in the midst a wedge-shaped elevation. The cheeks are in front separated by a furrow running from the front of the glabella to the edge-furrow.

The tail-shields are much more vaulted. This is especially the case with the rhachis, which broadens towards the back and stretches nearly as far as the edge. Consequently, the lateral parts of the pygidium, which are already narrow, become even more so towards the back part. They are not separated by a furrow, as it is the case with those of the head-shields. The pygidia have at the back-edge on either side a little cog pointing backward. The rhachis of the pygidia is clearly divided into three parts. The back-part is the largest by far and is particularly swollen. The lateral furrows of one side do not meet those of the other, as they are separated by a wedge-shaped elevation passing on from the second part to the first and ending towards the back in a blunt point slanting upward.

From the properties mentioned it may be easily seen why this kind of Agnostus was described by TULLBERG [1]) as Agnostus pisiformis L. var. socialis. Pictures of it have been given by BRÖGGER [2]) and POMPECKI [3]).

Up to this time this erratic-block is the only piece of alum-slate with Agnostus pisiformis L. var. socialis that was found in our diluvial grounds. In Germany they also seem to be very rare. Only GOTTSCHE [4]) mentions such a piece from SCHULAU. This one also contains, however, remains of Olenus truncatus Brünn. As firm rock such alum-slate with this variety of trilobites occurs in Sweden (Oeland and Bornholm included), in different places, as I learned from Prof. MOBERG, to whom I showed a piece of the erratic-block.

Microbiology. — *"Accumulation experiments with denitrifying bacteria"*. By G. VAN ITERSON JR. (Communicated by Prof. M. W. BEIJERINCK).

The great signification of the denitrifying bacteria for the circulation of nitrogen in organic life and the important biochemisms to which they give rise, make the study of these organisms very attractive.

[1]) TULLBERG, Om Agnostus-arterna i de Kambriska aflagringarne vid Andrarum. Sveriges geologiska Undersökning. Ser. C. N° 42 pag. 25.

[2]) BRÖGGER, Die Silurischen Etagen 2 und 3 im Kristianiagebiet und auf Eker. Pag. 56. Taf. 1. fig. 10 a b c.

[3]) POMPECKI, Die Trilobiten-Fauna der Ost- und Westpreussischen Diluvialgeschiebe. Beiträge zur Naturkunde Preussens herausgegeben von der Physikalisch Oekonomischen Gesellschaft zu Königsberg. Pag. 15, Taf. IV, fig. 24 a b.

[4]) GOTTSCHE, Die Sedimentär-Geschiebe der Provinz Schleswig-Holstein, pag. 11.

In the first place it was necessary to subject their distribution in nature and their isolation to an investigation, because the literature thereon offers but very deficient data. The best way to attain this object seemed to try whether the method of "accumulation" gave in this case, as in so many others, any definite result, and that for the following reasons.

The character of this way of experimenting is the cause, that many biological properties of the species there by accumulated may be predicted;

it renders it possible, in a simple way, directly and with certainty to isolate from nature a determined species; this is of special interest inasmuch the cultures of most bacteria, by being kept in the laboratoria, change their character to such a degree as to become irrecognisable, so, that the descriptions, found in bacteriological literature, according as they are made after newly isolated or long kept material, may be wholly different;

it teaches us to recognise the sought- for species in the different varieties occurring in the material used for infection, as these varieties are bound to corresponding culture conditions;

the identification and synonymy of the bacteria, which are always extremely difficult, even in case we possess good descriptions, made of freshly isolated cultures, are much facilitated by good "accumulation experiments";

these may, moreover, be controlled by anyone, and render the investigator independent from material isolated by others.

For the arrangement of my experiments I have followed the example given by Dr. H. H. GRAN [1]) of Bergen in his researches in the Bacteriological Laboratory at Delft on denitrifying sea bacteria.

By exclusively using nitrate as source of nitrogen in the culture liquid, which was contained in a cotton-plugged flask, so that the air could freely enter, he succeeded to restrict considerably the number of developing species of bacteria, when taking fresh sea-water for infection, bringing the denitrifying species to vigorous growth. He furthermore selected, as source of carbon the calciumsalts of organic acids, by which the prejudicial alkaline reaction, which appears in bouillon in consequence of the decomposition of the alkalinitrate, was avoided. Mostly calciummalate was used, which is a very good bacterial food, and has moreover the advantage of solving only to 0,8 % at 25° C., so that it can be added to an excess, whence, as the salt is oxidised, a new quantity is solved.

[1]) Studien über Meeresbacterien I, Bergens Museums Aarbog 1901 N°. 10.

After 2 or 3 successive inoculations in the same liquid a constant bacterial mixture was obtained.

I tried to apply these principles to the isolation of denitrifying land-bacteria, and so-doing I succeeded indeed, when using calcium-tartrate as source of carbon, to accumulate *Bacillus vulpinus*, hereafter to be discussed.

It proved however to be a fundamental improvement wholly or partly to exclude the access of air as thereby the growth of the denitrifying bacteria is not in the least impeded, whilst a number of other aerobic bacteria are very much hindered in their development.

Of the numerous methods of culture under exclusion of air I have followed the simplest, namely the "bottle method", long since in use in the Bacteriological Laboratory at Delft for the examination of the sulphate reduction by microbes and the lactic-acid fermentation. For my experiments this method proved perfectly adapted, as the quantity of air which finds access, can thereby easily be regulated. An ordinary, narrow-mouthed stoppered bottle, with an exactly fitting stop, is quite or partly filled with the culture liquid, and after sterilising or not, according to circumstances, the bottle is placed in the thermostat for culture.

1. *Historical.*

The reduction of nitrates by bacteria constantly begins with the formation of nitrite. This may be further converted in five different ways, viz.:

1st. It may be reduced to ammonia.

2nd. It may be converted into unknown, nonvolatile nitrogen compounds.

3rd. If in the liquid acid is formed simultaneously, it may give rise to the development of nitrogen-oxygen compounds.

4th. It may be decomposed in alkaline solution under formation of nitrogen-oxygen compounds.

5th. The nitrite may, in alkaline solutions, give rise to the development of nitrogen without the production of nitrogen-oxygen compounds. This is *denitrification proper*, of which here is only question.

Already in 1814 DAVY [1]) states that during putrefaction of animal matter nitrogen as such is freed. "Here it is again seen," says in 1860 G. J. MULDER [2]), from whom I borrow this particular, "if one wishes

[1]) Elemente der Agriculturchemie, Berlin 1814, S. 309.

[2]) De Scheikunde der Bouwbare Aarde, 1860, dl. 3, blz. 58.

truly to give the cuique suum in this part of science, one often must retrograde half a century."

Not before 1856 the problem was again taken into research. In that year REISET [1]) pointed out, that at the putrefaction of dung and flesh free nitrogen is produced. Later investigators have not been able to observe free nitrogen under these circumstances, in asmuch as no nitrate or nitrite are present, but the putrefaction of albuminous matter as such has still remained an open question from this point of view.

It was PELOUZE [2]), who in 1857, for the first time, with certainty stated the disappearance of nitrate during the putrefaction of animal matter.

BOUSSINGAULT [3]) observed in 1858 the disappearance of salt-peter in the soil. He ascribed it "à une cause purement accidentelle, à une action reductrice, exercée par de la matière végétale morte".

From the year 1873 date very interesting observations of SCHLOESING [4]) on nitrification. By studying the influence of oxygen on this process, he was led to the examination of denitrification. He found that nitrification in the soil was still very active, when it was held in a current of gas, which contained but 1,5 % oxygen. If he worked in a current of pure nitrogen, there not only occurred no nitrification, but even the nitrate, originally in the soil, disappeared entirely. He furthermore proved that at this decomposition nitrogen is formed.

Experiments of PASTEUR and the well known investigation of SCHLOESING and MUNZ on nitrification, induced GAYON and DUPETIT [5]) to ascribe denitrification to the action of micro-organisms. In 1882 they communicated their first results and these put the bacterial nature of the process out of all doubt. Their elaborate and excellent researches on this subject were published in 1886 [6]).

Our compatriots GILTAY and ABERSON [7]) isolated, for the first time, in 1892 a denitrifying ferment, and the prescription given by them for the artificial culture liquid has been followed by various later investigators.

The attention of bacteriologists was again fixed on these ferments

---

[1]) Expériences sur la putréfaction et sur la formation des fumiers. C. R. 1856, T. 42, p. 53.

[2]) Remarques de M. PELOUZE. C. R. 1857, T. 44, p. 119.

[3]) Nouvelles observations sur le développement des *hélianthus* soumis à l'action du salpètre donné comme engrais C.R. 1858, T. 47, p. 807.

[4]) Etude sur la nitrification dans les sols, C.R. 1873, T. 77, p. 203.

[5]) Sur la fermentation des nitrates, C.R. 1882, T. 95, p. 644.

[6]) Recherches sur la réduction des nitrates par les infiniments petits. Nancy. 1886.

[7]) Recherches sur un mode de dénitrification et sur le schizomycète qui la produit. Arch. Neerl. T. 25, 1892, p. 341.

by interesting agricultural experiments of P. WAGNER [1]) in 1895, which seemed to point out a danger produced by these bacteria for agriculture.

His experiments gave direct cause to the research of BURRI and STUTZER [2]), who, in the same year, elaborately described two denitrifying bacteria.

From that time this group has been laboriously studied and at present a number of twenty denitrifying species have been described [3]).

To these I for my own part might add some ten species more, but of these I will only discuss those, for which I can point out an accumulation experiment, which gives a constant result.

2. *General considerations.*

The hitherto isolated denitrifying bacteria are all aerobic. In liquids containing nitrate or nitrite, they can, however, grow vigorously with a very slight or without access of air, so that in this case they behave like anaerobic bacteria. They then transfer the oxygen of the nitrate or the nitrite to the organic compounds present in the culture liquid. Thence nitrogen is freed and the metals of the salts pass into carbonates or bi-carbonates, which process may be represented by the formulae:

$$5\,C.. + 4\,KNO_3 + 2\,H_2O = 4\,KHCO_3 + 2\,N_2 + CO_2$$

$$3\,C.. + 4\,KNO_2 + H_2O = 2\,KHCO_3 + K_2CO_3 + 2\,N_2$$

The correctness of this representation has been proved by the observations of GAYON and DUPETIT, GILTAY and ABERSON, PFEIFFER and LEMMERMANN, AMPOLA and ULPIANI, and also by my own researches.

We see from this, that in a liquid, simultaneously with the nitrate, the rate of organic substances decreases, and accordingly also the permaganate number. From a practical point of view this must necessarily be of signification for the explanation of the processes on which is based the biological purification of sewage and water [4]).

My experiments have furthermore convinced me that denitrification is inseparably connected with the growth, for which traces of free oxygen are always necessary.

---

[1]) Die geringe Ausnützung des Stallmiststickstoffs und ihre Ursachen, Landw. Presse, 1895, S. 92.

[2]) Ueber denitrifizierende Bakterien. Centrbl. f. Bakt. Abt. II, Bd. 1, 1895, S. 257.

[3]) O. LEMMERMANN. Kritische Studiën über Denitrifikationsvorgänge. Jena. 1900. C. HÖFLICH. Vergleichende Untersuchungen über die Denitrifikationsbakt. etc. Centrbl. f. Bakt., Abt. II, Bd. 7, 1902, S. 245.

[4]) Dr. JENNY WEYERMAN. Biologische stelsels tot reiniging van rioolvocht, enz. Vragen des Tijds. Febr. 1901, Sep., blz. 38.

In 1897 WEISSENBERG [1]) pronounced the hypothesis, that, at the reduction of nitrate to free nitrogen, nitrite constantly appears as interphase. I can perfectly well share this view, and that for the following reasons:

1st. All denitrifying species which I have studied in the course of this research could, in as much as they produced free nitrogen from nitrate, do the same from nitrite.

2nd. Like BURRI and STUTZER (l.c.) I have been able to isolate a species, which does convert nitrite into free nitrogen, but leaves nitrate intact, so that from a mixture of nitrite with a little nitrate, all the nitrite is removed by this bacterium, whilst the nitrate remains unchanged.

Here I must however observe, that at the conversion of nitrate into free nitrogen, not always nitrite can be detected in the culture. This fact has already been stated by SEWERIN [2]) and KÜNNEMANN [3]). It is however by no means in contradiction with WEISSENBERG's hypothesis, for if the course of the second process: decomposition of nitrite, is quicker, or as quick as the first: reduction of nitrate, the nitrite-phase is no more to be demonstrated.

KÜNNEMANN observed this fact in a variety of *B. stutzeri*, which observation I have been able to confirm; however, in my opinion, the cultural conditions played in this experiment a much more important part than the character of the variety. In bouillon with 0,1 % KNO_3, I could often point out no nitrite, whilst here a strong development of gas took place. On the other hand, I obtained with 4 and 5 % KNO_3 only a slight gas development, but a strong reaction of nitrite.

For the investigation of a colony on its denitrifying power, sterile test-tubes were filled with 10 à 15 Ccm. of bouillon, as well with 0,1 % KNO_3 as with 0,1 % KNO_2 and then inoculated. Denitrifying bacteria grow therein sufficiently, after 24 hours to produce a distinct turbidity, whilst, at the surface they form a scum-layer. Sometimes the scum is wanting, but is produced at shaking the test-tube.

Besides were used solutions of calcium-salts of organic acids, decocts of pease-leaves with 2 % cane-sugar, and decocts of potatoes, likewise with 0,1 % KNO_3 or KNO_2.

In this case a control experiment was made to decide, whether without addition of nitrate or nitrite, these solutions might cause

---

[1]) Studien über Denitrification. Arch. f. Hygiene. 1897, Bd. 30, S. 274.

[2]) Zur Frage über die Zersetzung von Salpetersauren Salzen durch Bakterien. Centrbl. f. Bakt. Abt. II, 1897, Bd. 3, S. 504.

[3]) Ueber denitrifizierende Mikro-organismen. Landw. Versuchs-Stat. 1898, Bd. 50, S. 65.

development of gas, which proved never to be the case with denitrifying bacteria.

In order to obtain perfectly convincing results, to the said culture liquids 10 % gelatin often was added and, when in the boiled solution, at about 30° C., some of the culture had been suspended, it was poured into a test-tube and solidified. The developing gas then remains as bubbles, nearly at the place of its origin, in the gelatin. This method ("tube-culture") produces a sharp reaction on denitrification, especially when controlled by a parallel experiment, using the same culture gelatin, without nitrate or nitrite.

This principle may also be used for a rough computation of the number of denitrifying germs in any material. So it was proved that circa 2000 of these organisms occur in 1 gr. of garden soil, and circa 100 in 1 gr. of canal water.

In these experiments the potassium-salts may be replaced by natrium- or magnesium-salts; calcium-nitrate, on the other hand, prevents even in dilute solution the growth of many bacteria.

Before passing to the description of the different accumulation experiments, I have to make a general remark about their arrangement.

Which species finally becomes most common in the used culture liquid depends on many circumstances, difficult to control, in particular on the mutual numerical proportion of the individuals and the nature of the different species in the material originally used for the infection, and likewise on the condition of the microbes themselves in consequence of previous circumstances.

This explains why, when using different materials of infection for the accumulation of one and the same species, it is sometimes necessary to modify the cultural conditions in accordance with the nature of that material.

I insist on this circumstance in particular to explain the different accumulation experiments described under *B. stutzeri*, on the one hand from water by using tartrate, on the other hand from soil by using malate.

3. *Accumulation of Bacterium stutzeri*, LEHMANN and NEUMANN [1]).

This interesting bacterium was isolated in 1895 from straw by BURRI and STUTZER (l.c.), whilst in 1892 BRÉAL [2]) had already shown the presence of denitrifying bacteria thereon.

---

[1]) LEHMANN u. NEUMANN. Bakteriologie. München 1896, S. 237.

[2]) De la présence dans la paille d'un ferment aérobie, réducteur des nitrates. C.R. 1892, T. 114, p. 681.

In 1898 KÜNNEMANN (l.c.) isolated the same species from soil and a variety from horse-dung and straw.

By accumulation experiments, logically carried out, I have succeeded in obtaining this bacillus from soil, canalwater, sewage-water and horse-dung.

The following experiment always led practically to a pure culture from canalwater:

A bottle of about 200 Ccm. is partly filled with fresh canalwater with addition of 2% calcium-tartrate, 2% KNO_3 and 0,05% K_2HPO_4 [1]), computed after the whole capacity. Then the bottle is filled up to the neck with canalwater and the stop is loosely put in, so that a little water is pressed from the bottle. In this way it is filled without a single bubble of air and, after shaking, put in a thermostat of 25° of 28°. The calcium-tartrate solves at this temperature for only 1%, so that this salt remains for a great part at the bottom.

Commonly already after one day a feeble production of gas is to be observed, issuing from the non-solved calcium-tartrate at this bottom. The process gets into full course after three or four, sometimes only after five days. So much gas thereby is produced that a coarse, slimy scum originates at the surface and a great quantity of the liquid is pressed out of the bottle. The gas containing only nitrogen and carbondioxyd, the culture remains anaerobic. The liquid grows turbid by the growth of the bacteria and the fine, cristalline calcium-tartrate changes into coarsely granular calcium-carbonate. After a week, in consequence of the scum formation, the bottle is nearly half void, and after about 12 days the reaction is at an end, in as much, corresponding with the chosen quantity of tartrate, all nitrate has disappeared.

If a vigorously growing culture is sown on broth gelatin, a mixture is obtained of colonies of various different species, from which *B. stutzeri* can easily be isolated, if we are once acquainted with it.

From such a bottle some drops are inoculated into a bottle of about 50 ccm. capacity [1]), which, after sterilisation, is filled for $^3/_4$ with the following sterile culture liquid:

Tap-water, 2 % calcium-tartrate, 2 % KNO_3 and 0,05 % K_2HPO_4.

After inoculation the bottle is quite filled up with the same liquid in the above described way, and after the lapse of two or three days, the same phenomena appear as in the first bottle.

If now, once more, of this transport a plate culture on broth gelatin is made, the great diminution in the number of species is

[1]) The capacity of the bottle is not indifferent.

surprising. All liquefying colonies, and most fluorescents have disappeared, whereas, among two common and some less frequently occurring species, *B. stutzeri* developes in great numbers and is easily recognised by the characteristic properties of its colonies.

By repeating the said transport this bacterium may still be more multiplied, so that, after three or four successive inoculations, practically a pure culture of this species is obtained.

From soil of the garden of the Bacteriological Laboratory I regularly obtained the same bacterium, in the course of the winter of 1901—2 by applying the "bottle method" with this liquid:

Tap-water, 2 % calcium-malate, 1 % KNO_3 and 0,05 % K_2HPO_4.

In the spring, however, though there were constantly some colonies of the species, the number of its germs proved so small that they were replaced by other denitrifying bacteria, particularly *B. denitrofluorescens*, of which more presently.

A detailed description of *B. stutzeri* is given by BURRI and STUTZER (l. c.), as well as KUNNEMANN (l. c.). It will therefore be sufficient here to give the chief characteristics by which this species is directly recognised.

The bacterium is a short, thick rodlet with a peculiar vibrio-like motion.

The colonies on gelatin are extremely characteristic (see Plate). After three or four days they have a diameter of about 0.5 mm. and after a week they attain 1 to 1.5 mm. When magnified they then resemble a rosette, or have an irregularly folded or crispate, greyish surface. The peculiar structure appears only distinctly, when the glass-dish which contains the plate culture, is reversed and the colony is seen through the bottom with about a 30-fold magnification. The most frequent shapes are represented in figures 1—4.

But it may happen that the crispate character becomes still more conspicuous and then the image is as in fig. 5.

Commonly it seems as if regularly arranged smaller colonies are situated in the larger ones, which may often be observed till in the outer border, and points to a peculiar periodicity of the mucus secretion in the interior of the colony.

In the colonies moreover a fine deposit is observed, and sometimes very distinct crystals, which may also be found in the gelatin around.

All these characteristics are particularly marked when the cultures have been recently isolated, but they may in the course of time get lost or become indistinct. Another property however remains always quite distinct, i.e. the adhering to the gelatin. Young colonies can only be removed in one piece, and of the older always part remains behind.

Fig. 1.

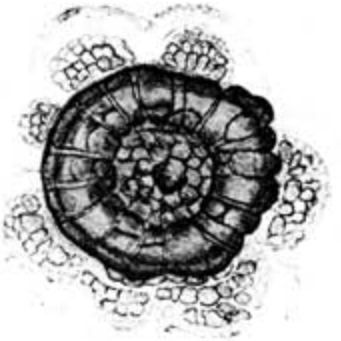

Fig. 2.

Fig. 3.

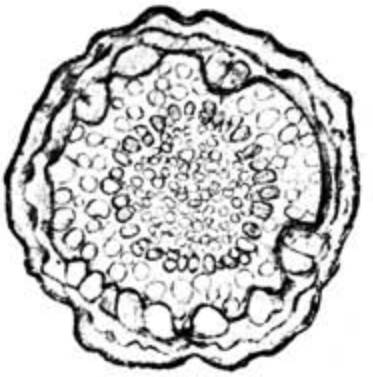

Fig. 4.

Fig. 5.

Very characteristic also is the growth of this bacterium on a sterilised slice of potato, where the curled and folded structure of the colonies is quite distinct, in consequence of the large dimensions they attain. The colour changes thereby into flesh-red. Old cultures grow soft in consequence of a dissolving process the slimy substance.

The compounds which can provide the carbon and nitrogen nutrition of this species were determined by means of the auxanographic method [1]), this giving in a simple way a measure for the difference in assimilability of the nutritive substances.

With KNO_3 as source of nitrogen, a feeble growth was observed with glucose and maltose. Kalium-succinate, malate, malonate, citrate and calcium-tartrate, gave rise to a vigorous growth. No growth was obtained with cane sugur, milk sugar, mannite, galactose and oxalic acid.

The auxanograms prove that tartrate belongs to the best assimilable substances, which explains why its use in the accumulation experiment with canal water produces such good results.

With kalium-citrate as source of carbon, NH_4Cl, KNO_3, KNO_2, asparagin, kalium-asparaginate and pepton, could serve as source of nitrogen.

B. stutzeri produces no invertin, does not split indican and ureum but it secretes diastase, although in very slight quantity. This latter fact explains the possibility of denitrifying by this species in solutions containing, besides the salts, only amylum and KNO_3. In broth no indol and no sulphureted hydrogen are produced.

B. stutzeri produces much alkali; even the presence of glucose does not prevent the production of it in a plate of broth gelatin.

Very remarkable is the behaviour of *B. stutzeri* towards free oxygen.

If the arrangement of the moving individuals under the influence of the oxygen of the air [2]) is examined in the glassroom, we find an accumulation in a line at rather great distance from the meniscus. On the other hand, growth is only observed [3]) in the meniscus itself. Hence, in this respect the bacterium behaves quite in accordance with the aerobic spirilla.

B. stutzeri is a very active denitrifying species; to broth could be added up to 4% KNO_3, and up to 1% KNO_2, without thereby preventing the development of gas. If in the before described way a "tube

[1]) Beyerinck. L'auxanographie ou la méthode de l'hydrodiffusion dans la gélatine appliquée aux recherches microbiologiques. Arch. Neerl. 1889, T. 23 p. 367.

[2]) Engelmann, Zur Biologie der Schizomyceten. Botanische Zeitung 1882, Bd. 40, S. 320.

[3]) Beijerinck, Ueber Atmungsfiguren beweglicher Bakterien. *Centr.bl. f. Bakt.* 1893, Bd. 14, S. 827.

culture" in broth gelatin is made, with 0,1% KNO_3, after two or three days the gas bubbles will appear over the whole length of the tube, and herein this species differs from *B. vulpinus*, where the gas bubbles originate at some distance from the meniscus only.

I will finally make mention of an instructive experiment I performed with *B. stutzeri*. Some garden soil was mixed with tapwater with 0,05 % K_2HPO_4, and a thin layer of this mixture in an ERLENMEYER-flask exposed to a temperature of 25° C. Under these circumstances the production of nitrate becomes very marked after two weeks. If now the whole content of the ERLENMEYER flask is poured into a stoppered bottle, which thereby is quite filled, whilst *B. stutzeri*, is used for infection, soon a development of gas sets in and the nitrate disappears completely. Hence it follows that according as the air enters our culture liquid well or not, nitrification or denitrification may occur. This is quite in accordance with older experiences described by SCHLOESING (l. c.) in regard to the soil in general.

4. *Accumulation of Bacillus denitrofluorescens n. sp.*

SEWERIN (l. c.) found in 1897 that *B. pyocyaneus* belongs to the denitrifying ferments. But the group of fluorescents proper was long fruitlessly examined as to their denitrifying power, first by LEHMANN and NEUMANN and afterwards by WEISSENBERG (l. c.). In 1898 KÜNNEMANN isolated for the first time a denitrifying bacterium, which liquefied gelatin and fluoresced.

Though in my experiments I often obtained fine cultures of a similar species, I did not succeed in finding a satisfactory accumulation experiment for it. On the other hand I found such an experiment for a non-liquefying fluorescent *Bacillus*, which I named *B. denitrofluorescens*.

The culture liquid for the accumulation of this species is:

Tap-water, 2 % calcium-citrate, 1 % KNO_3 and 0,05 % K_2HPO_4.

In a bottle of 50 Ccm. capacity, 1 to 2 gr. fresh garden soil is put; it is then quite filled up with the culture liquid, in the way described under *B. stutzeri*. The culture is made at 25° C.

When sowing on broth gelatin the 2nd or 3rd transport, successively kept in the same culture medium, I always obtained cultures containing almost exclusively colonies of that species.

In horse-dung, canal water and sewage water, I also observed this bacterium, but it is with more certainty to be isolated from soil.

In exterior appearance of the colony this species differs in no respect from one of the most common fluorescents, characterised by

lacking, on the culture gelatin, the smoothly spreading border. In young broth gelatin cultures, the pigment fluoresces blue, and after some time a white precipitate forms in the gelatin.

Examined auxanographically, KNO_3 as source of nitrogen proved to cause a feeble growth with mannite, a vigorous one with kalium-malate, citrate, malonate, succinate and tartrate, as well as with glucose and levulose. On the other hand no growth is seen with cane-sugar maltose, milk-sugar, and raffinose.

In broth, with 2 % glucose, this bacterium, like all fluorescents, produces acid. Broth with 2% cane-sugar, becomes however strongly alkaline, which is observed also in all other fluorescent secreting no invertin.

This bacterium neither produces diastase, nor can it hydrolise indican or ureum. In broth it forms no sulphureted hydrogen and no indol.

In its behaviour towards free oxygen it likewise corresponds with the fluorescents, i.e. with the cover-glass culture in the humid room, both motion and growth cause accumulation in the meniscus.

This makes the bacterium strongly contrast with *B. stutzeri* and *B. vulpinus*, whose motion figures show the spirillum type.

As to the energy of its denitrifying power *B. denitrofluorescens* corresponds with *B. stutzeri*. At the "tube experiment" with broth gelatin with 0,1 % KNO_3, the bubbles form over the whole length of the tube, quite in the same way as with *B. stutzeri*.

5. *Accumulation of Bacillus vulpinus n. sp.*

Already in my introductory observations I remarked, that an accumulation experiment with full access of air, when using tartrate and nitrate, produced this species, but the accumulation obtained in this way was still very imperfect. By cultivating under *partly* exclusion of air, I succeeded in improving the experiment very much. I obtained this result by enclosing in the culture bottle with the liquid a determined volume of air, and reinoculating from bottle to bottle under the same conditions three or more times. It is true that thereby not all other species are totally removed, but this is no obstacle to the recognition of *B. vulpinus*, whose colonies are extremely characteristic, possessing a quite unique brown-red pigment.

The experiment is as follows:

Into a bottle of 50 Ccm. 1 to 2 grams of fresh garden soil is put, and further it is filled up with the following culture liquid, whilst leaving on air bubble of 2 Ccm: Tap-water, 2 % Calcium-tartrate, 0.1 % KNO_2, and 0.05 % K_2HPO_4.

Here likewise the culture is effected at 25°.

With observation of the said proportion and operating as described under *B. stutzeri*, the different varieties of *B. vulpinus* can also be obtained from canal water.

The denitrification sets in very slowly and the development of gas gets not by far the intensity perceived in the preceding species. Here, too, by the complete disappearance of all liquefying bacteria already at the first transport, the isolation of the wished for species is much facilitated. Although at sowing the crude cultures on broth gelatin some *B. vulpinus* colonies may already be perceived, they multiply so much in the transports, that plates therewith prepared, appear, so to say, quite covered with the large, flatly spread, transparent, fox-coloured colonies of this species.

If for the accumulation other organic salts than tartrate are used, or a higher rate of nitrate than 0.2 %, not a single colony of *B. vulpinus* is detected, though it was certainly present in the infection material as it is universally, distributed in the soil.

By their growth the colonies strongly remind of the flatly spread variety of *B. fluorescens non liquefaciens*, but of fluorescence nothing is seen. In shape and motility the bacterium corresponds with *B. stutzeri*.

An interesting property of *B. vulpinus* is, that the brown pigment only develops under the influence of light. If simultaneously two cultures of this species are made on broth gelatin, and one, wrapped in black paper, is put in the dark, and the other in the light, for the rest in equal conditions, a great difference is perceptible. This becomes more obvious still, when making reinoculations or transports of either culture, likewise keeping these respectively in the dark and the light. So-doing a perfectly colourless culture can be obtained, but if this is again inoculated in the light, the brown colour returns. The pigment formation only takes place at growth, so that colonies, full-grown in the dark do not colour when exposed to light.

B. vulpinus belongs to the group of real chromophores [1]), i. e. the pigment is bound to the bacterial body, and the behaviour towards light is, in my opinion, another indication that in this group the pigment has a biological function.

The auxanographic examination proved, that with nitrate for nitrogen nutrition, a feeble growth is obtained with kalium-malonate, a vigorous one with levulose, glucose, maltose, kalium-citrate, succinate

[1]) See Beijerinck, La biologie d'une bactérie pigmentaire. Arch. Néerl. 1892, T. 25, p. 227.

acetate and tartrate, whereas cane-sugar, milk-sugar, mannite and raffinose produce no growth at all. Ammonium-chloride may also serve as source of nitrogen, when using tartrate for carbon nutrition.

Pepton, asparagin, and kalium-asparaginate may simultaneously serve as C and N-nutriment.

The bacterium secretes neither invertin nor diastase and does not split indican or ureum. In broth it produces no sulphureted hydrogen but a little indol.

In the "tube experiment" in broth gelatin with 0,1 % KNO_3, bubbles of nitrogen are exclusively seen to form at a little distance from the meniscus, and moreover, the culture of this species not succeeding in a bottle *wholly* filled with a culture liquid containing nitrate, we must needs conclude, that *B. vulpinus* wants considerable quantities of oxygen for the denitrification.

As regards the other species, which form gas bubbles also in the depth of the tube, I have come to the conviction that they too, want traces of free oxygen to this end.

Notwithstanding this different behaviour towards free oxygen, the motion figure, like that of *B. stutzeri*, shows the spirillum type.

By modifying the nutrient liquids and temperatures I have succeeded, as observed above, in accumulating various other denitrifying bacteria, beside those described. Thus I obtained, at 37° C. with calcium-citrate and 0,2 % KNO_3, under exclusion of air, and using garden soil for material of infection, the spirillum-like *B. indigoferus* Voges [1]), which denitrifies only feebly, but is interesting by its indigo-like pigment. When using sewage water, I obtained a strongly denitrifying, liquefying, blue pigment bacterium, not yet described.

Of all these experiments however, the result is not constant enough to be inserted here.

6. *Summary and conclusions.*

1st. The fundamental principle of my accumulation experiments was partly or completely to prevent the access of air. By this means I have succeeded, by cultivating in solutions of organic salts and nitrate, only by repeated transports in the same liquid, in bringing many denitrifying bacteria to a more or less perfectly pure culture.

[1]) Claessen. Ueber einen indigoblauen Farbstof erzeugenden Bacillus aus Wasser. Centrbl. f. Bakt. 1890, Bd. 7, S. 13.

Voges. Ueber einige im Wasser vorkommende Pigmentbacteriën, Centrbl. f. Bakt. 1893, Bd. 14, S. 301.

Of these experiments three always gave constant results, and produced respectively *B. stutzeri* NEUMANN and LEHMANN, *B. denitrofluorescens* n. sp. and *B. vulpinus* n. sp.

2nd. *B. stutzeri* deserves attention on account of the unique structure of its colonies, as seen in Fig. 1—5 on our Plate.

3rd: *B. denitrofluorescens* is the first example of a denitrifying, non liquefying fluorescent.

4th. *B. vulpinus* is a chromophorous pigment bacterium, whose pigment only forms at growth in the light.

5th. *B. stutzeri* and *B. vulpinus* behave towards free oxygen like aerobic spirilla, *B. denitrofluorescens* behaves like an ordinary aerobic bacterium.

6th. Like in soil and dung, in which it had also been found by other experimentators, I have established the general distribution of denitrifying bacteria in canal and sewage water.

7th. The denitrifying bacteria can, even with the slightest quantities of various organic substances, cause the disappearance of determined quantities of nitrate under development of free nitrogen.

8th. In one and the same culture medium, where nitrification is produced during aeration, denitrification may be caused by exclusion of air, this holds good also in regard to the soil.

At the end of this paper I want to express my sincere thanks to Professor Dr. M. W. BEIJERINCK for his kind, invaluable guidance and efficacious assistance, afforded me in these researches.

Delft, July 1902.

Physics. — *"An Hypothesis on the Nature of Solar Prominences."* By Prof. W. H. JULIUS.

The introduction of the principle of anomalous dispersion into solar physics makes it possible to form an idea of the Sun's constitution from which necessarily follow i.a. a great many peculiarities of prominences, which, until now, it has been impossible to deduct in a satisfactory manner from other physical laws. This I will show in the following pages.

In my paper on "Solar Phenomena, etc." read Febr. 24, 1900. I put forth the following hypothesis with respect to that part of the solar atmosphere, situated outside what is called the photosphere [1]):

[1]) W. H. JULIUS, Solar Phenomena, considered in connection with Anomalous Dispersion of Light, Proc. Roy. Acad. Amsterdam, II, p. 585.