerned in the formation of the second blanket from its start, a portion only having joined the upper aggregation.

It is believed that the effect of the nitrate or nitrite on *Polytoma* is through bacterial metabolism, oxygen being set free and made available. This possibility recalls the existence of aerobic organisms at the bottom of deep lakes where no oxygen in solution can be detected. Cole (1921) suggested the decay of plant tissues as being a possible source of atomic oxygen in these positions.

Other factors and rate of climb.

Light and darkness have no effect on the reaction. It works smoothly at all temperatures from freezing-point to 32° C. Most of the experiments have been carried out between 10 and 15° C., and within these limits variation in rate of climb due to temperature is slight.

A possible effect of varying hydrogen-ion concentration was looked for. A range of tap waters was adjusted from pH 3.8 to 9.0. Three series of tests were made with these and no variation in rate of climb was found.

Common salt, ammonium carbonate and ammonium chloride added to tap water in reasonable proportions have no effect on the climbing. Starch, cane sugar and peptone have likewise no effect directly but on the second day exert influence through the development of bacteria depriving the water of oxygen and the blanket hurries to the meniscus in consequence. Added egg albumen had no effect.

Grading of polluted waters.

A tentative attempt has been made to grade polluted waters by means of this reaction. Two tubes of each water examined and two control tubes of standing tap water are set up. Readings of the position and appearance of the blankets are made at one and four hours respectively and recorded in a graphic manner (Charts IV-VI). The progress of the blanket in each tube is represented by a column with bars showing the position of the aggregations at the readings and symbols indicating condition are added. Phases of disappearance are represented by broken lines. The tentative grade and the chemical analyses of the waters are shown above the columns. The grades are as follows:

- I. Climb slower than in tap water; highest quality.
- II. Climb at the same rate as in tap water; good quality.

Exp. 25. 26. i. 32. Culture m. Temp. 11-12° C.

	Parts per 100,000					
N as nitrates	0.68	0.72	0.62	0.58	0.24	—
O abs. 4 hours 80° F.	2.18	2.90	2.42	2.34	3.46	15.0
O in solution	1.15	1.10	0.65	0.85	0.40	0

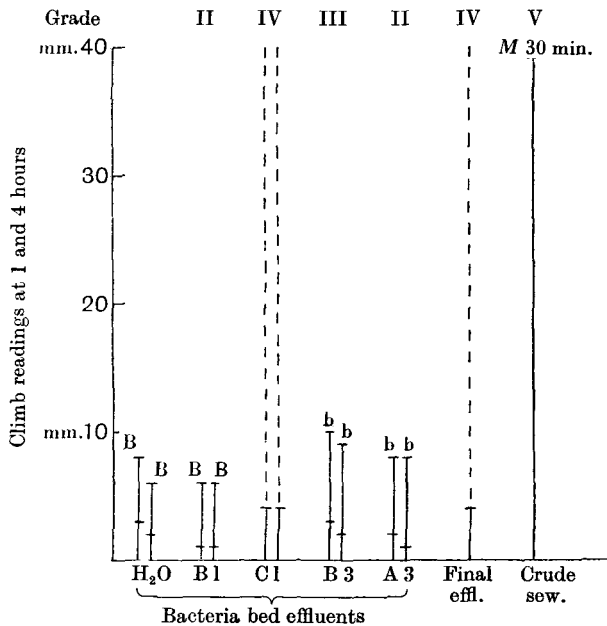


Chart IV. Contrasting a grading of polluted waters by the *Polytoma* reaction and analysis. Each column represents one tube. The lower bar is the position after 1 hour and the upper after 4 hours.

Exp. 44. 9. ii. 32. Culture Vi. Temp. 15° C.

	Parts per 100,000						
N as nitrates	0.18	0.22	0.20	0.26	0.36	0.28	0.24
O abs. 4 hours 80° F.	3.53	3.30	2.82	3.38	3.53	3.30	3.14
O in solution	1.2	0.9	1.0	0.95	0.9	0.9	0.25

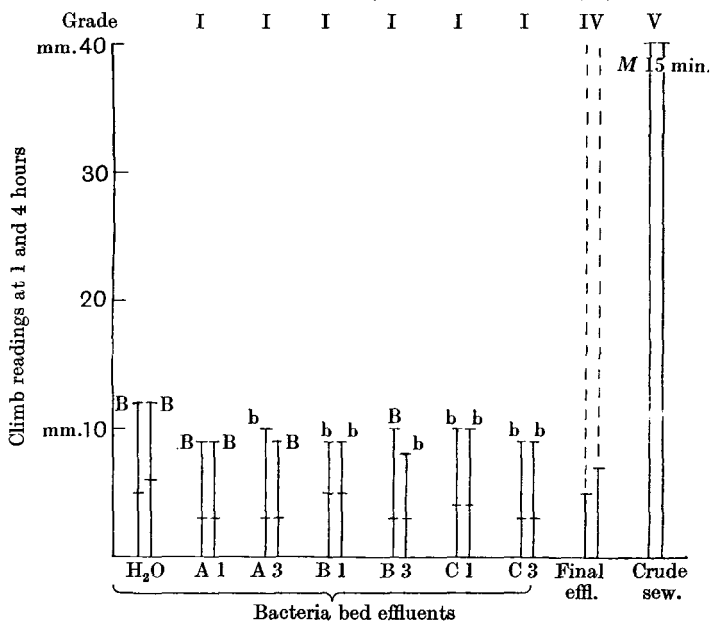


Chart V. Contrasting a grading of polluted waters by the *Polytoma* reaction and analysis. Each column represents one tube. The lower bar is the position after 1 hour and the upper after 4 hours.

- III. Climb more rapid than in tap water, the blanket persisting though faded; moderate quality.
- IV. Blanket disperses but does not re-gather at the meniscus in four hours; poor quality.
- V. Blanket disperses and re-gathers at the meniscus within 1 hour; bad quality.

Re-gathering of the aggregation between 1 and 4 hours has not been observed. It is a later phase, between 12 and 24 hours after the start. Grade V is very

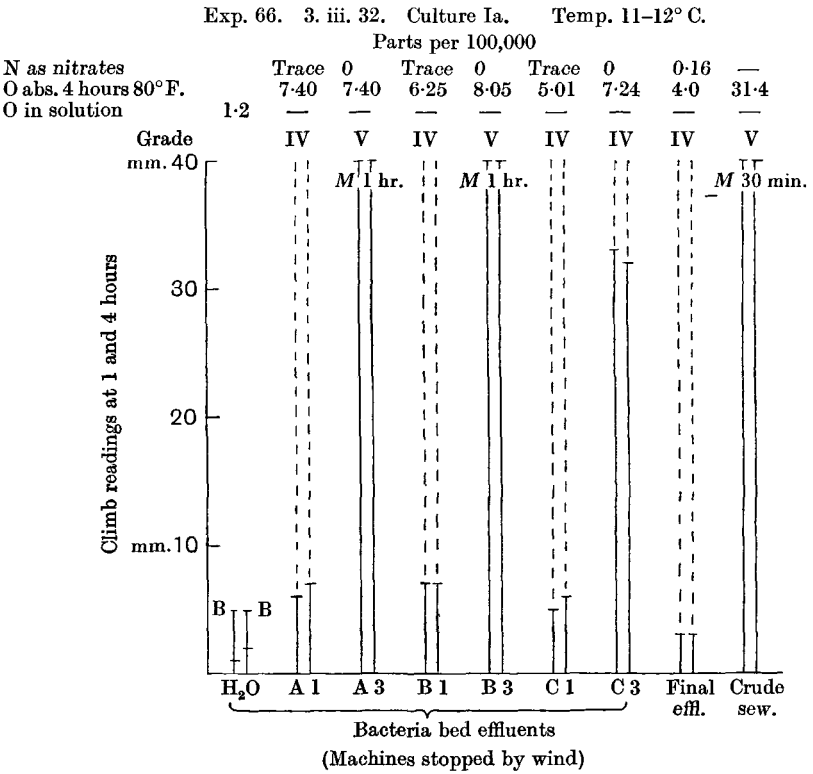


Chart VI. Contrasting a grading of polluted waters by the *Polytoma* reaction and analysis. Each column represents one tube. The lower bar is the position after 1 hour and the upper after 4 hours.

wide and can be subdivided by means of serial dilution of the polluted water but further study is necessary before the subdivisions of this grade are defined.

Twenty-two series of sewages and effluents involving 113 samples have been examined under this scheme. Three of the series are shown in the charts. The findings agree quite well with the results of the chemical analyses and the effect of the nitrate lag is often to be seen. It is not a delayed lag such as obtains in most cases when nitrate is added to tap water. It is more like the condition obtained with added nitrite but only minute traces of nitrite are said to be detectable in the Leeds sewage effluents.

The manner in which the biological reaction may have an advantage over the chemical tests for quality is shown in the following experiments (Chart VII). Old manure water which had sweetened through biological means and had become heavily infested with *Euglena viridis* and other photosynthetic organisms was examined by the *Polytoma* reaction and by chemical means on a dull day and on a bright day. No nitrates were detected; the Thompson-Miller test on the dull day gave a very low reading ($0 = 0.25/100,000$) and on

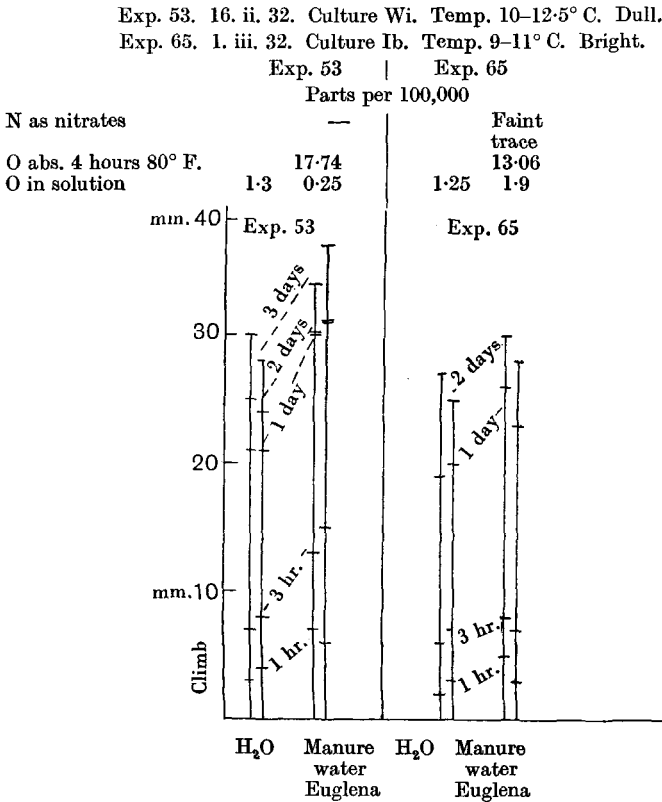


Chart VII. Showing that the *Polytoma* reaction takes cognisance of the presence of photosynthetic organisms in water but the oxygen absorption test does not indicate their benefit. Each column represents one tube.

the bright day a very high one ($0 = 1.9/100,000$); the oxygen absorption test, 4 hours at 80° F., indicated that it had the quality of a crude sewage, 17.74 parts of oxygen per 100,000 being absorbed on the dull day and 13.06 on the bright day. The *Polytoma* reaction on the bright day classed it in Grade II and on the dull day in Grade III. The case is of course deliberately made extreme but the photosynthetic organisms are present in bacteria bed effluents and are not allowed to make their presence felt in the chemical analysis as they do pronouncedly with the biological test.

Bacteria in the reaction.

It was soon realised that motile bacteria played an important part in the blanket formation. Through the first 50 subcultures *Spirillum* was the prominent member of this group, being present in vast numbers and an important element of the blanket. It then disappeared for reasons not yet known but the aggregations still retained the same appearance and behaved in the same manner. By making a fresh start *Spirillum* was re-introduced but did not again become dominant. During a brief period spent at Leeds Dr R. A. Q. O'Meara kindly gave some attention to the bacteriology of the culture. He was unable in the brief attempts made to isolate *Spirillum* which he found thrive in company but died out when isolated. He did isolate five organisms, identifying one provisionally as *B. terminalis* Migula. This and two of the others subcultured in nutrient broth gave very clear climbing blankets and differential rises in oxygen-adjusted tap waters, but the phases are slightly different to those obtained with the *Polytoma* culture and on the third day of climb results become anomalous owing to wastage on the climb and diffusion of the broth up the column. Another of these organisms climbed as a well-defined cloud with differential results. The fifth would not leave the inoculum in the tubes. It would be a waste of time to discuss these further at present. The experience with them indicates that the somewhat haphazard mixture of organisms with which the preliminary investigation of this phenomenon has been carried out may be bettered by one or other of the aerotactic bacteria if ever the reaction comes into use as a standard test.

CONCLUSION.

The writer would have been unable to carry the investigation of the reaction to this point without the assistance and advice given by Mr J. T. Thompson, Manager of the Leeds Corporation Sewage Works, and Mr A. Watson, Chemist at these works. He is grateful to Dr O'Meara for his interest in the reaction and to Dr F. C. Happold for providing subcultures of the bacteria isolated.

SUMMARY.

A small quantity of a culture of aerotactic organisms, especially *Polytoma uvella*, is placed in a narrow tube and a column of water superimposed. The organisms aggregate and rise in blanket-like formation at a speed which is inversely proportional to the amount of oxygen in solution. If nitrates or nitrites are present they slow down the rate of climb. The climb must be accelerated by the presence of substances and organisms with a pronounced affinity for oxygen and slowed down by the presence of photosynthetic organisms. The reaction may therefore be of use in estimating the quality of sewage effluents or pollution in rivers, having possibly some advantages over the present routine methods of chemical estimation. A tentative grading scheme for the quality of such waters is proposed.

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