Microbiology. *"On the Occurrence of Sulphate-reduction in the deeper layers of the Earth".* By C. A. H. VON WOLZOGEN KÜHR. (Communicated by Prof. G. VAN ITERSON JR).

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§ 1. *Introduction.*

The disappearance of organic matter at greater depths in the soil has since long occupied the minds of investigators. The difficulties associated with their inquiries regard especially that of obtaining sterile samples from such depths, which is essential to microbiological inquiry.

The process of oxidation, which causes organic matter to disappear, can be effected by free as well as by combined oxygen. When the air is shut off, as is the case in the lower strata, oxidation is of course brought about by combined oxygen.

Now the question is how this process can take place microbiologically.

The term sulphate-reduction designates the process by which, with the exclusion of air, organic matter in the soil is oxidized under the influence of combined sulphate-oxygen. This anaerobic process is effected by Microspira desulfuricans, discovered in 1895 by BEIJERINCK [1]). It being an exothermic process energy is set free through this oxidation, which is utilized physiologically by the sulphate reducing spirilla. The rough equation for sulphate-reduction gives the following formula:

$$2C\ldots + CaSO_4 + H_2O \rightarrow CaCO_3 + CO_2 + H_2S,$$

in which C... is the symbol for the source of carbon.

Microspira desulfuricans occurs in the mud of ditches and the ooze of the Dutch "Wadden". The grey, bluish-black to black colour of the soils in which sulphate-reduction takes place, must be ascribed to ferric sulphid, in which form the liberated hydrogen sulphid is combined by iron-compounds present in the soil.

The occurrence of the sulphate-reducing microbe at the greater depths in the terrestrial soil has been less frequently observed and,

1) Ueber Spirillum desulfuricans als Ursache von Sulfatreduktion. Verzamelde Geschriften. 3de deel, pg. 102.

to my knowledge, statements about it are few and far between. JENTZSCH [1]) e.g. records that in the deeper ooze-layers of the ocean of about 40 m. and more, reduction-processes occur, in which hydrogen sulphid and ferric sulphid are formed, which are ascribed by him to decomposition of proteins. It is more likely, however, that here also we have to do with sulphate-reduction, since it has been proved that this is of frequent occurrence under the circumstances alluded to.

Another statement is given by EUG. DUBOIS [2]) who observed the transformation of sulphate into ferric sulphid in the lower alluvial clay-layers underneath the Dutch Dunes.

An opportunity to ascertain the occurrence of sulphate reduction in deeper layers was offered, when in the autumn of 1921 a number of new wells were dug along the Sprenkelkanaal on the source of supply of the Amsterdam Dune Waterworks.

§ 2. *How the samples of sand, clay and peat were obtained from the well-shafts.*

In connection with the bacteriological sampling it will be well to set forth, in principle, the way in which the new wells were sunk.

A wide iron tube is driven vertically into a dug, shallow cavity. The sand is excavated from a greater depth than is at first reached by the tube, which can consequently sink gradually deeper. By means of a screw-thread one length of tube is screwed on to the other, so that a system of tubes is procured of the length necessary to reach a certain depth.

The masses of sand and the occasional lumps of clay and peat are removed from the tubes with a so-called "puls", consisting of a hollow iron cylinder of smaller diameter than the tube's. At the lower end it is sharp-edged to facilitate the sinking, while the bottom is provided with a valve, opening to the inside. By means of two iron bars that are fastened to the edge of the open top-part of the cylinder and are suspended on the same point of support, it is possible to connect the apparatus to a pulley-block. When moving the "puls" forcibly up and down in the wet mass of sand present in the shaft, it is ultimately filled with a pap of sand. The filled "puls" is then hoisted up and emptied by overturning it. This process of removing the sand from the well-shaft is briefly called "pulsen".

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[1]) Zeitschrift d. Geol. Ges. 1902. 54, p. 144. Cf. RAMANN, Bodenkunde, p. 180.

[2]) Het Leidsche Duinwater. Eene hydrologische studie. 1912, p. 19 en 20.

§ 3. *The examination of the sand- and clay-samples for sulphate-reduction.*

From a chemico-biological point of view it is interesting to ascertain the origin of the ferric sulphid, which gives a dull-grey, greyish blue to bluish-black colour to the soil-samples. The obvious hypothesis that the ferric sulphid was formed by sulphate-reduction, was in every respect substantiated by the examination of the many sand-, and clay-samples procured by means of the "puls". Thus the sulphate reduction in the deeper layers of the earth underneath the dunes appeared to be a bacteriological process of common occurrence.

The demonstration of sulphate reducing spirilla was performed after the accumulation-method of BEIJERINCK, the culture-medium [1]) used being:

Tapwater	100	
Na-lactate	0.5	
Asparagin	0.1	
$MgSO_4 . 7$ aq.	0.05	(or gypsum)
$Fe SO_4 . 7$ aq.	0.001	

with which sterile stoppered bottles of ± 150 cc. capacity were filled after infection with a quantity of the sand-, or clay-samples under examination. They were filled up to the neck, then cautiously stoppered and placed under 25° C.

BEIJERINCK [2]) showed that in this anaerobic procedure Microspira desulfuricans is exclusively the causative agent of the sulphate-reduction manifesting itself, as appears from the formation of hydrogen sulphid and the black ferric sulphid.

My culture bottles showed in every respect the same progress of the reduction process, so that hereby the examined sand-, and clay-samples gave evidence of the presence of Microspira desulfuricans.

The material used for infection of the medium was drawn from the inner portion of the sand-mass in the "puls" by means of a sterile spatula, and deposited in sterile wide-mouthed stoppered bottles. Directly when the samples were received at the laboratory they were subjected to investigation.

Sterile sampling could be effected to perfection only in clay-, and peat-samples. This was performed after BEIJERINCK's [3]) method. The sample was split in two. From the fracture laid bare, the required

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[1]) A. VAN DELDEN. Beitrage z. Kenntn. d. Sulfaatreduktion durch Bakt. Centralbl. f. Bakt. 2e Abt. 1903. Bd. XI, p. 83.

[2]) Verzamelde Geschriften. (Collected Papers) Vol. 4, p. 53.

[3]) Verzamelde Geschriften. (Collected Papers) Vol. 2, p 354. Note 2.

inoculation material was taken by means of a sterile spatula. The lumps of clay and peat suited our purpose well, since in the splitting the fracture was not contaminated by crumbling particles of the edges, which was owing to the solid structure of the samples resulting from their humidity.

The time in which the formation of hydrogen sulphid in the culture-bottles commenced was very different for the same inoculation-substance and especially for the sulphate reduction it largely depends on the number of viable germs present at the outset of the experiment.

The clay-, and the peat-samples dredged up with the "puls", were derived from the clay-, and the peat-banks underlying the dunes. They were all compact masses, in which the original stratified structure, arising from sedimentation, had been preserved. These clay-, and peat-layers being all but impermeable to water, their inside represents the original bacteriological condition of the stratum, from which the sample has been taken.

The clay- and peat-lumps were on the outside wet and on the inside, judging superficially at least, moderately humid. The water-content of the clay amounted to about 26%; in the clay-samples which contained peat in the stratified structure, the content of moisture was considerably higher, viz. about 50%. The peat-samples exhibited the largest amount of water, viz. rather more than 77%.

The clay- and the peat-lumps varied from very large ones to those of the size of a fist and appeared to meet the bacteriological requirements in every respect.

§ 4. *Summary of results and observations on the inquiry about sulphate-reduction.*

The number of soil-samples of the 9 wells which were examined for sulphate-reduction, have been summarized in the subjoined table.

The quantum of infection-material used for every sulphatereduction-test amounted to from 5 to 10 grs. of the soil-sample. After an interval of from 3 to 20 days sulphate-reduction revealed itself at 25° C., which period rose to 5 weeks in the case of the peat-sample B 31.

In every well, even the deepest of 34.50 m. below A.P., we chiefly found sand over the whole depth, in which irregularly spread lens-shaped clay-, and peat-layers occurred alternately.

With a few exceptions all the sand- and clay-samples indicated in the subjoined table, yielded on examination for sulphate-reduction a conclusive positive result. Consequently the dull-grey or grey colour of the sand-samples and the mostly blue to bluish-black colour of the clay-samples points to prevailing sulphate-reduction. This com-

B 22	B 24	B 25	B 26	B 27	B 28	B 29	B 30	B 31
21.6 M.	35 34 M.	35.30 M.	6.50 M.	*8.00 M.*		6.50 to 10.50 M.	*9.50 M.*	*8.00 M.*
							12.50 »	*14.00 »*
			15.10 »	*13.25*(*)»	*6.00 M.*	14.00 to 16.25 »	18.50 »	25.30 » (peat)
			17.50 »		6.50 »		32.50 »	
			28.50 »		16.50 »	*20.50*(*)»	34.50 »	
			32.50 »		*29.00 »*	*23.10 »*		
					34.50 »			

B 22, B 24, etc. = wells.

The values express in metres the depths below A.P. (= Amsterdam level) from which the soil samples have been drawn.

The figures in italics refer to clay-samples which enclose organic particles or peat-layers.

The figures in ordinary type are sand-samples.

(*) = no sulphate reduction in culture bottle.

mences at about 10 m. below the surface (7.5 m. —A. P.)[1]) to ± 37 m. (34.50 m.—A.P.) the largest depth examined here.

The conditions under which sulphate-reduction appears are:

1°. Absence of oxygen [2]).

2°. The occurrence of organic compounds.

3°. The presence of sulphate and the required mineral compounds.

The first condition, the absence of oxygen, is satisfied in consequence of the considerable depth below the level of the ground.

The second condition: the occurrence of organic compounds, is fulfilled already to the eye by the peat-sample and also by the clay-sample with enclosed peat-layers. That the sand-, and clay-samples, which do not enclose immediately distinguishable organic particles, also contain organic matter, can be demonstrated chemically, by the potassiumpermanganate method. This is conducted as follows: The soil-sample is boiled with diluted sulphuric acid and filtered. The filtrate is cooled down under the tap; now potassiumpermanganate (0.01 norm.) is instilled. The first drops are directly decolorized. which is owing to the oxidation of ferro- and mangano-compounds,

[1]) The grounds of the wells at the Sprenkelkanaal is lying at 2.5 M. above A. P.

[2]) Traces of oxygen are left out of consideration here.

Then a moment follows in which the colour of the added potassium-permanganate disappears only slowly: this is the oxidation of the organic matter, extracted by the diluted sulphuric acid, for in a drop of this extract, placed on a piece of filterpaper soaked with potassium ferrocyanid no ferro can be demonstrated any more.

The sand-samples are most often not so rich in organic compounds as the clay-samples, which often contain peat. Presumably this generates a stronger sulphate-reduction than is possible in the sand-samples, and this is probably the reason why clay can be darker in colour than sand.

VAN DELDEN [1]) has shown that for sulphate-reduction organic bodies are required which are easily oxidizable. This justifies the assumption that in the organic substances, demonstrated by us, there are some bodies difficult of oxidation and others again which are easily oxidizable, which is proved indirectly by the sulphate-reduction that manifests itself in the sand-, clay-, and peat-samples.

Also the 3rd condition, the presence of the required mineral compounds, was satisfied. In our examination for sulphate only small amounts could be demonstrated, which is explained by the disappearance of sulphate through sulphate-reduction.

One of the mineral combinations is that of the insoluble, black-coloured ferric sulphid, formed by the iron and the liberated hydrogen sulphid, as pointed out already in § 3.

From the foregoing we may deduce that the conditions of anaerobic life which we found in the deeper layers of the soil, fairly agree with the prevailing sulphate-reduction.

§ 5. *The content of „aerobic" and „anaerobic" germs of the deeper layers of the soil.*

Besides the demonstration of sulphate-reducing spirilla in the soil-samples, another question arises, viz. whether they contain other germs and whether these belong to the aerobes or the anaerobes. We examined the samples:

B 28	29.00 M — A.P.	(clay with peat)
B 29	6.50 — 10.50 M — A.P.	(clay).
B 31	25.30 M — A.P.	(peat).

The number of germs was ascertained in the way described in § 3. With a sterilized spatula inoculation-material was taken from the soil-samples, it was then shaken up in sterile tapwater and

[1]) Centralbl. f. Bakt. Bd. XI, 2te Abt. 1903, p. 83.

subsequently weighed. This material was used for making counting-tests by sowing the micro-organisms on nutrient gelatin. The counting of the microbe-colonies for the aerobic plate-cultures took place after 48 and 72 hours, after which there was hardly any increase of the colonies worth mentioning.

The anaerobic culture plates for the counting-tests were made after WRIGHT and BURRI's [1]) culture method, modified by me. As this strictly anaerobic method of cultivation yields very good results, it will not be amiss to state our procedure.

In a glass box closed tightly by a glass stopper with a ground rim a smaller petri-dish is placed containing a solidified culture-medium on which the anaerobes are sown in streaks. The circular open space left round the dish is first stopped up with non-absorbent cotton-wool on which a layer of absorbent cotton-wool is laid. The latter is soaked with 20 % potassium hydrate and finally with an equal volume of 20 % pyrogallic acid.

Throughout this procedure the petri-dish remains covered. After the cotton-wool has been soaked with pyrogallic acid the dishcover is removed, while the glassbox is closed by its cover-glass of which the glass-rim is smeared with vaselin. The rim of the glass-box may also be shut off with paraffin after the lid has been adjusted. In order to facilitate the opening of the glass-boxes, the wall is provided with a little hole which is shut off with paraffin and is opened again before taking off the lid of the box, in

Soil-sample.	Aerobes.		Anaerobes.	
	number of germs per c.c. of soil.		number of germs per c.c. of soil.	
	After 48 hrs.	After 72 hrs.	After 4 days.	After 12 days.
B 28 29.00 M. — A.P. clay + peat.	15400	20000	818	409000
B 29 6.50—10.50 M. —A.P. clay.	20	130	—	400
B 31 25.30 M. — A.P. peat.	—	7000	103600	160000

[1]) J. H. WRIGHT. A method for cultivation of anaerobic bacteria. Centralbl. f. Bakt. 1te Abt. 29, 1901, pg. 61. R. BURRI. 2te Abt. 1902. 8, pg. 533.

order to admit the air. Now the cover of the petri dish is easily removed.

The number of anaerobes was counted in the same way as that of the aerobes in the same sample.

Because we had determined the specific weight of the soil-samples, we could establish the number of germs per cc.

Our results we have tabulated on page 7.

The time in which the anaerobes yielded a constant number of colonies was considerably longer than that of the aerobes.

It strikes us that the anaerobic test yields a total of germs which is much greater than that of the aerobic one, while the amount of germs in B 28 and B 31 is much higher than that of B 29. The last-named fact is perhaps due to the higher content of organic matter in the first two soil-samples.

For the sake of comparison we may add that in raw water from the dunes the number of bacteria per c.c. varies in round numbers from 400 to 1800.

§ 6. *It appears that microbes derived from aerobic and anaerobic cultivation belong for the greater part to the facultative anaerobes.*

The number of species of bacteria obtained in the preceding paragraph by the method described, appeared to be only small when we examined their qualities. Generally the anaerobes and the aerobes [1]) were not identical. The following table shows the number of species of microbes we found:

Soil-sample.	Aerobes.	Anaerobes.
B 28 29.00 M. — A.P.	2 species	4 species
B 29 6.50 — 10.50 M. — A P.	2 »	1 „
B 31 25.30 M. — A.P.	1 „	4 „

As to their properties aerobes revealed some resemblance in acidformation from glucose, Berlin-blue formation from ferri-ferricyanid, the formation of hydrogen-sulphid from broth (lead-carbonate test), the splitting of aesculin, the formation of katalase, and most often in the inability to ferment glucose, to form lipase and diastase. Spores were not formed.

[1]) Probably B 29 anaerobe and one of the species B 29 aerobe were identic.

A difference in the properties of the two microbe-groups appeared from the following reactions: Anaerobes form nitrite from nitrate in a marked degree, indican is split extensively in most cases (oxidation of indoxyl to indigo-blue), a moderate amount of invertase is formed, a large amount of slime (wall-matter) is formed from saccharose. Aerobes lack these qualities. They liquefy gelatin, whereas the anaerobes do not.

My investigation into the properties of the microbes did not put me in a position to classify them.

When examining microbes derived from aerobic cultivation for their anaerobic behaviour, it appeared that only B 31 grew very well without air, those of B 28 and B 29, however, very badly. The occurrence of these aerobes seems to show that presumably very small quantities of air are to be found at larger depths in the soil, and that they are carried along with the rainwater that penetrates at a very slow rate into the deeper layers of the earth. If the layer, as is the case here with clay, is only sparingly permeable to water, the dissolved oxygen is allowed to diffuse to the places where it is to be consumed.

The microbes obtained from anaerobic cultivation developed enormously when living in air. This appeared conclusively when the anaerobic culture-boxes after being opened had been standing for some time exposed to the air. Then the microbe colonies grew larger and larger in a very short time. These bacteria grew very well as aerobes, also on nutrient agar-slants. Tested in this way the majority of the isolated bacteria appeared to belong to the *facultative anaerobes*, which is consistent with the occurrence of these microbes at greater depths.

§ 7. *Research for some other specific species of microbes.*

We endeavoured to ascertain the occurrence of obligate-aerobic nitrifying bacteria and of Azotobacter chroococcum, however, with negative result, as could be expected.

Nor could denitrifying microbes be demonstrated; no more could we detect anaerobic butyric bacteria and anaerobic bacteria which break down cellulose.

§ 8. VAN DER SLEEN'S *Manganese-Theory for the oxidation of organic matter at greater depths in the soil.*

The problem of oxidation of the organic matter in the deeper layers of the earth has been discussed by W. G. N. VAN DER SLEEN in his publication: „Bijdrage tot de kennis der chemische samenstelling van het duinwater in verband met de geo-mineralogische gesteldheid van den bodem." The writer says (p. 50) that at such a great depth bacterial influence on the oxidation of organic matter seems to be out of the question and he suspects manganese salts to act as oxygen-carriers. Further on (pg. 62) the writer says: „.... I do not think that Microspira desulfuricans occurs at such a depth as has to be assumed when ascribing sulphate-reduction only to this micro-organisme".

On pag. 51 the author records some experiments which go to show that manganese can transmit oxygen from the sulphates to an organic compound such as hydrochinon. To conclude from this that the oxidation of organic matter at the lower depths in the soil could occur in the same way, seems to me hardly admissible unless experimental evidence be brought forward that biological oxidation is out of the question. Such evidence has not been produced as yet. It may be deemed surprising that the author, who, as appears from the passage in his publication that we quoted just now, had taken cognizance of the bacteriological sulphate reduction has omitted to inquire into it. This is the more surprising since on the ground of its anaerobic behaviour Microspira desulfuricans is adapted to living at greather depths in the soil.

The evidence produced by our investigation set forth in the preceding paragraphs, by which it has been established that sulphate-reduction is of common occurrence at the greater depths underneath the dunes, warrant the conclusion that oxidation of organic matter can be effected by Microspira desulfuricans, without the additional influence of manganese compounds.

§ 9. *The transformation of sulphate in the clay-containing soil of the dunes and sulphate-reduction by Microspira desulfuricans.*

The "Koninklijke Academie van Wetenschappen te Amsterdam" [1]) has brought forth a report on the question to what the presence of so called Artesian water in the dune-soil is due, in a preliminary advice from G. A. F. Molengraaff and Eug. Dubois. In an enumeration of the chemical properties of dune-water the report contains the following statement:

[1]) Verslag v. d. gewone vergaderingen der Wis- en Natuurk. Afd. Vol. XXX, p. 212.

"From the surface downwards in and underneath the dune-masses the sulphuric acid content diminishes proportionally to the total thickness of the clay-layers occurring in them, i. e. in proportion to the increase of the volume of clay-soil, through which the water has percolated downwards.

This phenomenon is the result of the power of clay-soil to convert sulphuric acid and then retain it".

In the study by EUG. DUBOIS[1]) already quoted above, a detailed exposition is given of the transformation of sulphuric acid in the clay-layers, which consists in a reduction-process in the presence of organic matters, with formation of ferric sulphid.

It is evident from the foregoing that sulphate-reduction, which occurs not only in the deeper clay-layers, but also in the sand-soil, is brought about by Microspira desulfuricans. The life of this microbe, which is adapted to anaerobic conditions, accounts for the common occurrence of sulphate-reduction in the deeper layers of the earth and especially in the clay-soil, which generally has a higher content of organic matter.

So long as the conditions of this typically microbiological process are fulfilled, transformation of sulphate into ferric sulphid will hereby be generated, to which is to be ascribed the partial or total absence of sulphuric-acid salts in deep-dune water.

Heemstede, February 24 1922.

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[1]) „Het Leidsche Duinwater". Een hydrologische studie, 1912, p. 20.