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THE EFFECT OF ACIDITY ON THE DEVELOPMENT OF THE NITRIFYING ORGANISMS.[1]

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FOR nearly two decades, both lay and scientific minds have been constantly perturbed by frequent announcements of the discovery of some new microbe that is seeking the destruction of ourselves or of our domestic animals. We have been warned to be on the alert for these deadly foes in the food that we eat, in the water that we drink, and in the air that we breathe. This general alarm has caused us to overlook many of the other important discoveries in the world of microscopic organisms. Indeed, the rapid development of our knowledge of the disease-producing organisms has been accompanied by an equally important advance in our knowledge of that multitude of microbes that are not only our friends, but are necessary to our existence. It is to one group of these more friendly organisms that we wish to ask attention. Passing over a host of species that are of importance in various agricultural industries, including those organisms that enable the farmer to draw upon the uncombined nitrogen of the atmosphere for an increase of his available nitrogenous plant food, we desire to consider the group of organisms engaged in the final stages of the process of transforming the nitrogen of dead animal and

[1] Read before the Washington Section of the American Chemical Society, April 9, 1896.

vegetable matter into an inorganic form suitable for the nourishment of the higher forms of plant life. It will be remembered that before the nitrogen of the fallen leaf or of a scrap of meat can be readily assimilated by the higher plants, it must undergo three stages of preparation by as many sets of organisms: first, the process of "ammonization," in which nitrogenous organic matter is decomposed, yielding as final products water, carbon dioxide and ammonia; second, the process of "nitrozation," in which the nitrous ferments oxidize the ammonia to nitrous acid; third, the process of "nitration," in which the nitric ferments oxidize the nitrous to nitric acid. The organisms of the first class, the ordinary putrefactive ferments, have been known since the work of Schwann and Schultze, reported in 1839, and have been made familiar acquaintances by the more recent work of Pasteur and others. More than thirty species of bacteria and twenty species of molds and yeasts were isolated from the soil and studied in regard to their ammonizing power in 1893 by Èmile Marchale, a Belgian investigator, the detailed results of whose experiments one of us (Ewell) has had the pleasure of placing before American readers in the form of a translation published in *Agricultural Science* about a year ago. Although our knowledge of this class of organisms has been greatly enlarged, their study from the standpoint of the practical agriculturist is still a very promising field of investigation.

The two remaining stages of the process, in which the ammonia formed by the putrefactive ferments is changed to nitric acid, constitute the process of nitrification, a term that is often used to include all three of the above transformations. Until very recently this was generally supposed to be a purely chemical phenomenon and quite independent of the action of living organisms. Pasteur, reasoning from analogy, had expressed the opinion that the process was dependent upon the activity of a living ferment, but it was not until the investigations of Schloesing and Müntz in 1877 that the dependence of the phenomenon upon living organisms was given experimental proof. Warington, in England, at once repeated these experiments and obtained results that left no doubt as to the nature of the process.

The existence of a nitrifying ferment having thus been demonstrated, the contest that at once began for the honor of its isolation was such a one as has rarely been seen in the scientific world. Warington and the Franklands, in England ; Heraeus, Frank, Hueppe, Celli and Zucco, on the continent of Europe ; Jordan and Richards, in America ; all of these were prominent in this strife. As late as 1887, Frank asserted that the failure of the numerous attempts to isolate the ferment should be interpreted as evidence of its non-existence. For thirteen years the contest continued. The efforts of Warington, of the Franklands, and of Jordan and Richards, were just beginning to lead to tangible results, when, in 1890, a new worker appeared on the field in the person of Winogradsky, a Russian, working at Zurich. While many facts had been learned and many important observations had been made by the previous investigators, it was left for the worker last named to effect the first thoroughly satisfactory isolations of the nitrifying bacteria and to study them in pure cultures. These results were made possible by his finding a solid culture medium upon which these organisms grow well. Hosts of species had been isolated from the soil, from the air, and from natural waters by growth upon various solid media made by use of gelatine or agar-agar, but none of them possessed nitrifying power. Hence it had been concluded that the nitrifying ferment was unable to grow in these media, which were abandoned for the tedious dilution method. Winogradsky's medium was composed of gelatinous silica and nutrient salts. While its preparation is somewhat difficult and it apparently offers a pabulum for the development of a much greater number of microbian species than was at first supposed, nevertheless, it is the only medium available for the satisfactory isolation of the nitrous organisms. It is possible that this medium is also necessary for the isolation of some species of the nitric organism, but Burri and Stutzer[1] have recently studied a nitric ferment that thrives on ordinary peptone-gelatine. It is quite possible that these are the first workers that have ever given an organism growing upon peptone-gelatine an opportunity to grow in an inorganic solution containing nitrites ; *i. e.*, in such con-

[1] Centralblatt f. Bakterologie u. Parasitenkunde, 1895, 1, 721-740.

ditions as would determine its ability to change nitrites into nitrates. As the demonstration of the inability of the nitrous ferment to grow upon ordinary peptone-gelatine preceded the discovery that nitrification is accomplished in two stages and by the action of two distinct ferments, it seems to have been taken for granted that the nitric organism is also incapable of growing upon the organic jelly.

We have treated the history of this subject somewhat at length, yet very briefly considering the extent of its literature. This has been done in order that what is to follow may be better understood. In the light of the researches and discoveries just outlined, the farmer must regard his fields as immense bacterial cultures. He must study their needs as well as the needs of the cultures of the higher plants whose conditions of growth he has been studying so long. In order that he may expect prompt, certain and remunerative results from the use of fertilizers containing organic nitrogen, he must make sure that his soil contains ammonizing and nitrifying organisms of the highest grade of activity, and must establish the closest possible approximation to those conditions that enable these organisms to render the greatest service in their respective roles: the conditions must be such that the process of ammonization proceeds with the smallest possible loss of nitrogen from the volatilization of the ammonia formed, or from the formation of uncombined nitrogen instead of ammonia; on the other hand, the conditions should be as unfavorable as possible for the activity of the organisms of denitrification, which reduce nitrates with the liberation of uncombined nitrogen.

Bacteria are very sensitive to the conditions under which they are grown; not only do changes in these conditions alter the rate and nature of their growth, but they also change the quantity and quality of the products formed during this growth. The principal conditions that have been found to greatly influence the growth of bacteria, are amount and quality of food, supply of moisture, the proportion of oxygen in the surrounding atmosphere, temperature, the degree of acidity or alkalinity of the medium in which they are grown, the presence of substances having a retarding or accelerating action upon their growth, and the presence of rival or helpful species. Outside of the regions where irrigation is practiced, degrees of moisture and temperature are necessarily dependent upon meteorological conditions. In the case of the nitrifying ferments, increase of the aëration of the soil by thorough stirring has been shown to be very favorable to their highest activity. The most favorable quantity and quality of the various nutritive substances and of the other materials forming the soil, as well as the influence of the presence of other bacterial species, are still questions needing further investigation.

The proportion of alkali or acid in excess in the soil and in other media for the growth of bacteria has been shown to be a matter of the first importance. It is so constant a factor that it deserves consideration separately from the class in which it would logically fall, that of the substances having a retarding or accelerating action upon bacterial growth. The influence of the reaction of the medium upon the growth of bacteria has been very prettily demonstrated by various persons engaged in the quantitative determination of the bacteria in water. At the convention of bacteriologists, held in New York in June, 1895, George W. Fuller, of the Lawrence Experiment Station of the Massachusetts State Board of Health, presented a very interesting series of results upon this subject. We have taken the liberty to use an abridgement of one of Mr. Fuller's tables to illustrate the importance of this question.

Reaction. Cc. of normal alkali required to render one liter of the medium neutral to phenolphthalein.	Relative number of colonies per cc of water appearing upon plate cultures.		
	Sewage.	Merrimac River water.	Lawrence City filtered water.
40	6	1	2
35	16	3	3
30	45	4	8
25	64	55	46
20	100	100	100
15	106	89	92
10	101	54	60
5	98	46	43
0	86	38	35
—5	87	26	31
—10	82	21	26
—15	55	9	15
—20	48	3	8
—25	56	1	7

The marked effect of slight changes in the reaction of the medium is rendered very apparent by this table. The most favorable reaction is one that is slightly alkaline to litmus, but still requires ten to twenty cc. of normal alkali solution per liter to make it neutral to phenolphthalein. When the acidity is either greater or less than this amount, there is a rapid falling off in the relative numbers of colonies formed.

While it was known for many years before the discovery of the nitrifying organisms, that the presence of some base is necessary to the activity of nitrification, there is very little known in regard to the exact degree of variation that can take place in the reaction of the materials undergoing nitrification without causing an interruption of the process. In the cultivation of the nitrifying bacteria in artificial media, it has been customary to add some carbonate that will neutralize the nitric acid as fast as it is formed. For this purpose, the carbonates of calcium and magnesium have been much more used because they are without influence upon the reaction of the medium until acid has been formed to decompose them. Warington[1] has made some experiments to determine the proportions of sodium carbonate and bicarbonate that can be used for this purpose. He reports that sodium bicarbonate can be used in the proportion of one to four grams per liter, that six grams per liter retards the activity of the ferments, and that eight grams per liter stops it entirely. The use of sodium carbonate was not attended with as good results, since one gram per liter was found sufficient to greatly retard the vigor of nitrification. This is practically all that is recorded in regard to the effect of acidity and alkalinity upon these organisms, except some statements in regard to nitrification in peaty and other soils sufficiently rich in humus to be acid in reaction. It has been reported that some soils of this type contain no nitrates whatever while in place.[2]

There are several ways of testing the effect of acidity upon the nitrifying bacteria. The soil itself may serve as a medium without sterilization, or it may be used after sterilization, the seedings being made from pure cultures of the nitrifying organ-

1 *J. Chem. Soc.*, *1891*, 59, 529.
2 Chuard, *Compt. rend.*, 114, 181-184.

isms or from mixed cultures of the soil bacteria; artificial media may be seeded from pure cultures of the nitrifying organisms, from mixed cultures of the soil organisms, or with a small portion of the soil to be studied. Each of these methods has its advantages and uses. In any case, the supply of ammonia must be maintained until the process of nitrification is arrested by the acidity of the medium. When the soil is used as a medium or as the inoculating material, the experiment becomes a test of both soil and organisms, as the acid formed can have no retarding action until all of the readily salifiable base of the soil has been satisfied. When the mixed organisms of the soil are used in media containing organic matter, acid may be produced by other organisms besides the nitrifying bacteria.

As we had some forty samples of soil at our disposal during the last year for other purposes, it seemed wise to improve the opportunity to test the influence of acidity on the nitrifying organisms contained in soils from various parts of the country. The medium selected for this purpose had the following composition:

Ammonium sulphate	0.943 gram.
Dipotassium hydrogen phosphate	1.0 "
Magnesium sulphate	0.5 "
Calcium chloride	trace
Water	1000.0 cc.

One hundred cc. of this solution were used for each test. Before the addition of the ammonium salt, one liter of this medium requires two and six-tenths cc. of normal solution of sodium hydroxide to make it neutral to phenolphthalein. The titration was made without the ammonium salt, as the indicator is not applicable in its presence. As for every equivalent of nitric acid formed during the nitrification of this solution, an equivalent of free sulphuric acid is liberated; the nitrification of fourteen parts per million of nitrogen causes an acidity of the medium equal to two cc. of normal alkali per liter.

A summary of the results of forty-four tests is given in the following table. Twenty-two virgin soils and twenty-two cultivated soils are represented, coming from twenty-two states and territories. The tests were continued for two months and the figures recorded indicate the maximum number of parts per million of nitrogen nitrified during that time. In five cases no nitrification occurred; in four of these cases no nitrification occurred in the parallel tests made with the same soils and with the same medium to which calcium carbonate was added to neutralize the acid formed. In all but three of the tests the final product was entirely nitrate, showing that the nitric ferment was able to endure at least as much acidity as the nitrous ferment. In the three tests in which the nitrification was incomplete, there was only a small portion of the nitrite remaining unchanged to nitrate.

SUMMARY OF RESULTS.

In	5	cases	0	parts	per	million	of	nitrogen	were	nitrified.
"	1	case	11	"	"	"	"	"	"	"
"	2	cases	14	"	"	"	"	"	"	"
"	4	"	15	"	"	"	"	"	"	"
"	5	"	18	"	"	"	"	"	"	"
"	10	"	20	"	"	"	"	"	"	"
"	5	"	22	"	"	"	"	"	"	"
"	6	"	25	"	"	"	"	"	"	"
"	1	case	28	"	"	"	"	"	"	"
"	1	"	40	"	"	"	"	"	"	"
"	1	"	42	"	"	"	"	"	"	"
"	1	"	70	"	"	"	"	"	"	"
"	1	"	130	"	"	"	"	"	"	"
"	1	"	170	"	"	"	"	"	"	"

Averages:

Of	44	tests	28	parts	per	million	of	nitrogen	were	nitrified.
"	34	"	20	"	"	"	"	"	"	",

excluding five cases in which no nitrification occurred and five cases in which it was forty parts per million or more.

Remembering the numbers given above, we notice that the nitrification has stopped after the formation of an acidity equal to three to four cc. normal alkali, or when the reaction reaches five and a half to six and a half of Mr. Fuller's scale. It is quite possible that this extreme sensitiveness to acidity may account for the failure of attempts to grow these organisms upon ordinary peptone jelly.

In the case of the two peaty soils from the muck lands of

Florida, the results were twenty and twenty-two parts of nitrogen nitrified per million, which are very close approximations to the mean result of all the tests. The soils giving the excessive results of 130 and 170 parts per million were from Alabama, and examination has shown that they are both very rich in calcium carbonate. This, of course, explains the high results obtained.

We have "stock cultures" of the nitrifying organisms of all of these soils and hope to be able to report a repetition of these experiments with pure cultures, thus eliminating possible sources of error that may have resulted from the presence of the basic substances of the soils used as inoculating material in the series just reported. It is also desirable to determine the relative effects of various organic and inorganic acids upon the nitrifying organisms. This can only be satisfactorily accomplished by use of pure cultures. In regard to the effect of acidity upon the nitric ferments, but very little is known, except that, as we have observed above. they are not more sensitive to acidity than the nitrous ferments. The practice seems to have been to add an insoluble carbonate to the liquid media used for the growth of nitric organisms also, but we have found that this is unnecessary. The nitric organisms from all of the soils with which we have experimented, thrive well in a medium having the composition of the one given above in which sodium nitrite takes the place of the ammonium salt. There is, of course, no increase in the acidity of the medium during the growth of this ferment, as it merely changes nitrites to nitrates.

The organisms coming from various parts of the country seem to be very uniform in regard to their ability to endure acidity. If these results are again obtained when the tests are repeated with the pure organisms isolated from the different soils, the interpretation to be given to them is a very important one : these results are to be looked upon as evidence that we are to seek practical results in the study of the nitrifying organisms, not from a search for a peculiarly active species, but from a search for those conditions that are most favorable to the activity of these organisms in any given set of soil and climatic conditions.

These conclusions gain strength from the results of the recent experiments of Burri and Stutzer,[1] in which they found that the mixed organisms from several samples of soil from widely separated sources assumed an almost constant nitrifying power after a series of cultivations in an artificial medium. Whether their results are to be interpreted as an evidence of constant nitrifying power in organisms from widely different soils when these organisms are cultivated in the same medium, or whether they are to be regarded only as another example of the extreme variability that bacterial species have so often been observed to exhibit, is only to be determined by extended experiments. Of high value for the confirmation of these, or of any other results obtained by meansof artificial culture medium, is the re-inoculation of several soils with each of the organisms studied, noting the results during long periods of time. Both sterilized and unsterilized soils should be used for this purpose, as well as soils in which the chemical and biological conditions have been variously modified by artificial means.

We feel that the contribution that we have just made to the knowledge of this subject is almost lost in the vast unknown of the field that still remains to be explored, but this is offered as a note of the work that has been undertaken by us of attempting to make a comparative study of the microorganisms important to agriculture in typical soils from all parts of the United States. This note is not presented with any desire to preempt this field of study, for it is broad enough to monopolize the time and skill of many workers for many years to come.

STANDARD PRISMS IN WATER ANALYSIS AND THE VALUATION OF COLOR IN POTABLE WATERS.

BY ALBERT R. LEEDS.

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IN one of the first papers read before this Society and contained in its Proceedings, Vol. II, p. 1, for 1878, I have given an account of an instrument and a method for reading the quantities of ammonia obtained in nesslerizing, the instrument being termed a color comparator. It was designed primarily for this purpose only, and had its origin in the irregularities observable

1 *Centralblatt f. bakteriologie u. Parisitenkunde*, 1896, 2, 105–116.