BACTERIOLOGY OF ACTIVATED SLUDGE

H. W. VAN GILS

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PROEFSCHRIFT

TER VERKRIJGING VAN DE GRAAD VAN DOCTOR IN DE LANDBOUWKUNDE OP GEZAG VAN DE RECTOR MAGNIFICUS IR. W. F. EIJSVOOGEL, HOOGLERAAR IN DE HYDRAULICA, DE BEVLOEIING, DE WEG- EN WATERBOUWKUNDE EN DE BOSBOUWARCHITECTUUR, TE VERDEDIGEN TEGEN DE BEDENKINGEN VAN EEN COMMISSIE UIT DE SENAAT VAN DE LANDBOUWHOGESCHOOL TE WAGENINGEN OP MAANDAG 29 JUNI 1964 TE 16 UUR

DOOR

H. W. VAN GILS

DRUK: W. D. MEINEMA N.V. – DELFT

STELLINGEN

I

In publikaties over respirometeronderzoekingen met microörganismen dienen niet alleen de voor de endogene ademhaling gecorrigeerde waarden te worden opgegeven, maar ook die van de endogene ademhaling.

E. A. Dawes en D. W. Ribbons, Ann. Rev. Microbiol., 16, 241 (1962).

II

Hoewel bij het actief-slibproces het substraat vrijwel continu wordt toegevoerd, is de aard van het proces zodanig, dat niet kan worden gesproken van een continue bacteriecultuur.

III

Het op grote schaal produceren van biologisch onaantastbare chemische verbindingen (speciaal die welke in water oplosbaar zijn) kan gevaren in houden voor de volksgezondheid.

IV

De mening, dat door verlaging van de vetconsumptie atherosclerose zou kunnen worden voorkomen, is onjuist.

H. J. Thomasson, Ned. T. Geneesk., 107, 593 (1963).

V

De theorie, ontwikkeld door Bock, over de invloed van de extractiemethoden voor bietenpectine is onvolledig, aangezien geen rekening is gehouden met het verstorende effect van acetylgroepen op het geleervermogen van deze pectinen.

H. Bock, Diss. Techn. Hochsch., Karlsruhe (1943).

VI

De industrialisering van de landbouw is niet alleen een economisch en juridisch, maar ook een sociologisch probleem.

VII

Onder de huidige economische omstandigheden is het toepassen van kunstmatige beregening op bedrijven met overwegend akkerbouw niet rendabel.

VIII

Er is geen duidelijk verband tussen de spreiding in verblijftijd en de werkingsgraad van een bezinktank.

F. SCHMIDT-BREGAS, Institut für Siedlungswasserwirtschaft, Hannover 1958.
W. A. BEVERLOO, Water (1964).

IX

Aan composteren als middel tot verwijdering van stadsvuil dient de voorkeur te worden gegeven boven verbranding.

X

Bij de industrialisatie dient meer rekening te worden gehouden met de „industriële capaciteit" van de betrokken gebieden ten aanzien van de water-, bodem- en luchtvervuiling.

XI

Het afkeuren van astma(cara)patiënten bij sollicitatiekeuringen op grond van een algemeen reglement is een anachronisme.

XII

De nederlandse zachtmoedigheid jegens verkeersovertreders – uit roekeloosheid, onbegrip of onwil – dreigt een nationaal gevaar te worden.

De Groene Amsterdammer, 13 maart 1964.

XIII

Het succes van studententoneel is voornamelijk te danken aan de kwaliteit van de spelers en in mindere mate aan die van de regisseur.

Proefschrift van H. W. van Gils

Wageningen, 29 juni 1964.

Dit proefschrift met stellingen van Hans Willem van Gils, landbouwkundig ingenieur, geboren te Delft, 22 november 1933, is goedgekeurd door de promotor, Dr. Ir. E. G. Mulder, hoogleraar in de Microbiologie.

De Rector Magnificus der Landbouwhogeschool,
W. F. Eijsvoogel

Wageningen, 14 mei 1964.

Aan Tante Nel en Mama

Dit proefschrift dient als bijdrage tot de kennis van het actief-slib proces en werd uit dien hoofde gefinancierd door het Instituut voor Gezondheidstechniek T.N.O. Het onderzoek werd verricht op het Laboratorium voor Microbiologie der Landbouwhogeschool te Wageningen.

CONTENTS

CHAPTER 1

INTRODUCTION AND STATEMENT OF THE PROBLEM

A method extensively used for the stabilization of waste water is the activated sludge process in which sludge flocs are maintained in suspension in flowing waste water by air diffusion or mechanical agitation.

Activated sludge consists of yellow-brown to black coloured flocs, produced by the growth of bacteria and other micro-organisms in the presence of dissolved oxygen. Soluble and suspended matter are removed by the living mass of micro-organisms maintained under the mentioned aerobic conditions. After exhaustion of the waste material, the sludge flocs are allowed to settle down; they can be separated from the carriage water which, now freed for 90–95 per cent of its organic matter, leaves the settling tank as a clear liquid. Bacteria, which are present in high numbers in activated sludge, are primarily responsible for the purification of the waste water.

The activated sludge process is a most suitable method for the stabilization of waste water, because: a. the removal of the contaminating organic compounds is achieved rapidly; consequently, the aeration and detention periods may be short; b. due to the rapid and complete settling of the sludge mass, it may be separated from the carriage water by a simple process.

Since the English investigators Ardern and Lockett [4] developed the activated sludge process in 1914, its application, albeit with various modifications, has attained enormous dimensions. Accordingly, much empirical knowledge has been gathered about the technical operation of the process; however about the basic facts only scant information is available. Kluyver [55] in 1926 formulated this problem as follows: „What effects the stabilizing action that occurs when air is diffused into activated sludge? Evidently, it is worth while to consider this question at present, because the processes taking place, in fact, are the basis of a purification method for the application of which many plants are to be found all over the world, plants which have cost millions of guilders in some cases.

In attempting to answer this question on the strength of available literature, one may well be appalled by the almost incredible fact, that so little is known as to this. Besides numerous publications referring to the technical aspects of this method, only few are found in which the authors are concerned with the nature of the purification process".

Since Kluyver held the lecture from which this passage is derived, much

has been done to gain some more fundamental knowledge on the activated sludge process. Unfortunately, up to the present only a few basic facts have been found: as far as the ecology and metabolism of activated sludge are concerned a few data are available, but particularly little is known about the principles of the formation of bacterial flocs.

1.1 Statement of the problem

The present investigation attempts to add a few facts to the basic knowledge about the metabolic processes occurring in activated sludge. This aim has been approached in two ways *viz.* by ascertaining (a) the nature and (b) the function of the bacteria in activated sludge. To establish the importance of the bacteria isolated from the activated sludge floc, two criteria were to be used: a. the correspondence of their metabolic processes to those of the activated sludge, and b. their ability to flocculate.

CHAPTER 2

SURVEY OF LITERATURE

As stated by Hawkes [38] "bacteria can be regarded as the basis of the activated sludge floc both structurally and functionally, and are universally present in the traditional activated sludge". Therefore studies on the bacterial flora of activated sludge have already been the subject of a, though comparatively small, number of publications. However, the results of the various investigations are rather divergent, partly due to the use of different methods, but principally to the fact that completely different types of sludge have been examined. In this respect it is important to note that the bacterial flora of activated sludge is mainly dependent on the composition of the waste water to be purified.

2.1 Bacteriological examinations of activated sludge

Russel and Bartow [81] isolated thirteen varieties of non-nitrifying bacteria from activated sludge. All but four of these belonged to the *Bacillus subtilis* group of aerobic spore-formers, while most species formed a small amount of acid but no gas from glucose, and hydrolysed starch and casein. Kamm [54] carried out similar experiments and obtained substantially the same results. Harris *et al.* [37] found that 61 per cent of the organism in activated sludge were of the *Aerobacter aerogenes* type and 38 per cent of the *Proteus* type.

Bacteria entering the activated sludge plant with the sewage are from two main sources, firstly those present in water or in the infiltration water, and secondly, intestinal bacteria introduced with faeces. The results of earlier workers gave the impression that intestinal bacteria and spore-formers predominate in activated sludge and therefore played an important part in the purification of sewage [37].

Heukelekian [41], counting bacterial numbers in various types of settled activated sludge, found $9.5-48.5 \times 10^6$ bacteria per ml, or $3.7-73.5 \times 10^6$ per mg dry matter. Allen [1], after homogenizing the sludge, found 10^8-10^{10} bacteria per ml of non-settled sludge; he showed that: firstly, most of these were proteolytic, secondly, only a minor part was able to ferment glucose under anaerobic conditions and thirdly, most of the organisms were Gram-negative and rod-shaped, belonging to the genera *Pseudomonas*, *Flavobacterium* and *Achromobacter* [2]. Bacteria of faecal origin as well as spore-forming species

were found only in small numbers. Jasewicz and Porges [51] investigated activated sludge of dairy sewage and classified the strains found as follows: 26 per cent *Alcaligenes*, 34 per cent *Flavobacterium*, 14 per cent *Micrococcus* and 16 per cent *Pseudomonas*.

Literature nearly always mentions the occurrence of filamentous and sheath-forming organisms to a more or less extent. Especially in poorly settling sludge, many filamentous bacteria of the genus *Sphaerotilus* may be found [83]; in normal sludge they occur to a much less extent and consequently it may be assumed that these organisms are not essential for a good activity of activated sludge.

Moreover, there are protozoa in varying numbers in most sludges; opinions differ as to the importance of these organisms for a good clarification effect of activated sludge [52, 64]. Like filamentous bacteria they are considered to be indicators of the sludge quality [5].

Algae and fungi are observed in great numbers only under special conditions.

Formerly, it was assumed that activated sludge was formed by one special bacterium species. It was claimed that this bacterium could be frequently observed in activated sludge in so-called finger-like zoogloea (*i.e.* jelly-like matrices in which bacteria are separately imbedded). Buswell and Long [11] concluded on account of microscopic investigations that activated sludge consisted of zoogloeal masses mixed with filamentous bacteria. Winogradsky [95] and Butterfield [12] isolated zoogloea-forming organisms from activated sludge. Winogradsky classified her strain on account of nitrifying properties and of being chemoautotrophic as a *Nitrocystes* sp. Butterfield described his strain as *Zoogloea ramigera*. After the appearance of Butterfield's paper, *Z.ramigera* strains have been isolated by several investigators. The general characteristics of the strain were: rod-shaped; motile with one polar flagellum; aerobic; non-spore-forming; Gram-negative; capsule-forming; producing little or no acid from carbohydrates; producing ammonia from gelatin, casein and peptone; no nitrification; no formation of hydrogen sulphide; producing well-organized flocs when aerated in sterile sewage; growth on agar or gelatine was very scant, so that none of the isolated strains of *Zoogloea ramigera* has been kept alive for long. Hawkes [38] suggests that *Zoogloea ramigera* is no true species, but a growth form of various species.

From 1937 to 1943, Butterfield, Heukelekian, Wattie and co-workers investigated the purification of sterile sewage using pure cultures of *Zoogloea ramigera* strains. Butterfield, Ruchhoft and McNamee [13] found 50 per cent BOD-reduction *) after 5 hours aeration and 80 per cent after 24 hours. According

*) BOD means biochemical oxygen demand in 5 days at 20 °C.

Heuke lekian and Littman [42], activated sludge would mainly consist of flocculent masses of *Zoogloea ramigera*. Butterfield and Wattie [14) suggest that the active substance in purifying sewage in trickling filters as well as in activated sludge is formed by *Zoogloea* bacteria. Feldman [30] also found that *Zoogloea ramigera* was the main bacterium species in trickling filters. Because of its ability to flocculate and to stabilize nutrient substrates, it became generally accepted that *Zoogloea ramigera* was the primary organism in activated sludge.

The question of bacterial floc formation was again investigated by McKinney and co-workers. McKinney and Horwood [60] and McKinney and Weichlein [62] also isolated other bacteria from sludge, apart from *Zoogloea ramigera*. The bacteria apparently also had the ability to form flocs under suitable conditions in pure cultures. Among others they found *Escherichia coli*, *E. intermedia*, *Paracolobactrum aerogenoides*, *Nocardia actinomorpha*, *Bacillus cereus* and a number of strains of the genera *Pseudomonas*, *Alcaligenes* and *Flavobacterium* to possess this capacity. These floc-forming bacteria reduced the BOD in waste water by 66 to 88 per cent after 24 hours aeration.

Buck and Keefer [9] isolated an immobile Gram-negative rod, about $1 \times 5\mu$, with slime formation, but no capsules, reducing nitrate to nitrite and nitrogen, not affecting sugars and forming flocs under certain conditions. In sewage these bacterial flocs had the same purifying effect as activated sludge. Dugan and Lundgren [24] investigated a Gram-negative, rod-shaped bacterium with floc-forming properties, isolated from activated sludge. This bacterium did not affect carbohydrates; growth on agar seemed soft but actually the colonies were tough and firmly fixed to the agar.

These investigations into bacterial floc formation in pure cultures have until now contributed little to elucidate the mechanism of floc formation. Moreover, little is known about the importance of the various floc-forming bacteria in activated sludge formation under natural conditions and in stabilizing waste water.

2.2 Metabolism in activated sludge

Heukelekian and Schulhof [43] distinguished two processes in the activated sludge system *viz.* those of oxidation and those of floc formation. They suggested that different micro-organisms are involved in each of these processes and that biological as well as physical factors are effective.

As for the removal of organic matter, it may be assumed that many aerobic and also non-floc-forming bacteria may effect a considerable reduction of BOD in waste water [29, 45, 71] which is a biological process. The flocculation of the bacteria on the other hand could be mainly considered as a physical process [44].

With respect to transformations proceeding in activated sludge brought into contact with waste water, or more generally with organic material that may be biologically affected, it is known that: a. oxygen is needed; b. the amount of oxygen used is only a part of that theoretically needed for complete oxidation of the substrate; c. activated sludge first has to be adapted to some compounds; d. the removal of organic material by activated sludge elapses rapidly.

The removal of organic material by activated sludge can be achieved by adsorption followed by absorption and biochemical transformation (oxidation to water, carbon dioxide and ammonia, and assimilation into sludge material). According to Wuhrmann *et al* [97] compounds such as glucose, amino and fatty acids are removed by physiological processes rather than by physico-chemical processes (*i.e.* adsorption). When mixing sewage with activated sludge and excluding oxygen, Herb found [40] little removal of waste material. Placak and Ruchhoft [76] found more adsorption with carbohydrates as the polymerization degree was higher. Emulsified oils may be adsorbed for 100 per cent, since they are converted biochemically much more slowly than adsorbed by activated sludge.

Biochemical transformations are vital in the activated sludge process. For practical application it is important to know what part of the organic matter is broken down into water, carbon dioxide and ammonia, and what part, in whatever form, effects the growth of sludge. Placak and Ruchhoft [76] determined dissimilation and assimilation percentages for a great number of organic compounds after 24 hours of aeration (table 1).

Table 1. Respiration and assimilation in activated sludge (after Placak and Ruchhoft [76]).

Class of compounds	Percentage oxidized		Percentage converted to organized sludge
	Range	Mean	
Carbohydrates	5–25	13	65–85
Alcohols	24–38	30	52–66
Amino acids	22–58	42	32–68
Organic acids	30–80	50	10–60

A similar relation between dissimilation and assimilation of substrates was found by Wuhrmann, Von Beust and Ghose [97]. These investigators found that in nitrogen-free media during the absorption of glucose and sucrose, 16.6 and 13.6 per cent, respectively, were oxidized, and with acetic and butyric acids 32 and 29 per cent. On the other hand the amino acids aspartic acid and tryptophane were oxidized for 95.6 and 78.8 per cent respectively, *i.e.* almost completely.

The dissimilation percentages as recorded for carbohydrates are extremely low as compared with those under optimal conditions of bacterial growth. It indicates that only part of the assimilated substrate has been converted into cytoplasm, while the remainder was accumulated as intra- or extra-cellular polymerization products.

Comparable, though less extreme values were found by Barker [6] with the alga *Prototheka zopfii*. Such metabolic processes were called by this author „oxidative assimilation", which means „the primary conversion of the substrate to a primary product of assimilation, the raw material for all subsequent secondary synthesis within the cell" [7]. (See also the summarizing articles of Clifton [15, 16]).

A low dissimilation percentage was found also by Porges, Jasewicz and Hoover [78] in activated sludge grown in dairy waste water after nutrition with skim milk. These investigators distinguished increase in polysaccharides and protein synthesis (cell growth) in sludge growth. After four hours of aeration at 20 °C, all skim milk (with COD*) 1125 mg) added to 1000 mg of sludge (dry wt) appeared to have been diminished as follows: 13 per cent oxidized, 58 per cent stored as polysaccharides, and 22 per cent used for cell growth. These figures were calculated by means of a carbon balance and by performing an elementary analysis of the sludge. It was shown that the storage compounds occurred as polysaccharides consisting of glucose.

O'Brien [73], using sludge adapted to glucose, determined the oxygen uptake with Warburg respirometers during the removal of glucose in the presence of ammonium nitrogen. The removal of the substrate was followed by means of COD determinations. From the difference between the COD decrease and the oxygen uptake he calculated what part of the substrate was transformed to activated sludge. From the decrease of ammonium nitrogen in the medium he calculated the synthesis of cytoplasm, *i.e.* cell growth. O'Brien added 2000 mg glucose and 114 mg ammonium nitrogen to 860 mg (dry wt) glucose-adapted activated sludge; after 8 hours of aeration (no temperature is recorded) 93.4 per cent of the glucose was found to have been removed and transformed as follows: 15.2 per cent oxidized, 62.2 per cent stored as polysaccharides and 22.6 per cent synthesised into protein. After complete removal of the substrate the amount of stored polysaccharide decreased slowly. During the period of endogenous respiration O'Brien did not find any increase in soluble inorganic N-compounds in the liquid. From these results he concluded that amino acids did not act as an energy source for endogenous respiration, but that stored polysaccharides were used.

Gaudy and Engelbrecht [34] examined the transformation of glucose by

*) COD means chemical oxygen demand.

activated sludge grown on a mineral medium with glucose and ammonium chloride. By adding 500 mg glucose and 41 mg ammonium nitrogen to 565 mg (dry wt) activated sludge, they found that all glucose had been removed within 25 hours and converted as follows: 16 per cent oxidized, 20 per cent stored as polysaccharides and 16 per cent synthesised into protein. The dry matter increase was 66 per cent of the added glucose. These data were determined by chemical analysis: polysaccharides by means of anthrone and cell growth by means of biuret. Upon continued aeration, Gaudy and Engelbrecht found an almost complete removal of the polysaccharides stored during the uptake of glucose followed by a sharp increase in cell protein, until a maximum was reached after six hours; approximately 50 per cent of the added glucose had then been utilized in protein synthesis. With the same sludge the investigators also examined the conversion of glucose without a nitrogen source. Evidently, the rate of removal of the glucose by the sludge was only slightly lower than in the presence of ammonium nitrogen. After 2.5 hours all glucose had been removed and converted as follows: 17 per cent oxidized, 46 per cent stored as polysaccharides and approximately 13 per cent synthesized into protein; the dry matter increase was 63 per cent of the added glucose. With continued aeration the stored polysaccharides as well as the protein decreased slowly. These authors gave no explanation of the incompleteness of the dry matter balance.

Washington and Symons [93] found an increase in biologically inert material in activated sludge supplied with glucose, acetic acid or glycine. Next to fatty acids and organic nitrogen this material consisted mainly of polysaccharides. After exhaustion of the substrate, part of the stored polysaccharides disappeared from the sludge by endogenous respiration. Principally, however, organic nitrogenous compounds were used as substrate during endogenous respiration, giving rise to the accumulation of ammonia in the medium. These investigators accordingly concluded that the polysaccharides formed were no true reserve compounds.

Symons and McKinney [88] observed that in activated sludge at least part of the polysaccharides is formed extra-cellularly. Wilkinson [94] has brought forward that most bacteria cannot decompose their own extra-cellular storage compounds. Mulder *et al* [68] showed that *Arthrobacter globiformis* can decompose only 40 per cent of its polysaccharides during endogenous respiration. Yeast on the other hand may break down, during endogenous respiration, all intra- and extra-cellular lipids originally formed as storage compounds [20]. When ammonium nitrogen was supplied to lipid-rich yeast cells, the decomposition of the lipids induced a considerable increased number of yeast cells [68].

Several investigators mention a stimulating effect of ammonium nitrogen on the uptake of carbon compounds by activated sludge and by some micro-

organisms in pure cultures. Wuhrmann [96] found that the uptake of glucose and acetic and butyric acids by activated sludge in the presence of ammonium nitrogen was 1.2, 1.3 and 1.6 times, respectively, as rapid as in a nitrogen-free medium, while oxygen uptake per unit of substrate during this period was considerably higher. McLean and Fisher [65] obtained a similar effect with glycerol and citric acid, using *Serratia marcescens* as the test organism. Gaudy and Engelbrecht [35] found also a stimulating effect of ammonium nitrogen on the uptake and respiration of glucose by activated sludge. Yemm and Folkes [98] described the same phenomenon for a yeast species and Moses [67] for a fungus; both organisms had been cultivated in a nitrogen-deficient medium.

2.3 Endogenous metabolism of activated sludge

Fews facts are known about the endogenous metabolism of activated sludge. Only the investigations of Washington and Symons [93], cited earlier, demonstrated to some extent that endogenous energy is mainly derived from the break-down of nitrogenous organic compounds. Ribbons and Dawes [80] in experiments with *Sarcina lutea* cultivated in a glucose peptone medium, established that during endogenous respiration, stored polysaccharides as well as amino acids (hydrolysed and non-hydrolysed) decreased. On closer examination, only the fraction of polysaccharides soluble in hot water was proved to have contributed to endogenous respiration. When the cells had been cultivated on peptone only, endogenous respiration mainly took place at the expense of the amino-acidpool. Even when the cells had been cultivated on a nitrogen-deficient medium, endogenous respirations proceeded chiefly at the expense of the amino-acid pool and only partly of the accumulated polysaccharides. The oxidation of added glucose was depressed during the period of high endogenous respiration, while transformation of this glucose into polysaccharides did take place. The same investigators carried out a similar research with *Escherichia coli* and found that this organism first used the stored polysaccharides for endogenous respiration; not until all glycogen had been used up was the production of free ammonia observed. Glycogen was produced only when the medium was supplied with glucose [80], while other investigators found that *E.coli* mainly produced this polysaccharide during the lag-phase [74] and immediately after the completion of the logarithmic growth phase [47] when glucose was still available. Ribbons and Dawes [80] demonstrated that cells of *E.coli*, grown on a glucose-free medium, began to produce ammonia as soon as the endogenous respiration started. When glucose was supplied, however, the ammonia production was limited. This retarding effect was not observed when gluconic, succinic or acetic acid were added.

Due to the great differences in endogenous metabolism between the various

bacteria [19, 17] it will be difficult to anticipate the phenomena in activated sludge. The results of the investigations of Washington and Symons [93] correspond with those of the investigations of Ribbons and Dawes [80] with *Sarcina lutea.*

2.4 Theory on bacterial floc formation

In order to find more fundamental data on flocculation, many attemtps have been made in the course of the years to grow pure cultures of bacteria in nutrient solutions in such a way that the cells agglomerate to flocs that settle afterwards. More or less successful attempts to obtain "activated sludge" from pure cultures were carried out with the above-mentioned *Zoogloea ramigera* strains, while also other bacteria could be induced to form flocs under suitable conditions. However, up to the present only a few basic facts have been found concerning this property, forming one of the pillars of the activated-sludge process.

Some theories dealing with floc formation have been published [11, 28, 90] of which McKinney's is the most recent [61, 63]. This author suggests that the low energy level in the activated sludge system due to the low substrate/bacterial-mass ratio supplies the bacteria with inadequate amounts of motility energy for counteracting their mutual attraction. The electric surface charge is supposed to be an important factor in flocculation, while the formation of capsules or slime is also of importance. On account of observations by Porter [79], Dubos [23], Knaysi [56] and others, who established that capsules and slime consist of polysaccharides with changing contents of acetyl and amino groups, McKinney assumes that the chemical composition of the capsules and slime determines the nature of this electric charge. Peptide, direct salt or ester linkages are considered to be of minor importance for the flocculation of bacteria. Once the floc has started to form, some bacteria die and lyse; the insoluble polysaccharides would remain in the floc and entrap the less active bacteria. The size of the floc is limited owing to agitation of the system. The rate of settling of the flocs, according to McKinney, depends on their density and composition. The density is supposed to be determined by the bacteriological composition: long, filamentous, possibly branched organisms as well as a high content of living bacteria and a low content of inorganic or inert material would have an unfavourable effect on the rate of settling.

As yet many data have not been collected to support this theory. One of the few facts known about the formation of capsules and slime by bacteria in activated sludge is that polysaccharides occur in the sludge and that part of these compounds is extra-cellular [88]. Little is known about the structure and

composition of these polysaccharides and about their influence on the formation of bacterial flocs.

2.5 The substrate/bacterial-mass ratio related to the maintenance energy of bacteria

It has been found that in the phase of endogenous respiration most bacteria have lost their mobility [63] and that some species in this phase are more liable to form storage compounds than in the logarithmic phase [47, 89]. In activated sludge the bacteria are in the phase of endogenous respiration, probably due to the fact that soon after mixing of the sludge with aerated waste water, the carbon compounds present in the water are removed. After this the aeration of the activated sludge is continued for some time before mixing it again with fresh substrate. In spite of the presence in domestic sewage of surplus amounts of nitrogen, which would suggest the utilization of the carbon compounds in protein synthesis, the majority of the former compounds are converted into storage products.

Depending on the activated sludge system used, the substrate/bacterial-mass ratio is low to very low. In mixing activated sludge with waste water, approximately 2×10^{12} bacteria (2000 to 3000 mg dry wt of sludge material) per liter are supplied with an amount of organic matter with a COD varying from 300–1500 mg.

The investigation into the growth of bacteria at low substrate/bacterial-mass ratios has only started recently. Generally the relationship between the amount of substrate and bacterial growth is studied by using a small inoculum in relatively excessive amounts of substrate. A linear relationship was found between the yield of bacteria and the amount of substrate [66]; growth at a very low level of substrate is mostly determined by extrapolation (usually through the origin). According to Mallette it has to be taken into account that with very low substrate levels cells increase in size, but do not divide. As a consequence, bacterial counts do not show this type of growth, especially if the bacteria concerned are not in the logarithmic phase [Mallette, 58]. Therefore, determination of the dry weight or turbidity of the bacterial suspension is a more sensitive method for estimation bacterial growth than counting of cell numbers [26].

McGrew and Mallette [59] added small amounts of glucose to a moderately dense suspension of *E.coli* (5×10^8 cells per ml) in a mineral medium, and determined the increase in turbidity. The relationship between turbidity and amount of glucose was extrapolated through the point representing the size of the inoculum. By adding the glucose every six hours, these authors were able to determine the amount required for maintaining the culture on an equal

turbidity. Evidently, 5 μg of glucose per ml each time was exactly sufficient to maintain the level of turbidity during the experimental period. However, for keeping the number of viable bacteria at the initial level, a considerably higher amount of glucose had to be supplied than was necessary for maintaining only the turbidity at an equal level.

The experimental design of the investigations of McGrew and Mallette [59] approximates the conditions met with in the activated sludge system. Expressed in quantities customary in the purification of waste water, their substrate/bacterial-mass ratio varied from 10–800 mg COD per 2000 mg cell material (dry wt), with a threshold value for maintaining the level of turbidity of 130 mg glucose per 2000 mg cell material (6.5×10^{12} coli cells). These values are quite normal in the activated sludge process.

CHAPTER 3

MATERIAL AND METHODS *)

The activated sludge employed in the present investigation, came partly from the return conduit of the sewage purification plant at Zeist and was partly produced in the laboratory. The installation at Zeist works up sewage from this village having a strength of 30,000 population equivalents; since no significant industries are found in Zeist the sewage is mainly of domestic origin.

Activated sludge fed with sewage from the small town of Wageningen or with synthetic waste water was produced in the laboratory on a small scale. The Wageningen sewage is also mainly of domestic origin; the synthetic waste water consisted of a basal solution of mineral salts, enriched with a carbon source and a nitrogen source in such a way that the COD/N ratio approximated 36. Glucose was mostly used as the carbon source, in some instances acetate or lactate was used; ammonium sulphate and sometimes peptone served as the nitrogen source.

A COD/N ratio of about 36 was chosen, because the data presented by Helmers *et al.* [39] show that waste water having a BOD/N ratio > 32 brings about nitrogen deficiency of activated sludge, which results in a poor settling of the flocs. On the other hand waste water containing excessive amounts of nitrogen (BOD/N ratios below 17) produced sludges with increased capacity for nitrification. The generally observed COD/BOD ratio in domestic waste water is about 1.6; Porges *et al* [77] found a value of 1.2 for lactose, of 1.8 for casein and of 1.6 for skim milk. Transforming the above-mentioned limits of BOD/N ratios for a nutritionally well-balanced synthetic waste water and using an average COD/BOD value of 1.6, the COD/N ratio limits become about 25 and 48.

In the laboratory, activated sludge was produced either in an installation fed continuously or in two batch installations fed by fill and draw. A flow sheet of the former installation is shown in figure 1. The apparatus consisted of two aeration flasks 1 and 2, and a sedimentation flask 3. Both aeration flasks were trade bottles with a gradual change from bulge to neck, turned upside down. The liquid volume of the first aeration flask was 100 ml and that of the second 500 ml. The liquid in the apparatus was circulated by means of two air-lift pumps (see inset figure 1) in the following sequence: aeration flask 1,

*) Composition of culture media and analytical methods are reviewed in chapter 7.

sedimentation flask, aeration flask 2 and again aeration flask 1. The air-lift pumps were located in the neck of each flask, so that the air, used for circulating the liquid, bubbled through the liquid in the flask, achieving an excellent aeration and turbulence. The air necessary for these air-lift pumps,

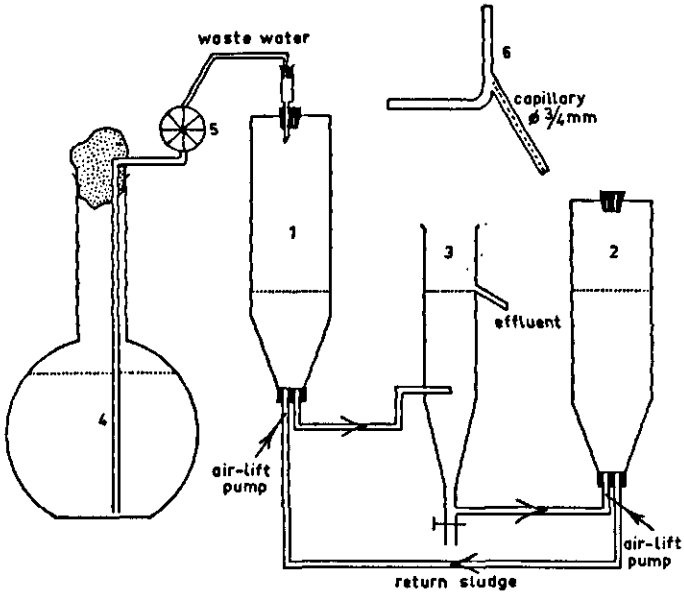

Figure 1. Continuously fed apparatus for the production of activated sludge under laboratory conditions; 1. and 2. aeration flasks, 3. sedimentation flask, 4. supply flask, 5. pump and 6. air-lift pump (enlarged).

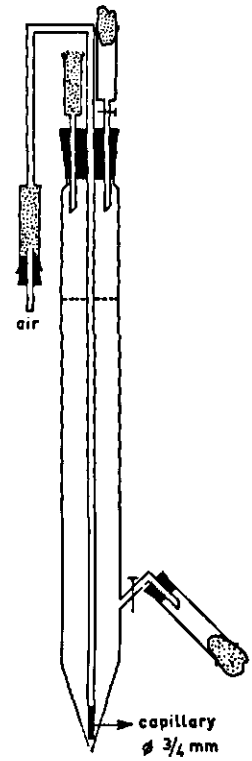

Figure 2. Apparatus to grow activated sludge or pure cultures of bacteria in batch culture fed by fill and draw.

was blown into the liquid through a capillary (⌀ 3/4 mm). The waste water was pumped from the supply-flask into aeration flask 1 with a rate of 100 ml per hour. The synthetic waste water used had a COD value of about 600 mg per liter; every day about 100 ml activated-sludge liquor was drawn off from aeration flask 2. When Wageningen sewage having a COD value of about 250 mg per liter was supplied, every day about 40 ml sludge liquor was withdrawn from the system.

Both the batch installations consisted of a glass cylinder (⌀ 6 cm) containing one liter liquid. In each cylinder aeration was obtained by blowing air into

the liquid through two capillaries (⌀ 3/4 mm) These batch installations were fed every day with synthetic waste water by fill and draw; to the first cylinder waste water having a COD value of approximately 725 was added and to the second waste water having a COD of about 1450 mg. From the first cylinder about 50 per cent of the sludge liquor was discarded once in a fortnight; from the second about 20 per cent was discarded every day.

In figure 2 a tapered glass cylinder (⌀ 5 cm) has been drawn in diagram, provided with an equipment for the cultivation of pure cultures of bacteria. The liquid volume was 750 ml; at about two-third of the height a plug was present, enabling the withdrawal of 500 ml liquid. Fresh medium could be introduced through a funnel in the stopper on the cylinder. However, this cultivation method suffered too much from infections. Aseptic conditions could be maintained more satisfactory when no liquid was drawn off and concentrated substrate was added only. Aeration was obtained by blowing air into the liquid through a capillary (⌀ 3/4 mm). The air was sterilized by passing through sterile cotton-wool and a solution of 1 ml concentrated sulphuric acid and 1 gram sublimate in one liter water.

Pure cultures of bacteria were grown in 100, 300, 500 or 1000 ml Erlenmeyer flasks containing 25, 60, 75 and 150 ml culture solution, respectively. Aeration was achieved by shaking the flasks. Additional substrate in concentrated form was added in some instances.

Both activated sludge and pure cultures of bacteria were grown at room temperature, approximately 20 °C. The aeration capacity *) at this temperature, estimated according to the sulphite method of Cooper *et al.* [18], was found to amount to 50–70 mg O_2/hour/100 ml water. This value is 1.4–5.0 times higher than the highest oxygen demand of substrate–supplied activated sludge found in the present investigation, so that the conditions for optimal aeration were fulfilled. In this respect the investigations of Pirt and Callow [75] may be recorded who found that the aeration capacity as estimated by the sulphite method has to be at least 1.4–2.0 times as high as the oxygen demand of the bacteria to assure optimal oxygen supply to the micro-organisms.

Unless stated differently, the sludge flocs or the bacterial cells were washed twice by centrifuging and resuspending in distilled water or in a solution of mineral salts, before determinations were carried out. Determinations in which living cells were involved were carried out within two hours after sampling. If no time was available to perform the necessary chemical determinations immediately, the samples were quickly frozen and stored at −20 °C.

The uptake of oxygen and the production of carbon dioxide by sludge or bacterial suspensions were determined at 20 °C by the direct manometric

*) Aeration capacity = the rate at which oxygen dissolves in oxygen-free water, expressed as mg O_2/hour/100 ml liquid.

method of Warburg. Suspensions were made in 0.033 M Sörensen phosphate buffer solution, pH 7.2, to which 5 mg $FeCl_3 \cdot 6H_2O$ and 50 mg $MgSO_4 \cdot 7H_2O$ per liter were added.

Dry weight of activated sludge or bacterial suspensions was estimated after washing by centrifuging twice, drying at 105 °C and weighing until constant weight; the ash content could be estimated after heating the same sample at 600 °C during one hour.

Organic and ammonium nitrogen were determined by a micro-Kjeldahl method; the amount of protein in cell suspensions was calculated from the organic nitrogen by multiplication by 6.25. In some instances protein was estimated directly by the biuret method [36].

Organic matter was determined by digestion with dichromate-sulfuric acid mixture and determination of the used dichromate. The amount of organic matter is expressed as COD.

Carbohydrates in clear solutions as well as in bacterial suspensions were determined colorimetrically by the anthrone method; reducing sugars were also determined colorimetrically according to the method of Nelson and Somogyi [21].

Bacterial methods are reviewed in section 4.1.

CHAPTER 4

BACTERIOLOGICAL INVESTIGATIONS

In preliminary investigations it was found that the bacterial flora of activated sludge consists mainly of aerobic, Gram-negative, non-sporeforming rods, an observation wich is in agreement with those of the literature. For the classification of this group of bacteria various schemes have been proposed [49]. In the 7th edition of Bergey's Manual [8] an important distinction is made between organisms with polar flagella and those with peritrichous flagella, the former being placed in the order *Pseudomonadales* and the latter in the *Eubacteriales*. For the differentiation of the genera, use is primarily made of biochemical characteristics. The polarly flagellated genera *Pseudomonas* and *Xanthomonas* are defined as attacking carbohydrates oxidatively with the formation of acid; *Aeromonas* and *Zymomonas*, also having a polar flagella, attack sugars fermentatively with the formation of acid or ethanol, and gas. For the detection of *Pseudomonas* two further tests are recorded to be available: the (cytochrome-) oxidase test [87] and the test for the anaerobic production of ammonia from arginine [91].

The peritrichously flagellated *Achromobacteraceae* with the genera *Achromobacter*, *Flavobacterium* and *Alcaligenes* are characterized by attacking carbohydrates (if at all) oxidatively (with or without the production of acid) which distinguishes them from the fermentative *Enterobacteriaceae*.

Bacteria which do not utilize glucose are often classified as *Alcaligenes*, therewith overlooking the fact that these organisms should have peritrichous flagella. The only genus mentioned in Bergey's Manual, satisfying the description: polar flagella and no action on carbohydrates, is the genus *Zoogloea*. Galarneault and Leifson [33] created a new genus *Lophomonas* for their lophotrichously flagellated bacteria which do not utilize carbohydrates. According to the description of the species *Zoogloea ramigera* in Bergey's Manual, the characteristics of *Lophomonas* are much like those of *Zoogloea*, except that the latter do not have the ability to form flocs.

The *Vibrio* group, as it occurs in Bergey's Manual, contains a heterogeneous collection of species of non-pigmented rods with polar flagella, some of which, because of the unreliability of the somatic curvature as a taxonomic character [49], probably ought to be near *Pseudomonas* [82] or *Lophomonas* [57].

The aim of the bacteriological inventarisation of the activated sludge floc

as carried out in the present investigation was, first, to gain an impression as to total numbers and types of bacteria present, second, to establish the morphological and physiological characters of the most frequently occurring types, and, third, to trace the role of the latter in the functioning of the sludge.

Identifying all the bacteria isolated from the activated sludge seemed to be an impossible task. It turned out that even a tentative identification of the Gram-negative bacteria, which occur most frequently in activated sludge, would be a time-consuming work that moreover would encounter many difficulties, since in some cases the boundaries of the genera are ill-defined. Owing to the latter, in a number of instances, characters of isolated organisms rather than well-defined species names had to be recorded.

In addition to organisms of the *Pseudomonas-Achromobacter* type, a number of aerobic, mostly non-motile rods with marked diversity of form were found to occur in activated sludge. Because of their morphology these organisms seemed to belong to the family of the *Corynebacteriaceae*. In this investigation they will be named „coryneforms".

4.1 Methods

Three types of activated sludge were investigated bacteriologically. These sludges were obtained from a. a laboratory installation continuously fed with sewage from the town of Wageningen, b. the sewage-purification plant at Zeist and c. a laboratory installation fed with synthetic waste water by the fill and draw system.

The isolated bacteria were classified according to morphology, attack of carbohydrates and ability to utilize ammonium nitrogen as the sole nitrogen source. In order to facilitate the classification of the isolated micro-organisms, a number of well-defined bacteria of the above-mentioned types, obtained from various culture collections, have been tested and used for comparison. Breakdown of glucose, employing the Hugh and Leifson technique (cf page 29), formed the base of the classification (table 2). According to Hugh and Leifson (48), group I includes strictly aerobic bacteria forming no acid from glucose, group II strictly aerobic bacteria producing acid from glucose, and group III facultative anaerobic bacteria forming either acid without gas (III^1) or acid plus gas (III^2) from glucose.

4.1.1 Viable counts

After 30 minutes settling down, the activated sludge was washed by decanting the supernatant, after which the sludge was suspended in a solution of 8.5 g sodium chloride per liter; this procedure was repeated once. The sludge suspension was then homogenized during two minutes in a blendor in order

Table 2. Behaviour of a number of well-defined bacterial strains in the test of Hugh and Leifson [48].

Group *)	Response to glucose		Organisms
	Aerobic	Anaerobic	
I	n	–	*Alcaligenes faecalis* (NCIB 8156) (Prague A 192)
			Vibrio percolans (NCIB 8193)
			Flavobacterium aquatile (NCIB 8535) (Prague A 208)
			Arthrobacter globiformis (Mulder st. 1 en 166)
			Brevibacterium linens (ATCC 8377)
			Mycobacterium phleï (ATCC 354)
II	A	–	*Pseudomonas fluorescens*
			Pseudomonas putida
			Pseudomonas denitrificans
			Xanthomonas phaseoli
			Xanthomonas hyacinthii
			Agrobacterium tumefaciens
			Chromobacterium violaceum
			Achromobacter lacticum (NCIB 8208)
			Flavobacterium spec.
			Corynebacterium bovis (ATCC 7715)
			Arthrobacter simplex (Mulder st. 251 en 252)
			Cellulomonas biazotea (NCIB 8077)
			Microbacterium lacticum (NCIB 8540)
			Micrococcus agilis
III[1]	A	A	*Serratia marcescens*
III[2]	AG	AG	*Escherichia coli* (NCTC 9001)
			Aerobacter aerogenes
			Aeromonas liquefaciens **)
			Proteus vulgaris

– = no reaction; n = neutral or alkaline reaction; A = acid formation; AG = acid and gas formation.

*) I and II strictly aerobic, I no acid, II acid from glucose; III facultative anaerobic, III[1] acid, III[2] acid and gas from glucose.

**) Data from Hugh and Leifson [48].

to disperse the flocs, so that after several hours settling of the sludge, clarification of the supernatant was not achieved. The aim of this procedure was to break up as much as possible the bacterial clumps, which would falsely reduce the apparent count. Allen [1] who introduced this procedure, found viable counts in various types of activated sludge after homogenizing 10–100 times higher than those in untreated samples.

From the homogenized sludge samples, dilution series were made for counting numbers of organisms. The agar plates used were of different composition, but mostly they contained tryptone and glucose. Relatively poor agar

plates were employed because earlier investigations into the bacterial vegetation of water [31] have shown that higher counts may be obtained on poor media than on the usually recommended rich media [86], owing to the fact that the pin-point colonies, which only develop after a prolonged incubation time are included in the countings on the former media.

Facultative anaerobic bacteria were counted in triplicate by transferring appropriate dilutions to a modified Allen's broth containing glucose; production of gas was observed by trapping in a Durham's tube and production of acid by adding bromo-thymol blue as indicator. Strictly anaerobic bacteria were counted in deep tryptone agar tubes. *Enterobacteriaceae* were counted in triplicate in McConkey broth and bacteria of the coli-aerogenes group in duplicate on eosin methylene blue agar (EMB-agar).

Counts of aerobic nitrogen-fixing bacteria were made on nitrogen-free glucose agar plates.

Inspection and countings of tubes and plates were generally made after 6 days' incubation at 25 °C. Prolonged incubations did not increase countings or alter reactions in tubes.

4.1.2 Isolation and maintenance of cultures

Inocula from colonies on the count plates corresponding with dilutions higher than 10^6, were tranferred to yeast-extract glucose agar slants. From the plates with the highest dilution, inocula were taken from all the colonies, while from the more crowded plates, inocula were taken from all the colonies on a sector of the plate. The slants were incubated at 25 °C until good growth was obtained and then they were stored at 4 °C.

With some of the isolated bacteria, considerable difficulties were encountered in keeping them alive on agar slopes. To avoid this difficulty in later experiments, the inocula from the count plates were first transferred into dilute nutrient broth. After growth had been obtained, this broth culture was used to make the stock culture on yeast-extract glucose agar, and used for a number of tests described below.

4.1.3 Screening tests

The isolated bacteria were examined microscopically after being incubated for 48 hours (or longer if necessary) in dilute nutrient broth at 25 °C. At first Gram-stains were made of all the bacteria, but it soon became obvious that most of the isolated strains were Gram-negative, so that no further Gram-stains were made, except when deviating forms were encountered. Irregular rod-shaped bacteria (coryneforms) often did not retain the Gram-stain. To identify bacteria of the genus *Pseudomonas* in a simple way, the cytochrome-oxidase test [32] was applied. One set of count plates was covered with a

mixture of *p*-aminodimethylaniline–2HCl and α-naphtol, and the colouring of the colonies observed.

The presence of catalase was demonstrated by covering the second set of count plates with 3 per cent hydrogen peroxide solution and by observing the production of gas.

To test the break-down of glucose by the isolated strains, stab cultures were made in the O-F medium *) according to Hugh and Leifson [48]. This O-F medium consists of a semisolid agar, which in comparison with the tryptone content is rich in sugar, so that a possible slight production of acid from the sugar will not be obscured by the production of ammonia from the tryptone. Any production of acid will be indicated by a change in colour of the bromothymol blue indicator. The test can be performed aerobically as well as anaerobically; anaerobic conditions prevail in the lower part of the tube, but may be obtained more completely, if necessary, by covering the agar with a layer of sterile melted petrolatum.

The ability of the isolated strains to grow on inorganic nitrogen was tested by using the multi-plate method according to Den Dooren de Jong [22]. A comparison was made of the growth on agar containing 0.1 per cent ammonium sulphate and 0.1 per cent tryptone, respectively, in the presence of different carbon sources. By using large agar plates a considerable number of bacteria, inoculated in small streaks, were tested on one plate. The following carbon sources were tested: lactose, glucose, glycerol, citric acid, lactic acid and acetic acid.

To test the ability to hydrolyse gelatin or casein, a number of bacteria were inoculated in small streaks on large plates containing tryptone glucose agar enriched with 5 per cent gelatin or casein. Hydrolysis of gelatin was shown by covering the agar with a solution of sublimate in hydrochloric acid. The unaltered gelatin precipitates, but around a colony of a hydrolysing bacterium the agar remains clear. Hydrolysis of casein could be observed directly by the presence of a clear zone around the colony of the hydrolising bacterium.

All the test cultures were incubated at 25 °C and inspected after six days.

4.2 Preliminary investigations

The microbial flora growing on tryptone glucose agar plates seeded with various samples of activated sludge consisted almost entirely of bacteria and only for a very small proportion of molds and yeasts.

Almost all the bacteria grown on these plates of the dilutions higher than 10^6 appeared to be Gram-negative and catalase-positive; only a few colonies

*) O-F medium means oxidation-fermentation medium.

coloured blue when tested on cytochrome oxidase indicating that representatives of the genus *Pseudomonas* were present only in small numbers.

Pasteurisation of a sample of activated sludge from the Zeist plant for ten minutes at 80 °C and subsequent plating gave a count of no more than 100–1000 aerobic spore-forming bacteria per ml, while cultures of successive dilutions of these pasteurized samples in deep tryptone agar tubes showed that strict anaerobic spore-forming bacteria were present only up to about 1000 per ml.

On tryptone glucose agar containing 0.2 per cent sodium azide inoculated with activated sludge from Zeist, Gram-positive cocci were counted in numbers corresponding to about 100–1000 per ml of sludge.

Nitrogen fixers were counted in numbers lower than 100 per ml.

4.3 Activated sludge grown in Wageningen sewage

After settling of the sludge, the supernatant still contained a number of cells. In section 4.1.1 a description is given of a washing procedure of activated-sludge flocs to be applied before estimating bacterial numbers contained in the flocs. This procedure will presumably remove part of the bacteria present in the sludge samples, because not all the cells are bound to the floc.

4.3.1 Effect of washing of the flocs on bacterial numbers

To see what percentage of the bacteria in the samples are removed by the washing procedure, bacterial numbers of untreated and washed flocs were estimated. The sludge samples were taken from the laboratory installation which was fed continuously with Wageningen sewage. Tryptone glucose agar and sewage agar *) were used for counting bacterial numbers. Additional estimations were carried out with the modified Allen's broth (see chapter 7).

From the results (see table 3) it will be seen that without washing, plate counts of activated sludge were considerably higher than when washed twice. About 60 to 70 per cent of the living bacteria were removed by the two washings. The counts in Allen's broth, McConkey broth and on EBM-agar were about proportionally decreased by the washing procedure.

Total plate counts of washed sludge corresponded with approximately 10^9 bacteria per ml, 10 per cent of which were able to form acid from glucose and about 0.1 per cent acid and gas. Only a small portion of the bacteria in the sludge appeared to belong to the *Enterobacteriaceae* and a still smaller portion to the coli-aerogenes group.

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*) Sewage agar consisted of screened Wageningen sewage solidified with 1 per cent Davis agar.

Table 3. Bacterial counts of untreated and washed activated sludge grown in Wageningen sewage (numbers per ml; dry weight of the sludge 12.0 mg per ml, sludge volume index *) 85).

	Activated sludge					
	Untreated			Washed twice		
Count medium	n	A	AG	n	A	AG
Allen's broth	10^9	10^8–10^9	10^6–10^7	10^8–10^9	10^7–10^8	10^6
Tryptone glucose agar	15×10^8	–	–	6×10^8	–	–
Sewage agar	7×10^8	–	–	1×10^9	–	–
McConkey broth	–	10^6–10^7	10^5–10^6	–	10^5–10^6	10^5
EMB-agar	12×10^4	–	–	7×10^4	–	–

n = total count; A = acid formation; AG = acid and gas formation.

4.3.2 Characterization of bacteria isolated from Wageningen activated sludge

Since the purpose of the bacteriological part of this investigation was to collect information about the nature of the micro-organisms in the activated-sludge floc, bacteria were isolated from the count plates of washed samples. From the count plates of the highest dilution, 100 strains were transferred to slants: 59 from the tryptone glucose agar plates and 41 from the sewage agar plates. These strains were tested for morphological and physiological characters according to the scheme given in section 4.1. The results of the various tests applied are given in tables 4a en 4b. As to the growth of the strains with an inorganic nitrogen source, this ability was considered to be present if a strain developed on a medium containing ammonium nitrogen plus one of the tested carbon sources.

From the results reported in table 4, it is obvious that the bacterial flora of the examined activated sludge can be mainly classified into three groups of aerobic bacteria: a. not affecting glucose, 42 and 33 per cent, respectively; b. affecting glucose without production of acid, 21 and 27 per cent, respectively; c. attacking glucose with production of acid aerobically, 30 and 15 per cent respectively. Moreover, one strain, isolated from sewage agar, was able to grow facultative anaerobically on glucose, whereas from tryptone glucose agar plates 12 such strains (23 per cent) were isolated. This gives the impression that these facultative anaerobics were unable to grow in sewage and it seems likely to suppose that they did not play an important metabolic role in the removal of waste, but were accumulated in this typical sludge only because of their abundant presence in domestic waste water.

A few strains could be classified as „coryneforms" according to their mor-

*) Sludge volume index = $\frac{\text{per cent settling by volume}}{\text{per cent suspended matter}}$

Table 4. Characteristics of bacteria isolated from Wageningen activated sludge (a) on sewage agar and (b) on tryptone glucose agar.

a. Number of strains isolated from sewage agar.

Group *)	Total	Yellow colonies	Motile	No growth with						
				NH_3-N	lactose	glucose	glycer.	citric acid	lactic acid	acetic acid
I a. Rods	20	5	15	0	19	13	1	0	8	7
b. Coryneforms	1	0	0	0	0	1	0	0	1	1
II a. Rods	10	5	6	0	8	0	0	1	4	3
III[1] a. Rods	1	0	0	1	1	0	0	0	1	1
III[2] a. Rods	1	0	0	0	0	0	0	0	0	1
Spore-forming bacteria	0									
Total	33	10	21	1	28	14	1	1	14	13
Died on slant	8									

b. Number of strains isolated from tryptone glucose agar.

I a. Rods	27	8	19	7	24	17	7	11	14	11
b. Coryneforms	4	2	0	1	0	0	1	0	1	1
II a. Rods	8	6	3	0	6	0	1	1	2	2
III[1] a. Rods	12	3	2	1	6	0	1	5	8	9
III[2]	0									
Spore-forming bacteria	1	0	1	0	1	0				
Total	52	19	25	9	37	17	10	17	25	23
Died on slant	7									

*) I and II strictly aerobic, I no acid, II acid from glucose; III facultative anaerobic III[1] acid, III[2] acid and gas from glucose.

phology; physiologically they mostly belonged to the group of aerobic bacteria that could utilize glucose without formation of acid. Because of their ability to transform into spheres on ageing, they were considered to belong to the genus *Arthrobacter.*

Hydrolysis of gelatin was observed with 40 of the 85 strains tested; 75 strains were able to grow on ammonium nitrogen in the presence of a suitable carbon source.

4.4 Activated sludge from the Zeist purification plant

Bacterial counts of homogenized sludge were made in duplicate. To diminish the selectivity of the agar media, count plates of various composition were used. All media contained the same mineral base (see chapter 7) to which 0.1 per cent ammonium sulphate or 0.1 per cent tryptone was added as the nitrogen

source and 0.6 per cent Na-citrate or 0.4 per cent glycerol, glucose or lactose as the carbon source.

To count coliform organisms, counts were made on EMB-agar, while facultative anaerobics were counted in modified Allen's broth enriched with glucose or lactose.

The results of the various counts are given in table 5.

Table 5. Bacterial counts of activated sludge from the sewage purification plant at Zeist (numbers per ml; dry weight of the sludge 2.4 mg per ml, loss on ignition 76 per cent and sludge volume index 135).

	Mineral medium +0.1 per cent ammonium sulphate			
Dilution	+Na-citrate	+glycerol	+glucose	+lactose
10^6	110	> 200	> 200	80
10^7	8	40	73	10
10^8	2	3	9	5
	Mineral medium +0.1 per cent tryptone			
	+Na-citrate	+glycerol	+glucose	+lactose
10^6	105	> 200	> 200	75
10^7	12	40	100	9
10^8	2	4	15	2

		Allen's broth enriched with					
		glucose			lactose		
	EMB-agar	n	A	AG	n	A	AG
10^6	4	+	+	+	+	+	+
10^7	1	+	+	+	+	+	+
10^8	0	+	(+)	-	+	-	-
10^9		(+)	-	-	(+)	-	-

n = growth; A = acid formation; AG = acid and gas formation; + = positive reaction in all tubes; (+) = positive reaction in part of the tubes.

Bacterial counts on plates with ammonium nitrogen were about 75 per cent of those on plates with tryptone as the nitrogen source; with glucose as the carbon source the highest counts were obtained, *viz.* approximately 10^9 per ml. In broth, about the same counts were obtained as on tryptone glucose agar plates. On plates with glycerol, citrate and particularly lactose the counts were considerably lower than on those with glucose. Facultative anaerobic bacteria were counted up to 10^7 per ml, while on EMB-agar about 4×10^6 bacteria per ml were counted, indicating, that coliform organisms formed a minor part of the facultative anaerobics, which formed a few per cent of the total number of bacteria present in this typical activated sludge.

From each of the tryptone glucose agar plates seeded with 10^8 and 10^7 times diluted sludge, 56 subcultures were collected and kept on slants. These strains were examined morphologically and physiologically for their response to glucose. The results are given in table 6.

Table 6. Characteristics of bacteria isolated from Zeist activated sludge.

Group *)		Number of strains, subcultured from tryptone glucose agar; dilution: 10^8	10^7
I	a. Rods	13	22
	b. Coryneforms	1	4
II	a. Rods	0	2
III[1]	a. Rods	0	8
III[2]	a. Rods	0	2
Total		14	38
Died on slant		2	2

*) I and II strictly aerobic, I no acid, II acid from glucose, III facultative anaerobic, III[1] acid, III[2] acid and gas from glucose.

Five strains (about 9 per cent) morphologically belonged to the genus *Arthrobacter*; they did not produce acid from glucose. The remainder of the strains were aerobic, Gram-negative, and rod-shaped; about 68 per cent of the tested strains formed no acid from glucose and belonged to group I, 4 per cent formed acid oxidatively (II) and 19 per cent fermentatively (III).

4.5 Activated sludge grown with glucose and ammonium sulphate

Subsequently the bacterial flora of two types of activated sludge, both grown in the laboratory and fed daily by fill and draw with mineral medium containing glucose and ammonium sulphate in two different concentrations (sludge type 2 was fed with twice as much glucose and ammonium sulphate as sludge type 1, see chapter 3), was examined. After homogenization of the sludges, appropriate dilutions were seeded on count plates of tryptone glucose agar and ammonium sulphate glucose agar. The bacterial counts are given in table 7.

Counts on ammonium sulphate glucose agar were slightly higher than those on tryptone glucose agar, *viz.* for sludge type 1: 21×10^7 and 14×10^7 bacteria per ml respectively, and for sludge type 2: 33×10^7 and 22×10^7 bacteria per ml.

From high-dilution count plates of sludge type 1: 105 and of sludge type 2: 125 strains were subcultured in nutrient broth. As soon as growth was visible, the strains were examined morphologically, checked for the absence of infections and inocula transferred to slants.

Table 7. Bacterial counts of two types of activated sludge grown with glucose and ammonium sulphate (numbers per ml; type 1: dry weight 5.4 mg per ml, loss on ignition 83 per cent, sludge volume index 28; type 2: dry weight 3.9 mg per ml, loss on ignition 90 per cent, sludge volume index 26).

Activated sludge	Dilution	Bacterial counts on	
		Tryptone glucose agar	Ammonium sulphate glucose agar
Type 1	10^6	141	218
	10^7	14	12
	10^8	1	3
Type 2	10^6	208	328
	10^7	31	40
	10^8	3	4

Many of the isolated strains were Gram-negative rod-shaped bacteria, but an even larger number had more or less spherical cells, partly $< 2\mu$ and partly 2–4 μ. The cocci $< 2\ \mu$ appeared to be Gram-positive and since at closer examination no pleomorphy was observed, it was concluded that these strains belonged to the genus *Micrococcus*. About 24 per cent of the isolated strains from sludge type 1 were *Micrococci* and about 27 per cent from sludge type 2. The irregular egg-shaped cocci, 2–4 μ large, had thick capsules, even in very young cultures; sometimes these bacteria were laying in tetrads embedded in a common capsule. The Gram-stain was not retained by the cells, though this might be rather erratic because of the thick capsules. It was not possible to classify these large bacteria as far as their genus with the help of Bergey's Manual. Some photomicrographs of these large coccoid bacteria are given in plate I; to show the capsules, photographs are given of suspensions mounted in Indian ink according to Duguid [25].

At closer microscopic examination it was possible to observe similar coccoid bacteria, often laying in tetrads in various types of activated sludge, particularly in sludge grown on dairy waste water.

From the two types of activated sludge grown on glucose and ammonium sulphate a few filamentous bacteria were also isolated, one of which could be classified as belonging to the genus *Nocardia*. No *Sphaerotilus*-like bacteria were isolated.

The classification according to morphological characters of the bacterial strains isolated from both types of sludge is given in table 8.

All strains isolated from sludge type 2 were tested in the O-F medium for their response to glucose and by the multi-plate method for their growth on mineral medium with ammonium sulphate and glucose or lactose. The results of these tests are reviewed in table 9.

Table 8. Classification according to morphological characters of bacteria isolated from activated sludge grown with glucose and ammonium sulphate.

Morphology	Number of subcultures from count plates seeded with dilutions			
	Sludge type 1		Sludge type 2	
	10^6	10^7	10^6	10^7
Gram-negative rods	2	33	4	40
Gram-positive cocci $< 2\mu$	2	18	2	30
Egg-shaped Gram-negative cocci $2–4\mu$	2	26	5	36
Filaments	0	1	0	3
Total	6	78	11	109
Not grown in nutrient broth	2	19	0	5

Most of the Gram-negative rod-shaped strains from sludge type 2 were able to grow on glucose-containing mineral medium without production of acid; only a few strains were able to grow on mineral medium containing lactose. Some strains attacked glucose oxidatively with production of acid, but none was able to grow facultatively anaerobically on glucose. About 20 per cent of the rod-shaped strains, mostly having uncoloured colonies, were unable to grow on the mineral medium with glucose; in the sludge these strains apparently utilized decomposition products of glucose and dead bacteria. Approximately 60 per cent, half of them having yellow colonies, grew on the mineral

Table 9. Response to glucose and ability to grow with ammonium nitrogen of bacteria isolated from activated sludge grown with glucose and ammonium sulphate.

Group *)		Numbers of strains			
		Total	Yellow colonies	No growth on $(NH_4)_2SO_4$	
				+glucose	+lactose
I	a. Rods	35	12	8	29
	b. Cocci $< 2\mu$	12	0	0	9
	c. Egg-shaped cocci $2–4\mu$	17	0	0	10
	d. Filaments	0			
II	a. Rods	9	2	0	7
	b. Cocci $< 2\mu$	20	0	0	19
	c. Egg-shaped cocci $2–4\mu$	24	0	0	20
	d. Filaments	3	0	1	0

*) I and II strictly aerobic, I no acid, II acid from glucose.

medium with glucose without production of acid, while the final 20 per cent produced acid from glucose oxidatively. Of the last group, comprising 9 strains, two had yellow colonies and three were positive in the oxidase test.

Most of the isolated coccoid strains were able to grow in mineral medium with glucose and about half of them also with lactose; many of these cocci produced acid from glucose oxidatively and only a few strains were facultative anaerobes.

No catalase-negative strains were encountered and about 74 per cent of the strains was able to hydrolyse gelatin, although some, particularly the large coccoid strains, only very weakly.

4.6 Discussion

Due to the variations in composition of different waste waters, it may be expected that activated sludges growing in these waters, vary in bacterial flora. Although soil, aerial and intestinal forms of micro-organisms may be found in sewage, it is reasonable to expect that the organisms responsible for the formation and the operation of the activated sludge flocs represent a more or less specialized group of (water-)organisms. Isolation and identification of the predominant types of organisms as well as studying their physiological characters was the aim of this part of the investigation. Pure cultures of these organisms were employed to gain information concerning the floc formation and the metabolism in activated sludge (cf chapter 5 en 6).

Moulds, yeasts, spore-forming bacteria and bacteria of faecal origin were found to be present in minor numbers in the three different types of activated sludge analysed bacteriologically in the present investigation. Calculated per gram washed activated sludge (dry wt), bacterial counts of the three types of sludge tested were approximately 10^{11}. These counts were considerably higher than those reported by Heukelekian [41], presumably because the sludges applied for the countings were not homogenized as contrasted with the present investigation.

In the activated sludge from Zeist the highest bacterial numbers were obtained when the dilute suspensions were seeded on tryptone glucose agar, although those on ammonium sulphate glucose agar and on tryptone glycerol agar were only slightly lower. This indicates that most of the bacteria in the sludges concerned were able to use glucose for growth, but did not require this carbon source. Most strains were able to grow on a mineral medium containing ammonium nitrogen and glucose.

In activated sludge grown in such a complex waste water as sewage, a pronounced contamination of the bacterial flora of the sludge with relatively large numbers of bacteria conveyed by the domestic wastes may be expected

to occur. Although the microbial flora of fresh waste water consists mainly of facultative anaerobes, including a certain number of intestinal bacteria, only moderate numbers of bacteria of the latter type have been found in the present investigation in the sludges grown in Wageningen or Zeist sewage. Allen [2] has already shown that intestinal and even facultative anaerobic bacteria form a minor part of the bacteria present in activated sludge. In this respect the results of the present investigation are in agreement with those of Allen.

The bacteria found in the activated sludge flocs grown on sewages of domestic origin belonged predominantly to the family of the *Achromobacteraceae*. In addition, members of the *Pseudomonadaceae* and *Corynebacteriacea* were found to occur. These results partly agree with those of Allen [2], who found that the majority of the bacteria in this type of activated sludge consisted of Gram-negative, non-spore-forming rods, not affecting sugars and tentatively determined as belonging to the genera *Achromobacter, Flavobacterium* and sometimes to the genus *Pseudomonas* (because of the formation of a diffusable green fluorescent pigment). Although also in the present investigation the genera of the isolated bacteria were not identified exactly, a sufficient amount of information was gathered to show that *Pseudomonas* constitutes only a minor part of the predominant bacteria of activated sludge grown on domestic sewage. Members of the genera *Achromobacter, Alcaligenes, Flavobacterium* and possibly *Lophomonas* were found to be the main constituents of the bacterial flora of such sludges. Most of the Gram-negative rod-shaped strains, isolated from the domestic types of activated sludge did not produce acid from glucose. A large part of these strains was not able to affect glucose at all, thus presumably belonged to the genera *Alcaligenes* and *Lophomonas*. The strains attacking glucose without production of acid apparently belonged to the genus *Achromobacter* or if they had yellow colonies to the genus *Flavobacterium*. A smaller number of the Gram-negative rod-shaped strains utilized glucose aerobically with production of acid; many of these strains had yellow colonies and probably were representatives of the genus *Flavobacterium*, while of the remainder a few had a positive oxidase reaction test *(Pseudomonas)* and the others were considered to belong to the genus *Achromobacter*. (According to the 7th edition of Bergeys' Manual the genus *Achromobacter* as well as the genus *Flavobacterium* includes organisms which oxidize glucose oxidatively; both genera may comprise either strains which form acid from glucose or strains forming no acid). Most of the strains not tested because of their early death, gave yellow colonies on the slant and probably were also *Flavobacterium* species.

Only a few strains were able to grow anaerobically in the presence of glucose; their number varied with the composition of the count plate. On sewage agar plates they were found in considerably lower numbers than on tryptone glucose

agar plates, which may indicate that though these organisms occur in moderate numbers in the flocs, they do not grow very well on sewage.

Only a few "coryneforms", all belonging to the genus *Arthrobacter*, were isolated from domestic activated sludge.

From the two types of activated sludge produced in the laboratory in mineral medium with glucose and ammonium sulphate also many strains belonging to the *Achromobacteraceae* were isolated. Most of these Gram-negative rod-shaped strains utilized glucose only aerobically without the production of acid; about half of these strains formed yellow colonies and probably belonged to the genus *Flavobacterium*, while the other half belonged to the genus *Achromobacter*. Representatives of the genera *Alcaligenes* and *Lophomonas* were present to a small extent in this type of sludge. Only a few of the isolated rod-shaped strains attacked glucose oxidatively with production of acid.

The majority of the bacterial strains isolated from these types of laboratory sludges consisted of coccoid strains. Part of these presumably belonged to the *Micrococcaceae*, but the majority were large, Gram-negative, capsulated, egg-shaped bacteria (about 2–4 μ), of which most strains could utilize glucose aerobically with or without production of acid. The genus of these large coccoid bacteria could not be ascertained with the aid of Bergey's Manual. Morphologically they looked much like the *Nitrocystes* described by Winogradsky, but because of their unability to oxidize ammonia they are physiologically entirely different.

A review of the relative numbers of predominant bacteria isolated from the various types of sludge examined, is given in table 10. The high percentages of species unable to utilize glucose in both the domestic types of activated sludge, may indicate that the Wageningen and Zeist sewages contain no or only low amounts of sugars as a result of which the relating sludges are not adapted to those compounds.

Several bacterial strains isolated from the four types of sludge could not be tested. After tranfer of these strains to the yeast-extract glucose agar slants, some of them did not grow at all, while others after initial satisfactory growth on the slants could not be kept alive. The latter sometimes were kept alive by transferring the inoculum from the count plate directly into nutrient broth; nevertheless the growth on agar of most of these strains was not improved by this treatment.

It may be concluded that the growth conditions on agar media are not suitable for a number of bacteria living in the activated sludge. This could mean that in activated sludge more bacteria are present than are counted on agar plates. On the other hand the counts in liquid media were only slightly higher than those on count plates. Therefore, it may be assumed that the predominating bacteria in activated sludge can be counted on count plates as well.

The method of isolation applied in the present bacteriological investigation is probably not suitable for the isolation of representative strains of the genus *Zoogloea*, principally because it is stated in literature that *Zoogloea* strains grow poorly on agar media. It may be that the slight difference between counts in liquid media and on plates resulted from the presence in the sludges tested of *Zoogloea ramigera*. If this were true it must be concluded that only a minor part of the bacteria in activated sludge belong to the genus *Zoogloea*.

Table 10. A review of the relative numbers of genera of predominant bacteria isolated from various types of activated sludge.

	Percentage of strains isolated from activated sludge grown in:			
	Wageningen sewage		Zeist sewage	Mineral medium with glucose and $(NH_4)_2SO_4$
Genera of the strains	I	II	III	
Alcaligenes / *Lophomonas*	40	33	45	7
Achromobacter	16	6		16
Flavobacterium	30	27	23	12
Pseudomonas	5	2	4	3
Arthrobacter	3	7	9	
Micrococcus				27
Egg-shaped cocci				34
Fac. anaerobic rods	6	23	19	

I isolated on sewage agar plates; II isolated on tryptone glucose agar plates.

CHAPTER 5

FLOCCULATION OF BACTERIA IN PURE CULTURE

In the bacteriological examination as discussed in chapter 4, data were obtained on the nature of the bacteria occuring most frequently in activated sludge. To analyse the function of these bacteria in the sludge, it appeared to be desirable to investigate which of these bacteria in pure culture were able to form flocs showing the same properties as activated-sludge flocs, *i.e.* rapidly settling and leaving a clear supernatant liquid. It therefore seemed most significant to examine the conditions under which bacteria will flocculate.

According to McKinney and Weichlein [62] many bacteria flocculate, provided conditions are suitable, *e.g.* when the substrate/bacterial-mass ratio is low and the surface charge is within a range favourable for flocculation [63]. Especially *Alcaligenes faecalis* is supposed to be a bacterium which easily flocculates in pure culture. Accordingly, the floc-forming property of the following bacterium-species was examined: *Alcaligenes*, *Achromobacter*, *Flavobacterium*, *Lophomonas*, *Pseudomonas* and the large egg-shaped capsulated coccoid bacterium described in section 4.5. In other words those strains belonging to the groups I and II (strictly aerobic bacteria, either forming acid or not from glucose) in the classification system used in this investigation.

5.1 Screening of bacteria on floc-forming ability

The ability of bacteria to flocculate in pure culture was examined by growing bacterial strains in nutrient solutions in Erlenmeyer flasks which were rapidly shaken mechanically. Every day, shaking was stopped for three hours in order to observe any possible flocculation. This was considered to take place when the bacteria settled within three hours, leaving the supernatant liquid clear.

5.1.1 Screening of strains isolated from activated sludge grown in sewage of Wageningen.

Some twenty bacterium strains isolated from activated sludge grown on sewage from Wageningen were tested for flocculation. All twenty strains were Gram-negative, rod-shaped and were able to grow in a mineral medium with a small quantity of yeast-extract, ammonium sulphate and Na-lactate. The bacteria were inoculated in three different nutrient solutions, consisting of a basal solution of mineral salts (see chapter 7) and a. peptone 2 g/l, b. yeast-extract 1.5 g/l and Na-lactate 1.5 g/l, or c. yeast-extract 1.5 g/l and glucose 1.5 g/l.

The tested strains showed good growth in these nutrient solutions. No true flocculation was observed with any of these strains, although the cell masses of a number of bacteria did not grow completely dispersed in the nutrient solution, but initially were attached for the greater part to the glass wall at the interface of air and liquid, from which they were removed by shaking later on.

5.1.2 Screening of identified strains.

The following strains, obtained from various collections, were tested similarly to the strains isolated from sludge: *Pseudomonas aeruginosa*, *Ps. fluorescens*, *Achromobacter lactium* (NCIB 8208), *Flavobacterium aquatile* (NCIB 8535), *Fl. aquatile* (Prague A208), *Alcaligenes faecalis* (NICB 8156), *Alc. faecalis* (Prague A192) and *Vibrio percolans* (NICB 8193). Furthermore, a strain was included, isolated from activated sludge, grown in dairy waste water, which had the following properties: Gram-negative, rod-shaped, monotrich, liquified gelatin, used glucose to a very slight extent, formed no acid from glucose, grew poorly on agar media and not at all in mineral nutrient solutions with glucose, glycerol or Na-lactate. In view of these characteristics and of its floc-forming ability, this strain was considered to belong to the genus *Zoogloea*.

The bacteria were inoculated in a nutrient solution of 1.5 g/l of yeast-extract and 1.5 g/l of Na-lactate. After incubation for six days, only three strains showed some flocculation. The *Zoogloea* strain formed a reasonable amount of slowly settling flocs; the supernatant, however, was not entirely clear. The strains *Alc. faecalis* (Prague A192) and *V. percolans* (NCIB 8193) formed also a small amount of slowly settling flocs, while the supernatant after settling was completely turbid. All the remaining bacterial strains showed a completely dispersed growth.

5.1.3 Screening of strains isolated from activated sludge grown with glucose and ammonium sulphate.

Fifteen bacterium strains isolated from activated sludge, grown on synthetic waste water (glucose and ammonium sulphate) were tested for flocculation. The strains were inoculated in nutrient solutions consisting of a basal solution of mineral salts and a. glucose 6 g/l and $(NH_4)_2SO_4$ 0.5 g/l, b. glucose 3 g/l and casamino acids 1.0 g/l. After six days of rapid mechanical shaking, three strains appeared to have flocculated in both nutrient solutions, although the supernatant was not entirely clear after the flocs had settled. Six other strains appeared to have formed bacterial masses which settled moderately rapid, but could not be considered as flocs, however; after settling the supernatant liquid was clear.

The three floc-forming bacterium strains were Gram-negative and rod-shaped; two of these could be described as belonging to the genus *Achromobacter*,

while the third, on account of its yellow colour, was considered to be a *Flavobacterium*. It was striking to observe that these bacteria occurred in the floc as short, thick rods, whereas in dispersed condition they appeared to be active motile, slender rods.

The six strains, forming non-flocculently settling masses belonged to the earlier described bacterium species with large egg-shaped capsulated bacteria. The liquid in which these bacteria had grown was quite clear after 5 to 6 hours; the settled bacterial mass, however, was considerably larger than that of activated sludge.

5.2 Influence of polyvalent ions on the flocculation of bacteria in pure culture

Most of the tested bacterial strains did not appear to be able to form settling flocs in a normal nutrient solution. The investigations of Eddy [27], and Jansen and Mendlik [50] into the flocculation of *Saccharomyces cerevisiae* showed that dispersed yeast cells could be induced to flocculate by adding calcium ions and to a smaller extent also by adding ferric and other bivalent or polyvalent cations. In the presence of about 10 g/l of calcium sulphate, a clear ageing effect in flocculation was observed. Cells 20 hours old did not flocculate, while 60 hours old cells did; when sugars were added, however, these yeast flocs disappeared again.

The following experiments were carried out to decide whether addition of calcium or ferric ions to bacterial suspensions would affect flocculation favourably, *i.e.* to decide whether more bacterium species would flocculate, leaving the supernatant after settling of the flocs completely clear.

5.2.1 Effect of calcium ions on the flocculation of bacteria in pure culture.

A. *Precultivated cells.* Various bacterium strains were inoculated in large aeration vessels containing 500 ml of a nutrient solution consisting of a basal solution of mineral salts and 2 g/l of tryptone. The following strains were inoculated: one *Achromobacter* strain and one *Alcaligenes* strain, isolated from activated sludge, grown in sewage of the town of Wageningen and furthermore the above-mentioned *Zoogloea* strain, *Alc. faecalis* (Prague A192) and *V. percolans* (NCIB 8193).

After five days, when the bacteria in all vessels showed good growth, 1 ml of a solution containing 50 mg of calcium chloride and 5 mg ferric chloride was added to each vessel during aeration (because of turbulence). One minute afterwards, aeration was stopped and the suspension was observed for one hour to see whether flocculation and settling occurred. The addition was repeated until flocculation or settling occurred. After the third addition, distinct

formation of settling flocs occurred in all vessels. However, only the supernatant in the vessel with the *Zoogloea* strain was clear after settling of the flocs.

B. *Growing cells.* Five series of four Erlenmeyer flasks, each containing 100 ml of a nutrient solution (tryptone 1.5 g/l and Na-lactate 1.5 g/l) were supplied with the following separately sterilized solutions:

a. 2 ml of $CaCl_2 \cdot 2H_2O$ (20 mg);
b. 2 ml of Sörensen phosphate buffer 0.067M, pH 7.2;
c. 2 ml of $CaCl_2 \cdot 2H_2O$ (20 mg) + 2 ml of phosphate buffer (b);
d. no addition.

Each series was inoculated with one of the following bacterium strains: *Zoogloea* spec., *Alcaligenes faecalis* (Prague A192), *Vibrio percolans* (NCIB 8193), *Flavobacterium aquatile* (Prague A208), and *Pseudomonas fluorescens.*

A distinct effect of the additional calcium chloride on flocculation was already to be observed after four days, though not with all five strains. Six days after inoculation, the *Pseudomonas* and the *Flavobacterium* strains showed completely dispersed growth with added calcium chloride, whereas the other strains formed flocs settling reasonably rapidly in two hours, leaving the supernatant clear. Without added calcium ions only the *Zoogloea* strain flocculated, the supernatant was, however, not clear after settling. The remaining bacterial strains showed completely dispersed growth. Addition of the phosphate buffer did not have any effect on flocculation.

5.2.1.1 Effect of increased amounts of calcium ions in the nutrient medium on the flocculation of a *Zoogloea* species.

In order to examine how much calcium chloride was needed to induce a suspension of *Zoogloea* spec. to flocculate, this bacterium was inoculated in Erlenmeyer flasks containing 100 ml of a nutrient solution, consisting of 1.5 g/l of tryptone and 1.5 g/l of Na-lactate and supplied with increasing amounts of calcium chloride, *viz.*: 0, 1, 2, 4, 6, 8, 10, 15, 20 and 30 mg per 100 ml. Four days after inoculation, flocculation was to be observed in the flasks with 6 mg of calcium chloride or more. After settling, the supernatant was not clear. Six days after inoculation, flocculation was to be observed in all flasks. In the flasks with less than 4 mg of calcium chloride, growth was still dispersed for the greater part; in the flasks with 6 mg or more, the supernatant was completely clear two hours after the shaking was stopped.

5.2.2 Effect of ferric ions on the flocculation of bacteria in pure culture.

The effect of ferric and calcium ions on the floc formation and settling of the flocs was studied with two strains of the large coccoid bacteria isolated from activated sludge (see page 35). Both strains were inoculated in 75 ml of a nutrient solution consisting of a basal solution of mineral salts, 1 g/l glucose

and 0.25 g/l ammonium sulphate; from two days after inoculation onwards, 2 ml of a sterile nutrient solution, consisting of 75 mg of glucose and 20 mg of ammonium sulphate, was added daily during nine days. The pH of the nutrient solution was kept at 7.2 by means of potassium hydroxide.

Flocculation was not observed in either of the strains during the cultivation period; the bacterial mass settled in about four hours, leaving the supernatant clear.

Eleven days after inoculation, the bacterial masses were separated by centrifugation, washed with distilled water, centrifuged again and afterwards suspended in distilled water. The suspensions of both strains were made up into two series of tubes containing 5 ml suspension per tube (10 × 120 mm). One series of tubes was supplied with increasing amounts of calcium chloride, the second with different amounts of ferric chloride. After shaking, the contents of the tubes were inspected for flocculation. It appeared that addition of 200 μg of ferric chloride or more per 5 ml suspension of both strains brought about flocculation and accelerated settling of the bacterial masses. Addition of calcium chloride up to 800 μg per 5 ml on the other hand did not appear to induce flocculation or to accelerate the rate of settling of the bacterial masses.

5.3 Effect of the C/N ratio of the nutrient solution on flocculation

Symons and McKinney [88], in experiments with batch cultures, found that activated sludge showed poor flocculation in a nutrient solution with a C/N ratio of over 32, so that bacteria were lost in renewing the nutrient solution. The data of Helmers *et al.* [39], mentioned in chapter 3, indicate also decreased flocculation at high BOD/N ratios.

In the present investigation, the effect of the C/N ratio of the nutrient solution on flocculation was examined by growing activated sludge and bacteria in pure culture in nutrient solutions with varying C/N ratios.

5.3.1 Effect of the C/N ratio on the flocculation of activated sludge.

Four sterile aeration vessels A, B, C and D were supplied with 750 ml of sterile nutrient solution with C/N ratios of 3, 10, 20 and 40, respectively. Na-lactate was used as the carbon source and ammonium sulphate as the nitrogen source. The lactate concentration was 2000 mg/l in all vessels. Inoculations were carried out with one ml of a 10^6 dilution of homogenized activated sludge grown in a laboratory apparatus and fed continuously with synthetic waste water (basal solution of mineral salts + 1 ml/l of Na-lactate (60 per cent) + 85 mg/l ammonium sulphate).

Five days after inoculation, some settling, but no flocculation was observed in all vessels. The cultures in the vessels A and B were green, those in C and D

white. From this day onwards, after an one hour's settling period, 500 ml culture solution was drawn off daily and replaced by fresh nutrient solution with the correct C/N ratio. On the eighth day, flocculation had occurred in all vessels, although after a settling period of one hour the supernatant was clear in none of the vessels. Twelve days after inoculation, a reasonable amount of flocs had been formed in all vessels which settled well; one hour after terminating the aeration, the supernatant in the vessels B, C and D was clear, but it was still turbid in A. The green colour in vessel B had almost disappeared, but it was still visible in A. In all vessels the flocs were coloured greyish white; in A they had a better structure and settled more readily than in the other vessels. After separation by centrifugation, the COD in the supernatant after substraction of the value for the basal solution with mineral salts only was nil. Accordingly, it may be assumed that all organic matter had been removed. After suspending the centrifuged floc mass, bacteria were counted on ammonium lactate agar plates. Furthermore, dry weights of the formed sludge masses and carbohydrate contents were determined (table 11). The results of

Table 11. Dry matter, carbohydrate content and number of bacteria in activated sludge grown in synthetic waste water with varying C/N ratios.

Vessel	C/N ratio	dry wt (mg/l)	carbohydrates (%, dry wt)	bacteria (numbers per ml)
A	3	1251	3.1	5.2×10^7
B	10	1324	3.3	4.7×10^7
C	20	1124	6.1	3.5×10^7
D	40	900	6.2	2.5×10^7

this experiment show that with increased C/N ratio of the nutrient solutions, sludge production was lower, while the carbohydrate content, as estimated by means of anthrone, was clearly higher. The highest values obtained were relatively low, however, as compared with values obtained in other experiments (cf tables 20 and 21). Bacterial numbers in the sludges grown at a low C/N ratio were about twice as high as those occurring at a high ratio.

5.3.2 Effect of the C/N ratio on the flocculation of Alcaligenes faecalis *(Prague A192).*

This experiment was also carried out in four large aeration vessels containing 750 ml of nutrient solution. In addition to a basal solution of mineral salts, the nutrient medium was supplied with peptone and glycerol in such quantities as to provide C/N ratios of about 5, 10, 20, 30. The glycerol concentration varied, while the peptone concentration remained constant at 360 mg/l, so that the COD values varied considerably in the different media.

From the third day after inoculation with *Alc. faecalis* (Prague A192) onwards, after an one hour's stopping of the aeration, 500 ml of the culture solution was drawn off daily and replaced by fresh medium with the correct C/N ratio. After six days, only little growth was observed in all vessels, presumably owing to the low peptone concentration in the media. No settling occurred in any of the vessels, so that due to the regular renewal of the nutrient solution, two-thirds of the bacterial cells were lost daily. Therefore, an additional amount of peptone was supplied once (3 g per vessel). After this the bacterial mass in all vessels rapidly increased, though flocculation was not observed in any of the vessels. Four days after the additional peptone supply, the nutrient solution was daily renewed again with substrate of the correct C/N ratio.

The experiment was terminated seventeen days after inoculation; none of the four vessels had shown any flocculation or settling.

5.3.3 Effect of the C/N ratio on the flocculation of a Zoogloea *species.*

This experiment was carried out in one liter Erlenmeyer flasks containing 100 ml of nutrient solution. The latter consisted of mineral salts (including 200 mg/l of calcium chloride), tryptone and Na-lactate in such amounts that the C/N ratios were about 5, 10, 30 and 50. The organic carbon content was kept constant at about 400 mg/l. The *Zoogloea* strain (cf page 42) isolated from dairy waste activated sludge was used for inoculation. From the second day after inoculation onwards, 10 ml of concentrated substrate (containing approximately 40 mg C) with the correct C/N ratio was added daily. The pH was corrected by adding dilute HCl when necessary.

Two days after inoculation, formation of very small flocs was observed in the flasks with the highest C/N ratio; one day later, flocculation had taken place in all flasks. The flocs settled well, leaving the supernatant clear after one hour. This continued during the entire experimental period of nine days. As to the influence of the C/N ratio in the substrate on flocculation, the results of this experiment did not allow any conclusions to be drawn.

5.4 Discussion

The experiments described in this chapter show that flocculation of bacteria in pure culture comparable to activated sludge is not easily achieved. Addition of calcium and ferric ions in relatively small concentrations in some cases did induce flocculation in suspensions of certain bacteria. In the case of calcium, this was particularly true when it was supplied in the nutrient solution in which these bacteria were growing. After settling of the flocs, however, in many cases the supernatant was not clear, *i.e.* was not free from cells. A number of bacterium species tested, *e.g.* those belonging to the genera *Pseudomonas* and

Flavobacterium did not flocculate, even after adding calcium and ferricions. With *Alc. faecalis* and *V. percolans* it was possible to form flocs after addition of extra calcium ions to the nutrient solution. The *Zoogloea* strain formed flocs in nutrient solutions without extra calcium added, but the flocculation was obviously more complete when extra calcium chloride was supplied. Addition of ferric chloride to suspensions of large egg-shaped cocci brought about flocculation and an increase in the rate of settling, whereas addition of calcium chloride had no such effect.

These results clearly show that polyvalent ions may be of significance for the formation of bacterial flocs, but the bacteria first have to meet certain conditions. One of the conditions presumably is the enclosure of the bacterial cells by a layer of slime. Though this slime layer probably consists of polysaccharides, moderate concentrations of these compounds were detected by means of the anthrone method in activated sludge grown on lactate and ammonium sulphate (table 11). In the sludges grown in sewage, about 12 per cent of the dry matter consisted of polysaccharides, while the cell masses of some of the large egg-shaped coccoid bacteria likewise contained no more than about 10 per cent of polysaccharide in the dry matter. These values are considerably lower than those obtained in activated sludges grown with glucose (tables 20 and 21) and those recorded in the literature for sludges grown with acetate or glycine [93]. Since the anthrone method, used in the present investigation for the determination of polysaccharides, detects reducing sugars and polymeric compounds liberating reducing sugars upon hydrolysis with sulphuric acid, slimy compounds of still unknown composition apparently occur in relatively large amounts in certain sludges and in certain bacterial capsules.

If it is assumed that slimy substances surrounding the bacterial cells contribute to floc formation, it could be expected that an increased C/N ratio of the nutrient solution by virtue of higher production of polysaccharides by the bacteria would promote flocculation. This suggestion, however, would be in contradiction with the experience [39, 88] that high BOD/N ratios of waste water deteriorate settleability of activated sludge. Experiments carried out in the present investigation with activated sludge and with pure cultures of *Alcaligenes faecalis* (Prague A192) and of a *Zoogloea* strain did not yield any indication that the C/N ratio of the nutrient solution affects the flocculation of bacteria.

CHAPTER 6

METABOLIC INVESTIGATIONS

6.1 Dissimilatory activity of activated sludge

Investigations recorded in literature have indicated that there is a relatively low uptake of oxygen during the removal of organic compounds by activated sludge. Moreover, it is stated that these organic compounds are assimilated to polysaccharides, which are only partly available as an energy source for the bacteria in the sludge [93]. A relatively small part of the substrate is supposed to be used for the increase in the number of bacteria. The slight oxydative decomposition and the formation of polysaccharides by activated sludge indicate a relation with the phenomenon, described *e.g.* by Barker [5, 6] as oxydative assimilation.

In the present investigation, the dissimilation percentage during the removal of various organic compounds by different types of activated sludge was examined. The effect of various nitrogen sources on the dissimilation of carbon compounds was also determined. To elucidate the bacterial processes going on in activated sludge, a comparison was made between dissimilation and assimilation phenomena as observed in activated sludge with those in pure cultures of bacteria.

6.1.1 Dissimilatory activity of washed suspensions of activated sludge from Zeist.

The oxygen uptake of activated sludge from Zeist *) was measured after adding various substrates. The following substrates were tested: lactose, sucrose, glucose, Ca-gluconate, ribose, glycerol, mannitol, ethanol, Na-acetate, Na-lactate, Na-pyruvate, Na-malate, Na-succinate and Na-citrate. Figure 3 reflects some of the oxygen uptake curves obtained.

In determining which part of the substrate is respired, the question arises whether the total oxygen uptake after addition of the substrate should be considered a standard or the so-called net-oxygen uptake (*i.e.* substrate-oxygen consumption minus endogenous oxygen consumption). As no data were available on the extent to which endogenous respiration is repressed by the added substrate, it was decided not to take the net-respiration as a standard for dissimilation, except when substrate respiration was only slightly higher than endogenous respiration.

*) Zeist activated sludge data: loss on ignition 75, nitrogen 6.7, polysaccharides 12 per cent of dry wt.

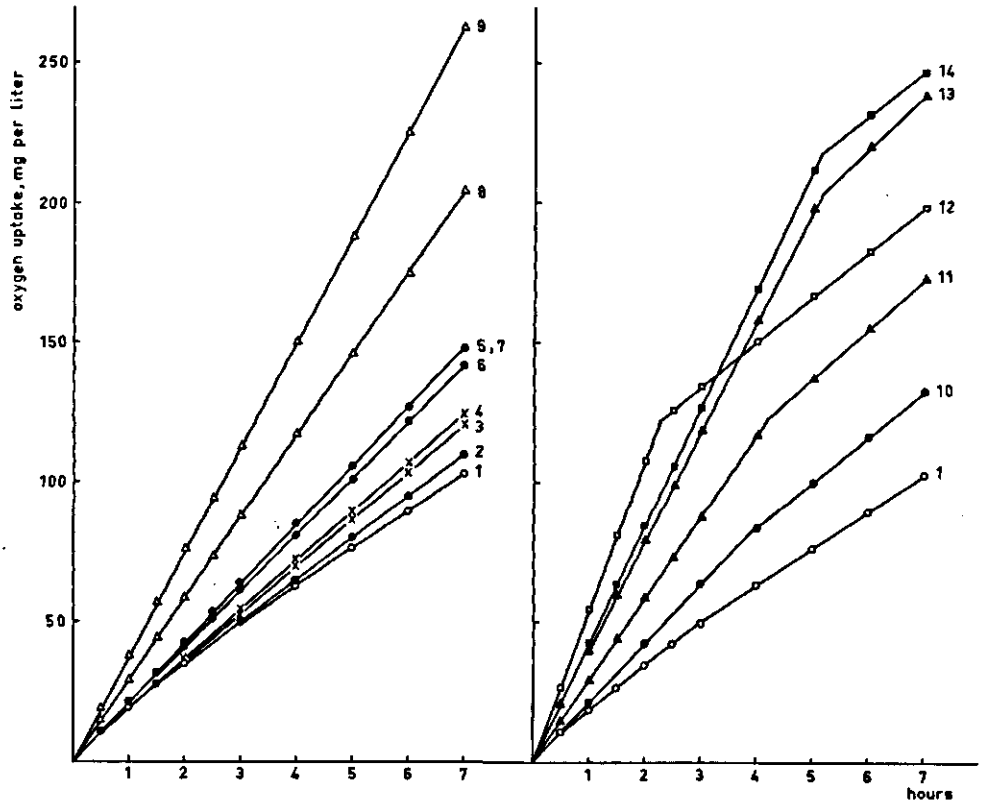


Figure 3. Oxygen uptake by washed suspensions of Zeist activated sludge after addition of various organic compounds (calculated as mg O_2 per liter sludge suspension of 2340 mg dry wt). 1. endogenous; 2. mannitol 660 mg/l; 3. Na-α-ketoglutarate 1500 mg/l; 4. Na-citrate 1470 mg/l; 5. glucose 900 mg/l; 6. fructose 900 mg/l; 7. glycerol 470 mg/l; 8. Na-malate 890 mg/l; 9. Na-succinate 1350 mg/l; 10. glucose 300 mg/l; 11. Na-pyruvate 550 mg/l; 12. Na-acetate 680 mg/l; 13. Na-lactate 560 mg/l; 14. ethanol 230 mg/l.

With most organic acids and ethanol a distinct increase in the rate of oxygen consumption was observed after addition to the activated sludge. After some time this increased rate of oxygen consumption decreased rather abruptly, indicating depletion of the substrate. This could be established by centrifugation of the sludge mass and reacting upon the substrate in the clear supernatant or by determining COD.

Table 12 presents a number of data calculated from the oxygen-uptake curves in figure 3.

Most of the organic acids and ethanol were respired readily by activated sludge from Zeist. The dissimilation percentage at the moment the rate of oxygen consumption decreased was always much smaller than 100 per cent. It may therefore be assumed that large parts of the substrate were assimilated. Only

Table 12. Rates of oxygen consumption and dissimilation percentages of washed suspensions of Zeist activated sludge after addition of various substrates.

Substrate	Q_{O_2}	Q_{O_2}'	Dissimilation percentage *)
Glucose, fructose	9.1	1.5	–
Ca-gluconate	7.5	0.0	–
Mannitol	7.6	0.1	–
Glycerol	9.1	1.5	–
Na-glycerophosphate	8.5	1.0	–
Na-acetate	23.0	15.5	38.2
Na-lactate	17.0	9.5	43.0
Ethanol	17.9	10.4	46.0
Na-pyruvate	12.6	5.1	30.5
Na-citrate	7.5	0.0	–
Na-α-ketoglutarate	7.7	0.2	–
Na-succinate	16.1	8.6	> 63
Na-malate	12.5	5.0	> 51
Endogenous	7.5	–	–

*) Calculated without correction for endogenous oxygen consumption.
Q_{O_2} oxygen uptake in mg per g sludge (dry wt) per hour;
Q_{O_2}' the same after substraction of the endogenous Q_{O_2}.

the sodium salts of succinic and malic acid were dissimilated for over 50 per cent; seven hours after addition of these substrates still no decrease was observed in the rate of oxygen uptake.

The rate of endogenous oxygen consumption of the activated sludge was relatively high during the Warburg experiments, though a gradual decrease was observed during the experimental period.

For the estimation of respiration quotients *), carbon dioxide production was measured in a number of separate Warburg vessels containing no potassium hydroxide solution in the central well. To liberate the CO_2 retained in the buffer solutions, the contents of the vessels were treated with dilute sulphuric acid at the termination of the experiments. The following RQ values were found: endogenous 0.70; supplied with glucose 0.95; and supplied with Na-acetate 0.89.

6.1.1.1 Uptake of glucose by activated sludge from Zeist.

It was striking that with the different sugars, gluconic acid, mannitol and glycerol, the rate of oxygen uptake was only slightly higher than without adding substrates. Besides the oxygen consumption by the activated sludge after addition of the glucose, the rate of uptake of the glucose by the sludge was determined as well. This was performed by means of a Warburg experiment in five-fold. At different periods of time after addition of the substrate, the contents of one vessel were separated by ice-cold centrifugation and the amount

*) Respiration quotient (RQ) $= \frac{\text{volume of carbon dioxide released}}{\text{volume of oxygen consumed}}$

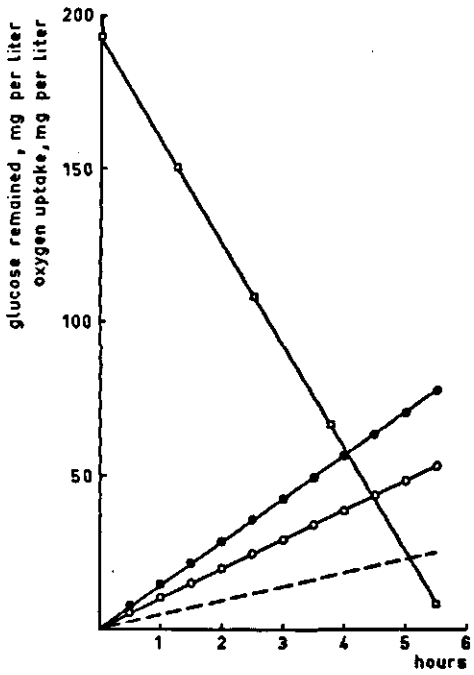


Figure 4. Removal of glucose by a washed suspension of Zeist activated sludge (1640 mg/l dry wt).
O———O endogenous oxygen consumption;
●———● oxygen consumption after addition of glucose 200 mg/l;
– – – – – – net-oxygen consumption;
□———□ glucose remained in solution.

of glucose in the clear supernatant determined. The result of this experiment is given in figure 4.

It will be seen that in spite of the low oxygen consumption of the sludge suspension with glucose (cf fig. 3 and table 12), uptake of this sugar by the sludge proceeded relatively fastly. The reason for the widely deviating results of both processes is that the major part of the absorbed glucose is not dissimilated to carbon dioxide and water, but is assimilated without uptake of oxygen.

From figure 4 it can be calculated that the rate of net-oxygen uptake per gram (dry wt) of activated sludge from Zeist during the removal of glucose was 2.8 mg O_2/g/hour, while the rate of uptake of glucose was 21.3 mg glucose/g/hour. After 5.5 hours, 96 per cent of the 200 mg of glucose had been removed, 13.5 per cent of which had been respired.

In comparison with the uptake of organic acids, in the preceding experiment the absorption of glucose in this experiment was relatively slow, too slow for practical purposes. This was presumably due to the fact that a large part of the bacteria in this sludge was not adapted to glucose or to carbohydrates in general.

6.1.2 Dissimilatory activity of different types of activated sludge from the laboratory apparatus.

Subsequently the oxygen uptake of activated sludge produced from Wageningen sewage was measured after addition of glucose and Na-acetate. The results obtained were almost identical to those obtained with the activated sludge from Zeist, *i.e.* there was hardly any increase in the rate of oxygen uptake after addition of glucose, whereas with Na-acetate added, the rate of oxygen consumption of the sludge was considerably increased, until about 35 per cent of the substrate had been respired.

In general, the results obtained with activated sludges grown on wastes of

domestic origin clearly indicate that such sludges dissimilate substrates like acetate much more readily than carbohydrates like glucose. This may be due, either to the fact that the majority of micro-organisms in these sludges is not adapted to utilize glucose, or belong to those types that are unable to affect carbohydrates. The bacteriological results obtained with these two types of activated sludge have shown that a considerable percentage of the isolated bacteria indeed belonged to the genera *Alcaligenes* and *Lophomonas*, which are unable to utilize carbohydrates.

From the investigations with washed suspensions of activated sludge described here and in section 6.1.1, it appeared that adaptation of the sludge to glucose utilization during the experimental period of seven to eight hours did not occur.

6.1.2.1 Dissimilatory and assimilatory activity of three types of activated sludge grown in synthetic media.

By growing activated sludge in synthetic media with glucose, Na-lactate or Na-acetate, it could be determined at what rate glucose and acetate were removed by adapted and non-adapted sludge types. Successively, originating from Zeist sludge, the three types of activated sludge were grown during three weeks in the continuously working laboratory apparatus in media consisting of a basal solution of mineral salts+ammonium sulphate 85 mg/l+(A)

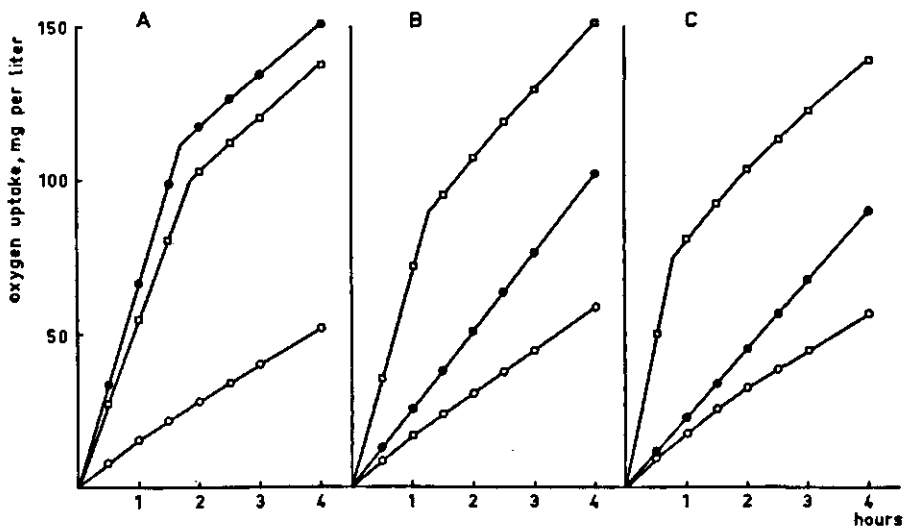


Figure 5. Oxygen uptake by washed suspensions of different types of activated sludge after addition of glucose and Na-acetate. Sludge type A. grown with glucose+ammonium sulphate, B. with Na-lactate+ammonium sulphate and C. with Na-acetate+ammonium sulphate; sludge concentrations A. 1320, B. 2040 and C. 1620 mg/l dry wt.
O——O endogenous; ●——● glucose 470 mg/l; □——□ Na-acetate 530 mg/l.

glucose 600 mg/l, (B) Na-lactate 750 mg/l, and (C) Na-acetate 1250 mg/l, respectively. Well-settling activated sludge flocs were obtained in all three cases.

The oxygen consumption of the three types of activated sludge was measured after addition of glucose and Na-acetate respectively. The Warburg experiments with glucose were terminated four hours after adding glucose; the contents of the Warburg vessels were transferred to ice-cold centrifugation tubes, and the sludge separated by centrifugation. The amount of carbohydrates (expressed as glucose) was determined in the sludge as well as in the supernatant by means of anthrone. By subtracting the amount of carbohydrates found in the sludge at the beginning of the experiment from that found at the end, the amount of glucose stored as carbohydrates in the sludge could be calculated. The oxygen uptake after addition of glucose enabled calculation of the amount of glucose that had been respired. The results of the Warburg experiments are recorded in figure 5 and table 13, the results of the glucose determinations in table 14.

Table 13. Rates of oxygen consumption and dissimilation percentages of different types of activated sludge after addition of glucose and Na-acetate. Sludge type A. grown on glucose+ammonium sulphate, B. on Na-lactate+ammonium sulphate, and C. on Na-acetate+ammonium sulphate.

Sludge type	Substrate	Q_{O_2}	Q_{O_2}'	Dissimilation percentage
A	glucose	47.5	35.9	24.2
A	Na-acetate	40.0	28.4	40.1
A	endogenous	11.6	–	–
B	glucose	12.5	4.2	–
B	Na-acetate	35.1	26.8	36.0
B	endogenous	8.3	–	–
C	glucose	13.8	3.3	–
C	Na-acetate	57.8	47.3	29.7
C	endogenous	10.5	–	–

Q_{O_2} oxygen uptake in mg per g sludge (dry wt) per hour;
Q_{O_2}' the same after substraction of the endogenous Q_{O_2}.

Table 14. Uptake, dissimilation and assimilation of glucose by washed suspensions of different types of activated sludge (per cents of added glucose).

Fate of added glucose	Activated sludge type *)		
	A	B	C
Remained	0	42	58
Respired **)	33	23	17
Stored in the sludge	52	19	10
Total	85	84	85

*) See table 13.
**) Calculated from the oxygen uptake without correction for endogenous respiration.

The oxygen uptake curves in figure 5 show that the sludge types B. and C. were not adapted to glucose; the rate of oxygen uptake after addition of glucose was much lower than that of sludge type A. (Q_{O_2}' values of 4.2, 3.3 and 35.9 respectively, cf table 13). With Na-acetate added, all the three sludge types showed an immediate and pronounced increase in the rate of oxygen uptake, accordingly it may be assumed that most of the micro-organisms occurring in these sludge types were able to break down Na-acetate without an adaptation period. However, the Q_{O_2}' value on acetate-grown sludge was almost twice that of glucose or lactate-grown sludges (47.3, 28.4 and 26.8, respectively), showing that either some adaptation or some selection of acetate-consuming organisms had taken place during the growth of the sludge on acetate.

When the sludge was adapted to the substrate, the rate of oxygen uptake was rapid for a short time, after which it decreased again rather abruptly to values somewhat above the level of the rate of the endogenous oxygen uptake. This decrease in the rate of oxygen uptake already occurred when only 30 to 40 per cent of the Na-acetate and 24 per cent of the glucose added were respired. An important part of these substrates accordingly must have been assimilated. This could be simply calculated for glucose and, as is shown in table 16, 33 to 40 per cent of the absorbed glucose was respired by all three types of sludge, while of the assimilated remainder 77.6 per cent in sludge A., 54.3 per cent in sludge B. and 40.0 per cent in sludge C. was recovered as stored carbohydrates. It may be concluded that adapted sludge will rapidly remove glucose, respiring only a small part, storing the greater part of the assimilates as carbohydrates. Evidently, a process is involved here that is generally known as oxidative assimilation.

6.1.3 The influence of different nitrogen sources on the dissimilatory activity of Zeist activated sludge.

So far, the oxygen consumption of activated sludge supplied with different substrates was measured without adding a nitrogen source. In the present experiment the effect of the addition of a number of nitrogenous compounds on the oxydative dissimilation of some carbon sources by activated sludge was investigated. The following nitrogenous compounds in the presence and absence of one of the following carbon sources were studied:

N-sources: glutamic acid 293 mg/l, arginine 174 mg/l, gelatin 200 mg/l, casamino acids (vitamin-free) 400 mg/l and ammonium sulphate 130 mg/l;

C-sources: glucose 600 mg/l, Na-acetate 680 mg/l and glycerol 460 mg/l.

The results of these respiration experiments are shown in the figures 6 and 7.

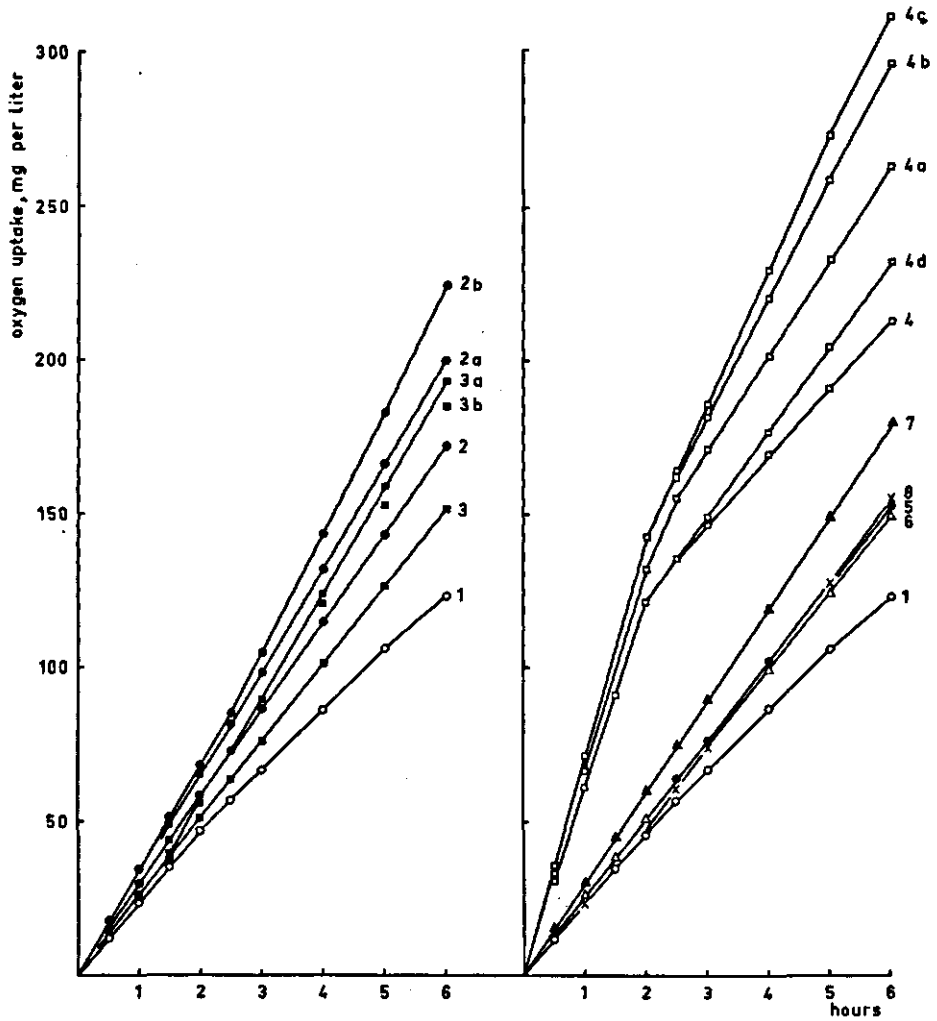


Figure 6. Oxygen uptake by a washed suspension of Zeist activated sludge after addition of various substrates in the presence or absence of an organic nitrogen source (calculated as mg O_2 per liter sludge suspension of 2560 mg dry wt). 1. endogenous; 2. glucose 600 mg/l, 2a. do+glutamic acid 293 mg/l; 2b. do+casamino acids 400 mg/l; 3. glycerol 460 mg/l, 3a. do+casamino acids 400 mg/l, 3b. do+gelatin 200 mg/l; 4. Na-acetate 680 mg/l, 4a. do +arginine 174 mg/l, 4b. do+glutamic acid 293 mg/l, 4c. do+casamino acids 400 mg/l, 4d. do+gelatin 200 mg/l; 5. arginine 174 mg/l; 6. glutamic acid 293 mg/l; 7. casamino acids 400 mg/l; 8. gelatin 200 mg/l.

The rates of oxygen consumption during the uptake of glucose, glycerol or acetate and during the subsequent period of endogenous respiration in the presence or absence of the nitrogen sources applied, have been recorded in table 15.

Figure 7.
Oxygen uptake by a washed suspension of Zeist activated sludge after addition of glucose and Na-acetate in the presence or absence of ammonium sulphate (calculated as mg O_2 per liter sludge suspension of 4020 mg dry wt).
1. endogenous;
2. glucose 600 mg/l, 2a. do + ammonium sulphate 130 mg/l;
3. Na-acetate 680 mg/l, 3b. do + ammonium sulphate 130 mg/l;
4. ammonium sulphate 130 mg/l.

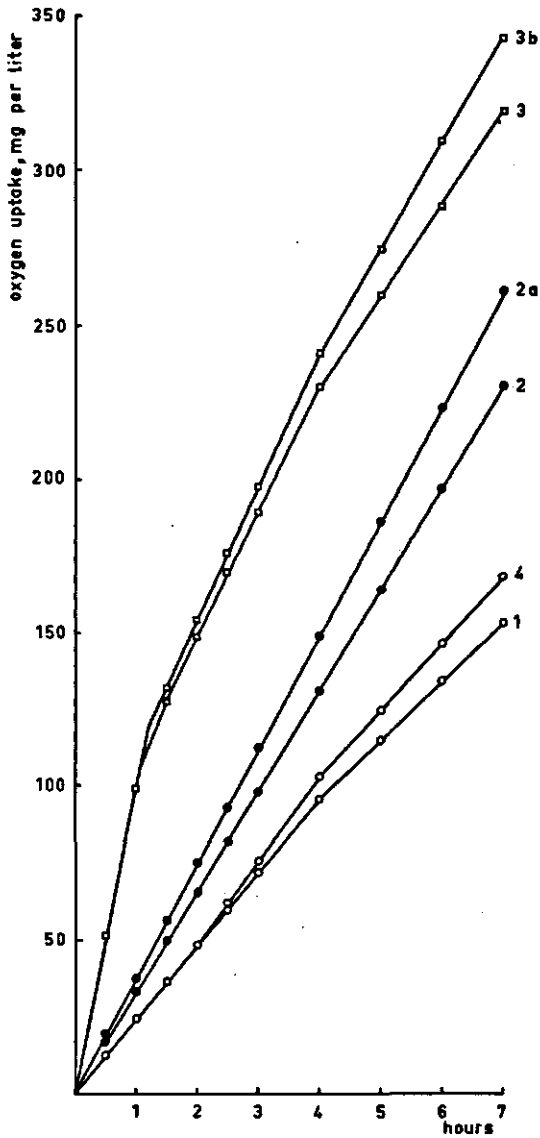


Figure 6 shows that addition of organic nitrogenous compounds as protein or amino acids had but a slight effect on the rate of oxygen uptake; only with casamino acids added did the rate of oxygen consumption by the sludge increase somewhat, particularly when Na-acetate was the carbon source (cf also table 15). To what extent the various nitrogenous compounds were absorbed by the activated sludge was not investigated. It is not known to what extent endogenous respiration was influenced by these amino acids.

Addition of ammonium sulphate resulted in a slight increase in the rate of oxygen uptake, probably due to nitrification. In more recent experiments with non-nitrifying activated sludge, the rate of oxygen uptake was exactly the same with or without ammonium sulphate.

No pronounced effect of the presence of the nitrogenous compounds only was observed on the rate of oxygen consumption with glucose or glycerol as the carbon compound (figure 6). However, a gradual increase in the rate of oxygen consumption was observed during the experimental period, when the activated sludge was supplied with a combination of glucose or glycerol and amino acids.

With acetate as the carbon source, the effect of added nitrogen compounds, particularly amino acids, on the rate of oxygen consumption during the absorp-

Table 15. Effect of different nitrogen sources on the rate of oxygen uptake by activated sludge from Zeist during and after the removal of glucose, glycerol and Na-acetate, respectively.

		During uptake phase		During endogenous phase	
Carbon source	Nitrogen source	Q_{O_2}	Q_{O_2}''	Q_{O_2}	Q_{O_2}''
Glucose	casamino acids	13.3	1.9		
	glutamic acids	12.8	1.4		
	none	11.4	–		
Glycerol	gelatin	11.4	0.0		
	casamino acids	10.2	0.1		
	arginine	11.4	1.3		
	none	10.1	–		
Na-acetate	gelatin	23.9	0.0	10.4	1.0
	casamino acids	27.9	3.9	17.1	7.7
	arginine	26.2	2.3	11.9	2.5
	glutamic acid	27.8	3.9	15.6	6.2
	none	23.9	–	9.4	–
None	gelatin	9.0	0.0		
	casamino acids	11.7	2.7		
	arginine	9.9	0.9		
	glutamic acid	9.9	0.9		
	none	9.0	–		
Glucose	$(NH_4)_2SO_4$	9.3	1.1		
	none	8.2	–		
Na-acetate	$(NH_4)_2SO_4$	24.8	0.1	11.0	0.6
	none	24.7	–	10.4	–
None	$(NH_4)_2SO_4$	6.0	0.0		
	none	6.0	–		

Q_{O_2} oxygen uptake in mg per g sludge (dry wt) per hour;
Q_{O_2}'' oxygen uptake in mg per g (dry wt) per hour after substraction of Q_{O_2} without added nitrogen.

tion of the substrate was more pronounced. However, most striking was that after the absorption phase, endogenous respiration was much more intensive in the presence of amino acids than in the absence of a nitrogen source; the same effect, be it to a much less extent, was observed with ammonium sulphate (table 15).

6.1.4 The influence of ammonium sulphate on the dissimilation of glucose by activated sludge from the laboratory apparatus.

In subsequent experiments the influence of ammonium sulphate on the dissimilation of glucose by activated sludge was investigated in more detail. The experiments were carried out with two types of activated sludge, grown in a laboratory culture which was continuously fed with yeast-extract+Na-acetate, and in a batch culture fed with glucose+ammonium sulphate by fill and draw.

A Warburg experiment was carried out with washed suspensions of the first-mentioned sludge with addition of glucose (470 mg/l) in the presence or absence of ammonium sulphate (300 mg/l). It was intended to carry out this experiment with activated sludge not adapted to utilize glucose. However, as seen in figure 8, the relatively high rate of oxygen uptake with glucose added ($Q_{O_2} = 27.1$) makes it highly probable that this sludge was adapted to glucose, presumably because the yeast-extract contained a small amount of carbo-

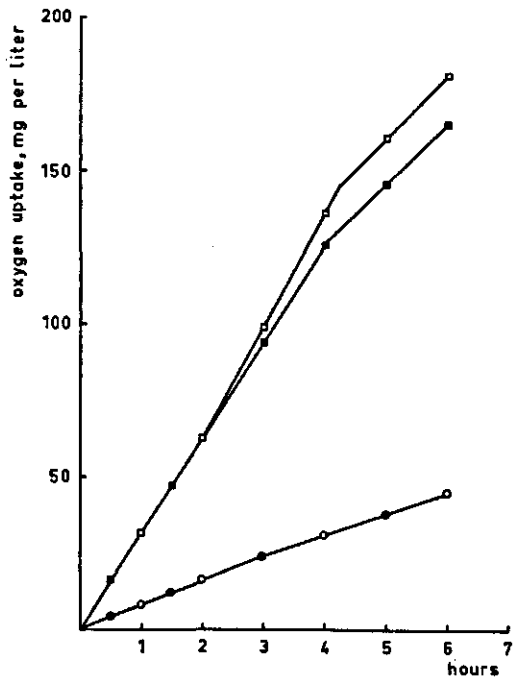


Figure 8.
Effect of ammonium sulphate on the oxygen consumption of activated sludge grown with yeast-extract+Na-acetate, after addition of glucose (sludge suspension of 1150 mg/l dry wt).
●——● endogenous;
■——■ glucose 470 mg/l;
□——□ do+ammonium sulphate 300 mg/l;
○——○ ammonium sulphate 300 mg/l.

hydrates. This adaptation was incomplete, however, as may be seen from the much higher Q_{O_2} values obtained with sludge grown in a glucose medium (see table 13).

No effect of added ammonium sulphate in the presence of glucose was observed during the first two hours of the experiment; thereafter a gradual increase of oxygen consumption occurred. This gradual increase may have been due to a physiological adaption and to a promotion of the multiplication of bacterial strains growing readily on glucose+ammonium nitrogen. The former supposes the development of adaptive enzymes for glucose decomposition in existing bacterial cells, whereas the latter represents a selection of bacterial strains.

The effect of ammonium sulphate on the oxygen consumption by activated sludge with glucose was also examined in an experiment with sludge grown on glucose+ammonium sulphate (a detailed description of this experiment will be given in the next section).

As may be seen from figure 9, the presence of ammonium sulphate had hardly any effect on the rate of oxygen consumption during the period of glucose uptake. The latter period lasted somewhat longer, however, than in the absence of added nitrogen, so that a slightly higher percentage of the glucose was respired during this period (cf table 16).

6.1.5 Influence of the sludge load on the dissimilation of glucose by activated sludge.

The sludge load is defined as the ratio between the amount of organic matter supplied (usually expressed in grams of BOD or COD) and the amount of activated sludge (expressed in grams of dry matter).

Table 16. Rates of oxygen consumption, dissimilation percentages and respiration quotients of washed suspensions of activated sludge at various glucose concentrations with or without ammonium sulphate.

Sludge load mgCOD/mg sludge (dry wt)	Glucose (mg/l)	$(NH_4)_2SO_4$ (mg/l)	Uptake phase				Endogenous phase Q_{O_2}
			Q_{O_2}	RQ	Dissimil. percentage	Duration (min)	
0	0	0	–	0.80	–	–	1.8
0	0	300	–	0.80	–	–	1.8
0.23	600	0	48	1.02	10.4	30	2.6
0.23	600	300	46	1.00	10.8	33	3.4
0.46	1200	0	51	0.99	10.4	57	3.5
0.46	1200	300	49	n.e.	11.9	66	4.7
0.92	2400	0	46	1.01	10.6	126	4.6
0.92	2400	300	47	1.00	11.2	128	8.0

Q_{O_2} oxygen uptake in mg per g sludge (dry wt) per hour.

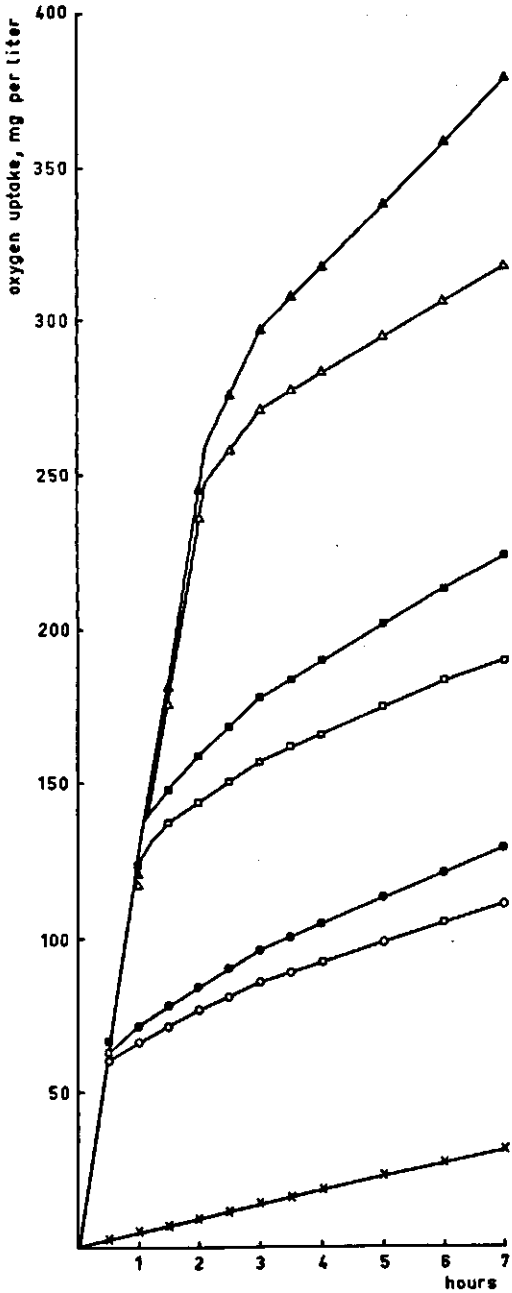


Figure 9.
Effect of different glucose concentrations, with or without ammonium sulphate, on the oxygen consumption by a washed suspension of activated sludge (sludge suspension of 2540 mg/l dry wt).
X——X endogenous;
O——O glucose 600 mg/l;
●——● do+ammonium sulphate 300 mg/l;
□——□ glucose 1200 mg/l;
■——■ do+ammonium sulphate 300 mg/l;
△——△ glucose 2400 mg/l;
▲——▲ do+ammonium sulphate 300 mg/l.

The influence of different amounts of added glucose on the oxygen consumption and the rate of glucose absorption was investigated with activated sludge grown in batch culture with glucose and ammonium sulphate. Glucose was

added in amounts corresponding with 600, 1200 and 2400 mg per liter, in the presence or absence of ammonium sulphate (300 mg/l). Oxygen uptake as well as release of carbon dioxide were determined. (figure 9, table 16). It is evident that with glucose added, the respiration quotient had increased as compared with that of the endogenous respiration.

As may be seen from figure 9, an increased glucose concentration did not affect the rate of oxygen uptake of the sludge, whereas the duration of the uptake phase increased almost directly proportionally to the sludge load (0.5, 1 and 2 hours respectively for 600, 1200 and 2400 mg glucose per liter). The percentage of the added glucose dissimilated at the termination of the uptake phase was independent of the sludge load (table 16).

Although hardly any effect of ammonium nitrogen on the rate of oxygen consumption was observed during the uptake of glucose, a clear effect of the sludge load and of added ammonium sulphate on the respiration rate was observed during the endogenous phase following the uptake period. The greater the sludge load, the greater the rate of oxygen uptake during the endogenous phase, particularly in the presence of ammonium sulphate. An approximately linear relationship existed between the increase of endogenous oxygen uptake and the glucose concentration.

The effect of the sludge load was also studied by varying both the sludge concentration and the glucose concentration. Activated sludge grown in batch culture with glucose and ammonium sulphate, was applied in two concentrations and supplied with glucose in two concentrations.

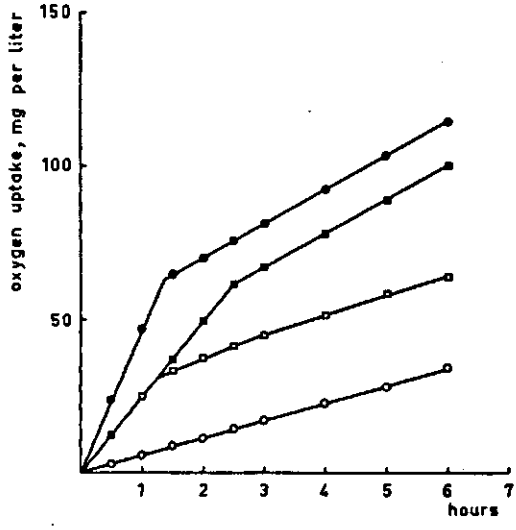
Table 17. Rates of oxygen uptake and dissimilation percentages of two different concentrations of activated sludge after addition of glucose in two concentrations.

Sludge load mg COD/mg sludge (dry wt)	Activated sludge (mg/l)	Glucose (mg/l)	$(NH_4)_2SO_4$ (mg/l)	Uptake phase			Endogenous phase Q_{O_2}
				Q_{O_2}	Dissimil. percentage	Duration (min.)	
0	1040	none	150	–	–	–	5.3
0.56	1040	600	150	45	10.8	80	10.8
1.12	520	600	150	48	10.6	150	20.2
0.56	520	300	150	48	10.6	73	15.4

Q_{O_2} oxygen uptake in mg per g sludge (dry wt) per hour.

Oxygen consumption per gram dry matter per hour (Q_{O_2}) appeared to be almost independent of the sludge load, when the substrate concentration or the sludge concentration were varied (table 17). In agreement with the similar results of the preceding experiment, it was shown that the rate of endogenous

Figure 10.
Oxygen consumption by washed suspensions of activated sludge in two different concentrations after addition of glucose in two concentrations.
□———□ 520 mg/l sludge + glucose 300 mg/l;
■———■ 520 mg/l sludge + glucose 600 mg/l;
●———● 1040 mg/l sludge + glucose 600 mg/l;
○———○ 1040 mg/l sludge, endogenous.
In all cases 150 mg/l ammonium sulphate was supplied.



oxygen consumption during the phase following the period of glucose uptake was higher, the higher the sludge load.

6.1.6 Effect of the oxygen tension on the dissimilatory activity of activated sludge.

The relatively low dissimilation percentage of glucose in activated sludge adapted to this substrate might be a result of an oxygen deficiency in the flocs. The bacteria present in these flocs thus could be induced to assimilate the glucose in a way demanding only little oxygen. To improve the oxygen level in the floc, the oxygen tension in the Warburg vessel has to be increased. Wuhrmann *et al* [97] have estimated that the oxygen concentration in the sludge liquor will be four times as high when pure oxygen is used than with air.

The influence of increased oxygen tension on the rate of oxygen consumption and the dissimilation percentage in activated sludge was examined in Warburg vessels after addition of glucose (430 mg/l) and ammonium sulphate (100 mg/l). Sludge grown in batch culture with glucose and ammonium sulphate was used in a concentration of 1130 mg/l. The experiment was carried out with oxygen percentages in the gas phase of 20, 60 and 100 per cent, respectively. An effect of the increased oxygen tension was observed neither on the endogenous oxygen uptake and the carbon dioxide release of the sludge, nor on the oxygen uptake and the carbon dioxide release during the uptake of glucose and the following phase of endogenous respiration. The dissimilation percentage neither changed under influence of the changed oxygen tension.

In another experiment the rate of uptake of glucose by a washed suspension of activated sludge was examined, while instead of air an oxygen-free mixture of nitrogen and 5 per cent of carbon dioxide was bubbled through the suspen-

sion. The gas mixture was freed from oxygen by leading it through a solution of 20 per cent potassium hydroxide containing 40 per cent pyrogallol. After supplying the gas mixture for half an hour to a liter of activated-sludge suspension, containing 1008 mg of Na-bicarbonate, 250 mg glucose was added to the suspension. Four hours after the glucose had been added, the gas mixture was replaced by air, so that oxygen was dissolved again in the suspension. Samples were regularly taken, in which the glucose concentration was determined by means of anthrone, after the sludge had been separated by centrifugation. The result of this experiment was as follows: under anaerobic conditions the glucose removal from the medium by the activated sludge proceeded at a rate of 5.8 mg/gram dry wt/hour, while immediately after the gas mixture had been replaced by air, glucose was taken up at a rate of 440 mg/gram dry wt/hour as a result of which within 20 minutes glucose was no longer detectable in the liquid. This result clearly shows that the uptake of glucose by activated sludge is an entirely aerobic physiological process.

6.2 The dissimilatory activity of pure cultures of bacteria

To study the physiological functioning of several bacterial strains in correspondence to the dissimilatory activity of activated sludge, the oxygen uptake by a number of bacterial strains, partly isolated from Zeist activated sludge and partly obtained from bacterial collections, was measured after addition of glucose or Na-acetate in the presence or absence of ammonium sulphate.

6.2.1 The dissimilation of glucose and Na-acetate by pure cultures of bacteria isolated from activated sludge.

The activated sludge strains studied, belonged to the groups I and II of the classification depending on physiological characteristics (see chapter 4). The strains obtained from the collections were *Arthrobacter globiformis* (strain 1, from the collections of Mulder and Antheunisse, [69]), *Flavobacterium aquatile* (NCIB 8535) and *Pseudomonas putida*. All strains showed good growth on a medium with Na-acetate and ammonium sulphate; all strains except strain 6 and the *Flavobacterium* strain showed good growth on a medium with glucose and ammonium sulphate.

The bacteria were inoculated in 500 ml Erlenmeyer flasks containing 75 ml of nutrient solution consisting of basal medium, yeast-extract 100, Na-acetate 800 and ammonium sulphate 500 mg/l. The flasks were mechanically shaken at about 20 °C. After four, seven and nine days respectively, 5 ml of a 1.2 per cent Na-acetate solution in Sörensen phosphate buffer (0.033 M, pH 7.2) was added to each flask. Eleven days after inoculation, the grown cell masses were separated by centrifugation and washed with water. Hereafter the oxygen

consumption of each washed bacterial suspension was determined in Warburg respirometers after adding glucose (516 mg/l) or Na-acetate (532 mg/l). Four hours after addition of the substrate, the bacterial masses of the Warburg vessels supplied with glucose were transferred to ice-cold centrifugation tubes and separated by centrifugation. The remaining glucose was determined in the supernatant, while the increase in carbohydrates in the bacterial mass was calculated by subtracting the amounts of carbohydrates in the cell masses, determined by means of anthrone, before and after the Warburg experiment. The respired part of the added glucose was calculated from the oxygen uptake determined during the Warburg experiment.

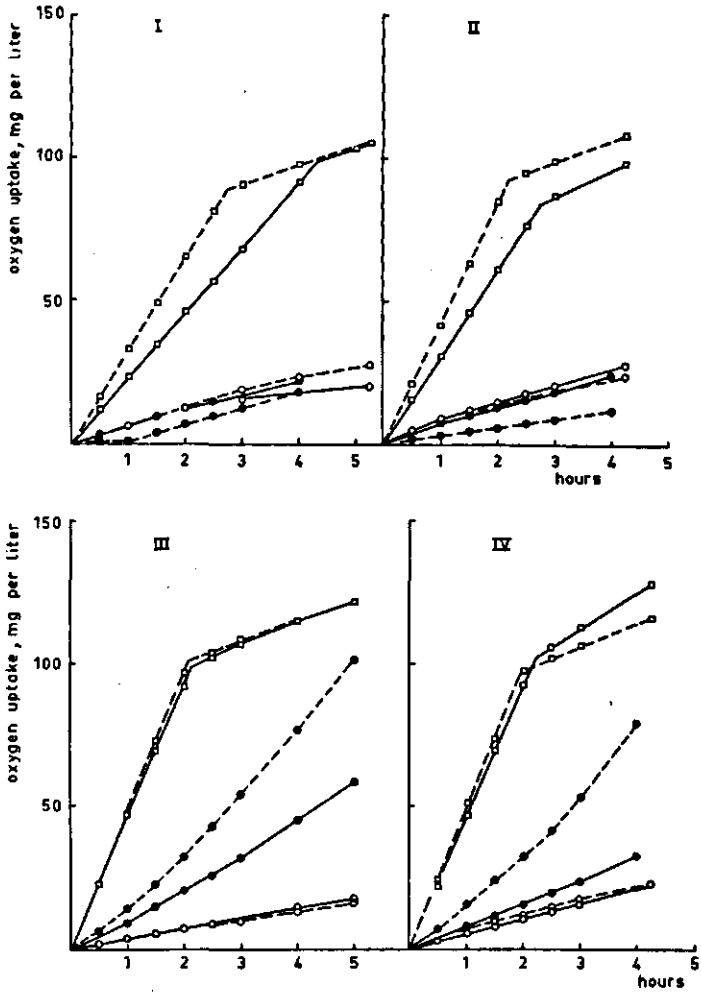


Figure 11.
Oxygen uptake by washed suspensions of bacteria, isolated from Zeist activated sludge grown with Na-acetate and ammonium sulphate, after addition of glucose and Na-acetate.
○ endogenous;
● glucose 516 mg/l;
□ Na-acetate 532 mg/l.
I. —— strain 3 (100 mg cell-N *)/1;
– – – strain 6 (83 mg cell-N/l).
II. —— strain 15 (61 mg cell-N/l);
– – – strain 17 (87 mg cell-N/l).
III. —— strain 16 (113 mg cell-N/l);
– – – strain 19 (104 mg cell-N/l).
IV. —— strain 1 (57 mg cell-N/l);
– – – strain 12 (89 mg cell-N/l).

*) Bacteria designated as cell-N.

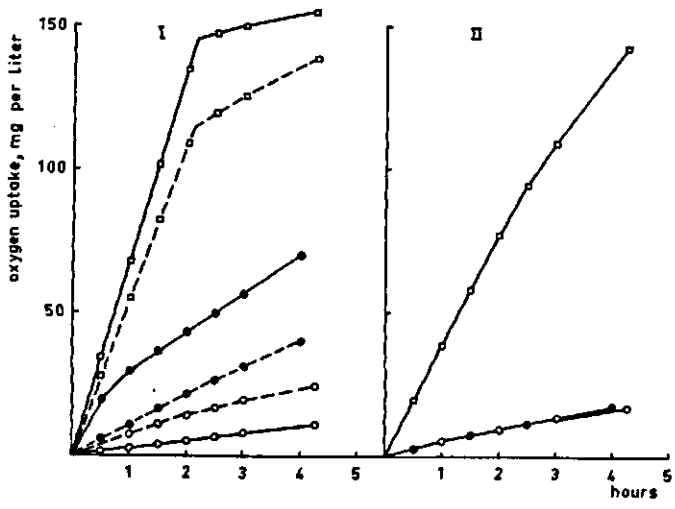


Figure 12.
Oxygen uptake by washed suspensions of *Pseudomonas putida*, *Arthrobacter globiformis* (strain 1) and *Flavobacterium aquatile* (NCIB 8535), grown with Na-acetate and ammonium sulphate, after addition of glucose and Na-acetate.
○ endogenous;
● glucose 516 mg/l;
□ Na-acetate 532 mg/l.
I. —— *Ps. putida* (57 mg cell-N/l);
– – – *Arthr. globiformis* (79 mg cell-N/l).
II. —— *Fl. aquatile* (77 mg cell-N/l).

Table 18. Dissimilation and assimilation of glucose by washed bacteria, grown with Na-acetate and ammonium sulphate.

		Percentage glucose after four hours				
Strain	Group *)	Left	Dissimilated	Assimilated to carbohydrates	Total	RQ
3	I	97.0	0.7	6.0	103.7	–
6	I	98.0	0.0	3.5	101.5	–
15	I	95.6	0.0	4.5	100.1	–
16	I	51.1	12.1	6.2	69.4	0.93**)
12	I	20.8	17.1	21.0	66.9	– **)
19	I	12.6	20.5	31.1	85.1	1.00**)
1	II	94.4	6.6	0.0	101.0	–
17	II	100.0	0.0	1.6	101.6	–
Arthrobacter globiformis		80.3	8.4	21.6	110.3	**)
Flavobacterium aquatile		97.2	0.3	4.0	102.5	
Pseudomonas putida		4.1	7.7	0.0	11.8	0.13&0.82

*) I and II strictly aerobic, I no acid, II acid from glucose.
**) analysed after five hours.

The oxygen uptake curves (figures 11 and 12) show that none of the bacterial strains, grown in a mineral medium with Na-acetate, were able to dissimilate glucose at a rate comparable to that of acetate; some strains showed adaptation to glucose utilization during the experimental period, others (strain 17) showed repressed respiration after addition of glucose. The respiration quotient of the bacteria, respiring part of the glucose, varied from 0.9–1.0, except for the

Pseudomonas putida strain tested. With the latter strain at first a considerable difference was observed between the amount of oxygen taken up and the amount of carbon dioxide released ($RQ = 0.13$), while from the glucose balance (table 18) it could be concluded that the glucose was rapidly oxidized, presumably to gluconic acid, that was only slowly broken down to carbon dioxide and water (during the second part of the uptake period $RQ = 0.82$). Possibly the formation of gluconic acid decreased the pH to such an extent that the total activity of the bacteria was limited. From the carbon dioxide released, it may be calculated that approximately 8 per cent of the glucose taken up by the *Pseudomonas* cells was dissimilated to water and carbon dioxide; the remainder of the glucose taken up was nearly quantitatively oxidized to gluconic acid as could be calculated from the oxygen uptake diminished by the oxygen consumption used for dissimilation. Gluconic acid does not give a colour with anthrone, so that this compound was not determined in this experiment.

When a comparison is made between the oxygen consumption curves of domestic activated sludge supplied with glucose or Na-acetate and those of bacterial strains, isolated from the sludges and grown in a mineral medium with acetate as the carbon source, a clear agreement may be observed. The strains of *Arthrobacter globiformis* and *Flavobacterium aquatile* behaved more or less similarly to the other strains tested. The exceptional behaviour of *Pseudomonas putida* is not in disagreement with the results of the sludge experiments, because the bacteriological analyses of the various activated sludge types gave relatively few *Pseudomonas* species (positive oxidase reaction) able to oxidase glucose to gluconic acid (O–F medium, group II).

6.2.2 · The dissimilation of glucose and Na-acetate by two Arthrobacter *strains.*

The results of the above-mentioned experiments have shown that the trend in the oxygen uptake by *Arthrobacter globiformis*, grown on Na-acetate, corresponds well with that of a washed suspension of activated sludge from Zeist after addition of glucose. Since a number of well-defined strains of this bacterium species were present in the Laboratory of Microbiology (collection of Mulder and Antheunisse, [69]) a more detailed investigation concerning the dissimilation of glucose by two strains (1 and 166) of the collection has been carried out.

By growing both *Arthrobacter* strains on mineral medium with and without glucose, bacterial cells were obtained which were either adapted to glucose or not. An investigation was made into the rate of glucose uptake, as derived from oxygen consumption of glucose-adapted cells, and into the rate at which non-adapted cells became adapted to this compound in the presence or absence of ammonium nitrogen.

The *Arthrobacter* strains were inoculated in 300 ml Erlenmeyer flasks containing 50 ml nutrient solution consisting of: mineral medium+ A. casamino acids 4000 mg/l, or B. casamino acids 2000 g/l+glucose 2000 mg/l. The flasks were mechanically shaken at 20 °C. Five days after inoculation, each flask was supplied with a quantity of the substrates equal to the original quantity. After a further four days the bacterial masses were separated by centrifugation, washed, suspended in a buffer solution and transferred to Warburg vessels for measuring the oxygen uptake after addition of glucose. Figure 13 shows that glucose may be readily respired by *Arthrobacter* strains, if the cells are adapted to this substrate. The adapted cells took up all the glucose added and during

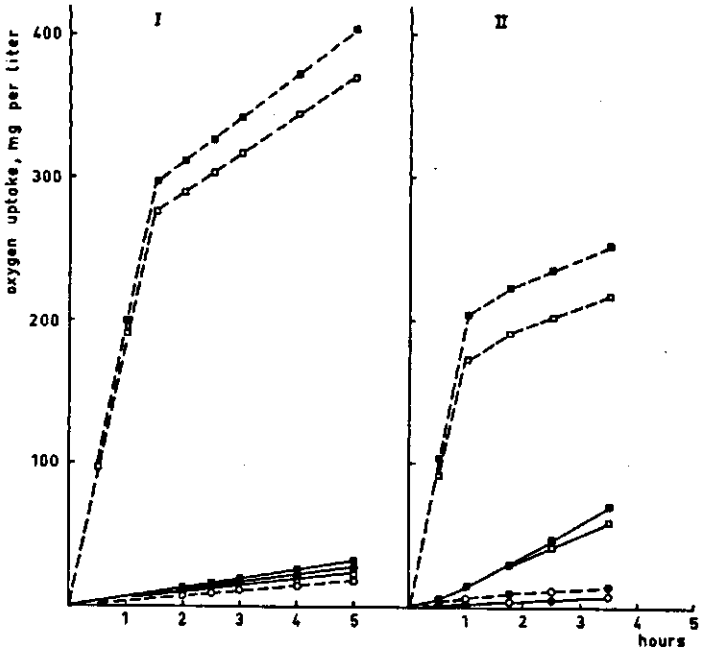


Figure 13.
Oxygen consumption by washed suspensions of two *Arthrobacter globiformis* strains after addition of glucose with or without ammonium nitrogen.
O endogenous;
□ glucose 600 mg/l;
■ do+ammonium sulphate 300 mg/l;
● ammonium sulphate 300 mg/l.
I. Strain no 1:
—— grown with casamino acids (417 mg cell-N/l);
– – – grown with casamino acids+glucose (339 mg cell-N/l).
II. strains no 166:
—— grown with casamino acids (283 mg cell-N/l);
– – – grown with casamino acids+glucose (348 mg cell-N/l).

the uptake phase respired 48.8 (strain No 1) and 29.4 per cent (strain No 166); in the presence of ammonium nitrogen these values equalled 50.9 and 35.0 per cent, respectively. When the cells were not adapted to glucose break-down, the bacterial mass of strain 1 did not store any glucose, respired about 1 per cent, whereas approximately 98 per cent of the glucose added was recovered in the medium at the end of the experimental period; the bacteria of strain 166 during the experimental period of 3.5 hours respired approximately 10 per cent

of the glucose added, stored about 6 per cent in the cell mass, whereas 88 per cent of the glucose was recovered in the medium at the end of the experiment.

In the presence of ammonium nitrogen the rate of oxygen uptake of adapted cells was the same as in the absence of a nitrogen source, but the uptake phase lasted somewhat longer, so that a slightly higher dissimilation percentage was found at the end of the uptake phase. Adaptation to glucose of strain 166 was somewhat more rapid in the presence of added ammonium nitrogen than in its absence; no influence of ammonium nitrogen on the oxygen uptake was found with strain 1.

Mulder and Antheunisse [69] used the same *Arthrobacter* strains and found dissimilation percentages varying from 25 to 60 per cent.

Compared with the oxygen uptake of washed suspensions of activated sludge from Zeist and from the laboratory apparatus (grown with glucose + ammonium sulphate) after addition of glucose, there is some correspondence with that of both *Arthrobacter* strains. Activated sludge not adapted to glucose takes up this compound relatively slowly, while a large part after its absorption is stored as polysaccharides. *Arthrobacter* cells, grown with casamino acids, reacted more or less similarly to activated sludge. Strain 166 utilized glucose slightly, whereas strain 1 was unable to utilize or absorb this sugar at all during the experimental period (figure 13). The former strain showed a slight adaptation to glucose break-down during the experimental period in the presence of both glucose and ammonium nitrogen.

Differences between the Arthrobacter strains and activated sludge were the much larger dissimilation percentages in *Arthrobacter* cells adapted to glucose and the lower rate of endogenous oxygen uptake of *Arthrobacter* cells as compared with activated sludge. As a possible reason for these discrepancies, the difference in cultivating the activated sludge and the pure cultures may be mentioned.

6.2.3 The dissimilatory activity of a Zoogloea *strain*

The *Zoogloea* strain recorded in chapter 5 was grown in the same way as activated sludge, be it under sterile conditions, in batch culture in a medium consisting of: a basal solution of mineral salts, 50 mg/l calcium chloride, 20 mg/l ferric chloride and 1000 mg/l peptone. Due to the floc-forming properties of this strain, from the second day after inoculation onwards 500 ml of the supernatant could be drawn off daily one hour after stopping the aeration, the removed solution being replaced by fresh medium. The supernatant never was entirely clear during the experimental period, as ought to be the case with activated sludge. Nine days after inoculation, a reasonable amount of well-settling flocs had been formed, part of which was used in measuring the oxygen uptake after addition of various substrates, as glucose (650 mg/l), glycerol

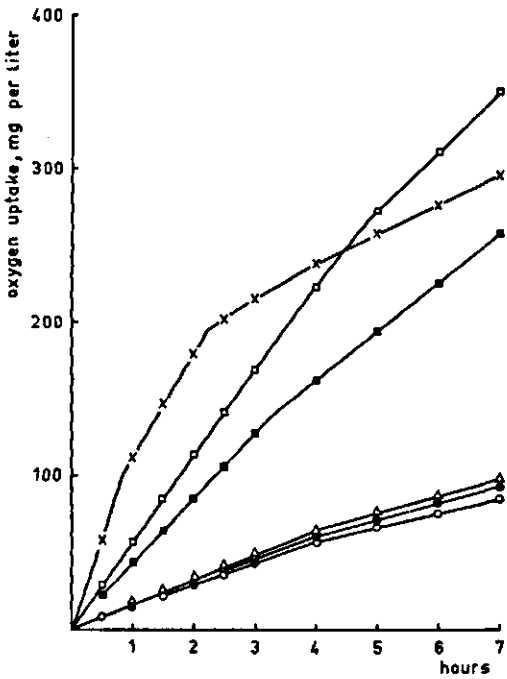


Figure 14.
Oxygen uptake by washed flocs of a *Zoogloea* strain grown in a peptone-containing solution after addition of various substrates (calculated as mg O_2 per liter floc suspension of 139 mg cell-N per liter).
O——O endogenous;
●——● glucose 650 mg/l;
△——△ glycerol 670 mg/l;
□——□ Na-acetate 1480 mg/l;
■——■ Ca-lactate 1560 mg/l;
×——× peptone 650 mg/l.

(670 mg/l), Na-acetate (1480 mg/l), Ca-lactate (1560 mg/l) and peptone (650 mg/l) (figure 14).

The oxygen uptake curves found resemble those of activated sludge. Both types of flocs consumed glucose and glycerol relatively slowly (domestic activated sludge) or not at all (*Zoogloea* flocs, cf figure 14). Acetate and lactate, however, were readily decomposed by activated sludge as well as by the *Zoogloea* flocs, dissimilation percentages in both cases being almost equal (table 19). Oxygen-uptake rates (Q_{O_2}) with acetate and lactate obtained with the pure-

Table 19. Rate of oxygen uptake of washed flocs of a *Zoogloea* strain, grown in a peptone-containing medium, compared with those of Zeist activated sludge after addition of various substrates.

	Oxygen uptake rate (Q_{O_2})		Dissimilation percentage	
Substrate	*Zoogloea* strain	Activated sludge from Zeist	*Zoogloea* strain	Activated sludge from Zeist
Glucose	8.7	9.1	–	–
Glycerol	9.3	9.1	–	–
Na-acetate	29.6	23.1	38	38
Ca-lactate	24.4	17.0	43	44
Endogenous	8.2	7.5	–	–

Q_{O_2} oxygen uptake in mg per g sludge (dry wt) per hour.

culture flocs were much in harmony with those of the activated sludge. For the *Zoogloea* flocs peptone, on which they were grown, formed a good substrate.

6.3 Assimilation of glucose by activated sludge

The respiration experiments described in the previous part of this chapter have shown that a major part of the substrate supplied to activated sludge is assimilated by the micro-organisms of the sludge. This would mean a. protein synthesis, b. storage of reserve compounds, *i.e.* compounds that may be used as an energy source later on, *e.g.* glycogen, and c. formation of compounds of no immediate importance for metabolism, *e.g.* slime and capsules that may, however, contribute to floc formation.

6.3.1 Protein synthesis and storage of compounds

The metabolism of two types of activated sludge (types 1 and 2) grown during more than two months in batch culture with glucose and ammonium sulphate was examined in more detail. The method of producing these sludges has been described in chapter 3. Sludge type 1, grown in cylinder 1, was fed with synthetic waste water with the concentrations of carbon and nitrogen sources half of those used for growing sludge type 2, grown in cylinder 2.

After adding the normal amount of mineral salts, certain amounts of glucose and ammonium sulphate to a known quantity of activated sludge contained in the two cylinders, the metabolism of both types of sludge was examined. This was done by determining the decrease in the glucose concentration (by means of the anthrone method or the Nelson-Somogyi method) or of the COD of the culture solution, and the increase in dry matter, total nitrogen (Kjeldahl) and carbohydrates (anthrone) in the sludges. The oxygen uptake of the sludges

Table 20. Metabolism of activated sludge (type 1) after addition of 600 mg of glucose and 100 mg of ammonium sulphate.

(A) Experimental results

Time from start (hours)	Glucose. H_2O in the nutrient sol. (mg/l)	Oxygen uptake (mg/l)	Sludge dry wt (mg/l)	Carbohydrates in the sludge		Nitrogen in the sludge	
				(mg/l)	(%, dry wt)	(mg/l)	(%, dry wt)
0	0+600	–	1330	410	30.8	81.6	6.1
0.5	350	30	1470	434	29.5	82.4	5.6
1	133	60	1610	490	30.4	84.5	5.2
1.5	0	88	1740	525	30.2	84.6	4.9
2.5	0	99	1720	520	30.2	88.3	5.1
12	0	–	1620	520	32.1	94.9	5.9
24	0	–	1610	504	31.2	92.1	5.7

(B) Glucose balance

Time from start (hours)	Glucose. H_2O Removed from the nutrient solution (mg/l)	Taken up by the sludge				Not accounted for	
		Respired (mg/l)	Assimilated to Carbohydr. (mg/l)	Protein *) (mg/l)	Total (mg/l)	(mg/l)	(%)
0.5	250	31	24	8	83	167	67
1.0	467	62	80	28	170	297	64
1.5	600	91	115	29	235	365	61
2.5	600	102	110	65	277	323	54
12	600	190 **)	110	128	428	172	29
24	600	300 **)	94	101	395	105	18

(C) Dry matter balance

Time from start (hours)	Glucose. H_2O removed from nutrient sol. (mg/l)	Respired (glucose) (mg/l)	Sludge increase (mg/l)	Loss of water ***) (mg/l)	Total (mg/l)	Not accounted for (mg/l)	(%)
0.5	250	28	140	23+2	193	57	23
1.0	467	56	280	43+7	386	90	19
1.5	600	83	410	55+10	558	42	7
2.5	600	93	390	55+10	548	52	9
12	600	175 **)	290	55+10	530	70	12
24	600	270 **)	280	55+9	614	–	–

*) Calculated by assuming a C/N ratio in protein of 3.5;
**) Calculated by extrapolating the oxygen uptake;
***) Molecular and condensation water.

was measured simultaneously in a separate experiment in Warburg respirometers, in which adequate amounts of mineral salts, glucose and ammonium sulphate were added to 2 ml of sludge.

Two experiments, both representative for a number of similar investigations, will be described (cf tables 20 and 21). In the first experiment, 600 mg of glucose and 100 mg of ammonium sulphate were added to one liter of sludge (type 1); in the second, 1500 mg of glucose and 250 mg of ammonium sulphate were added to one liter of sludge (type 2).

In both experiments the added glucose was partly recovered in the sludge as carbohydrates (for 19 and 31 per cent respectively). These carbohydrates were formed during the removal of the substrate, and they were only for a small part broken down during the subsequent endogenous phase; this part can be considered as a reserve compound (see b. page 71). However, a significant portion of the polysaccharides formed remained unimpaired during the endogenous phase; this part is considered to be useless for endogenous respiration (see c.

Table 21. Metabolism of activated sludge (type 2) after addition of 1500 mg of glucose and 250 mg of ammonium sulphate.

(A) Experimental results

Time from start (hours)	COD of the nutrient solution (mg/l)	Oxygen uptake (mg/l)	Sludge dry wt (mg/l)	Carbohydrates in the sludge		Nitrogen in the sludge	
				(mg/l)	(%, dry wt)	(mg/l)	(%, dry wt)
0	92.3+1451	–	2022	469	23.2	125	6.2
1	873.0	63	2510	713	28.4	131	5.2
2	187.5	125	2970	900	30.3	139	4.7
4	90.8	173	2935	893	30.4	149	5.1
9	91.3	–	2895	790	27.3	160	5.5
20	–	–	2880	787	27.3	167	5.8

(B) Glucose balance

Time from start (hours)	Glucose. H_2O removed from the nutrient sol. (mg/l)	Taken up by the sludge					
		Respired (mg/l)	Assimilated to		Total (mg/l)	Not accounted for	
			Carbohydr. (mg/l)	Protein *) (mg/l)		(mg/l)	(%)
1	690	65	244	58	367	323	47
2	1398	129	431	135	695	703	50
4	1500	178	424	231	833	667	44
9	1500	290 **)	321	336	949	553	37
20	1500	430 **)	318	404	1150	348	23

(C) Dry matter balance

Time from start (hours)	Glucose. H_2O removed from nutrient sol. (mg/l)	Respired (glucose) (mg/l)	Sludge increase (mg/l)	Loss of water ***) (mg/l)	Total (mg/l)	Not accounted for	
						(mg/l)	(%)
1	690	59	488	63+22	632	58	8
2	1398	117	948	127+39	1231	167	12
4	1500	162	913	136+39	1250	250	17
9	1500	260 **)	873	136+29	1298	202	13
20	1500	390 **)	858	136+29	1413	87	6

*) Calculated by assuming a C/N ratio in protein of 3.5;
**) Calculated by extrapolating the oxygen uptake;
***) Molecular and condensation water.

page 71). These polysaccharides, when secreted, may be a means of defence against protozoa, as has been suggested by Stacey [85].

Already during the uptake phase the nitrogen content of both sludges in-

creased slightly; this increase continued during the endogenous phase. It is suggested that this nitrogen increase indicates synthesis of cellular material (cytoplasm). The percentages of glucose utilized in protein synthesis were calculated for both experiments by assuming that the C/N ratio in protein is 3.5. At the end of the uptake phase it was found that in experiment 1, 4.8 per cent of the glucose had been transformed into cell protein and in experiment 2, 15.4 per cent; these figures were 17.0 and 27.0 per cent respectively at the end of the experiment.

The biuret method was not used in the determination of the protein synthesised. Though Gaudy and Engelbrecht [34] apparently obtained good results with this method, in the author's hand it yielded less reliable results than the nitrogen determinations by the micro-Kjeldahl method, when carried out with intact activated sludge.

It can be seen from the glucose balances of both experiments, that during and shortly after the uptake period, about half of the glucose added or more could not be accounted for as polysaccharides or protein. It is assumed that additional unidentified compounds have been formed by the sludges, that did not contain nitrogen and did not react with anthrone. During the endogenous phase after the removal of glucose was completed, the content of these unidentified substances in the sludge decreased gradually, while the protein content increased. Because of the relative constancy of the amount of polysaccharides in both sludges during the endogenous phase, it seems reasonable to assume that endogenous respiration mainly proceeded at the expense of the unidentified fraction, and not of the polysaccharides or proteins formed after the addition of glucose and ammonium sulphate.

The dry matter balance, derived from the results of both experiments, shows that only about 80 to 90 per cent of the added glucose was recovered by evaluating the oxygen uptake, the increase of dry weight of the sludge and the loss of water. The item „loss of water" was calculated from the amount of molecular water of the removed glucose, added to the amount of water derived from the condensation of glucose molecules to polysaccharides during the experiment.

6.3.2 Nature of the storage compounds

As shown in the previous section, a considerable part of the assimilated glucose could be recovered as polysaccharides. The nature of these polysaccharides was studied chromatographically. To elucidate the nature of the unidentified fraction accumulated during the uptake of glucose by both types of activated sludge, it was examined if lipids and poly-β-hydroxybutyrate could be constituents of this fraction.

6.3.2.1 Carbohydrate fraction

In the experiment described in section 6.4.1 it was found, by means of anthrone, that part of the glucose taken up was recovered in the sludge as a polysaccharide (tables 20 and 21). The composition of these polysaccharides was examined in more detail by chromatographical analysis. Glucose (1500 mg) and ammonium sulphate (250 mg) were added to one liter of activated sludge (type 2); samples were taken at regular intervals and the sludge separated by centrifugation. The COD of the supernatant and the dry weight of the sludge were determined. Furthermore, parts of the sludge were hydrolysed with 1 N, 4 N and 7 N sulphuric acid, respectively, in small air-proof tubes at 105 °C for 12 hours. Contrary to the anthrone determination, carried out with 96 per cent sulphuric acid in which the sludge was completely dissolved, part of the sludge remained undissolved during hydrolysis. This insoluble part of the sludge was discarded; it was separated by centrifugation together with the barium sulphate, originating from the neutralization of the sulphuric acid with a solution of barium hydroxide. Paper chromatograms of the hydrolysates were made by using butanol – acetic acid – water (4 : 1 : 5) as a solvent and an ammoniacal solution of silver nitrate (1 : 1) as a spray for the detection of polyalcohols. The following sugars were found in the 4 N and 7 N sulphuric-acid hydrolysates: glucose, fructose, glucosamine, xylose, ribose and rhamnose; only the glucose spots were large and clear, the others were small and more or less faint. In the 1 N sulphuric acid hydrolysate, glucose was only found in that of the sample taken two hours after the addition of the glucose; in the other hydrolysates no carbohydrates were found by paper chromatography.

In the hydrolysates the amounts of reducing sugars were determined by the method of Nelson–Somogyi and by means of the anthrone method. The results of the analyses are recorded in table 22. This table shows that less carbohydrates were hydrolysed with 1 N sulphuric acid than with 4 N and

Table 22. Reducing sugars in hydrolysed activated sludge after addition of 1500 mg of glucose and 250 mg of ammonium sulphate.

Time from start (hours)	COD (mg/l)	Sludge dry wt (mg/l)	Reducing sugars (mg/l)					
			I			II		
			1 N	4 N	7 N	1 N	4 N	7 N
0	94.5+1451	2408	52	456	452	474	488	456
2	181	3256	736	842	845	881	867	858
5	96	3320	440	790	786	770	778	791

I Determined according to the method of Nelson-Somogyi; II Determined by means of the anthrone method.

7 N sulphuric acid. In the latter two hydrolysates the same amounts of reducing sugars were analysed by both methods. A comparison of these values with the amounts of carbohydrate found earlier in the same type of sludge (type 2) by direct determination by means of anthrone (see table 21) shows that the latter gave somewhat higher carbohydrate contents. It is reasonable to suggest that this difference was due to the removal by centrifugation of part of the sludge insoluble in 4 N or 7 N sulphuric acid, which apparently contained approximately 4 per cent of the polysaccharides.

6.3.2.2 Formation of lipids

To investigate whether lipids belonged to the assimilation products from glucose in activated sludge, extractions with ether and chloroform have been performed. One liter of activated sludge (type 2) was supplied with 1500 mg of glucose and 250 mg ammonium sulphate. At different periods of time after starting the experiment, samples were taken and successive extractions with ether and chloroform carried out. The weight loss of the samples by extraction with ether, 10.4 per cent of sludge dry wt at the beginning of the experiment, decreased to 4.5 per cent when all glucose had been taken up; afterwards it increased regularly until after 20 hours a value of 8.0 per cent was found. The weight loss after extraction with chloroform, which indicates the presence of poly-β-hydroxybutyrate, was: at the beginning 4.4 per cent, at the end of the uptake phase 2.0 per cent and after 20 hours 5.5 per cent. From these data it may be concluded that under the conditions of the experiments the formation of lipids from glucose by activated sludge is of minor importance.

6.3.2.3 Fractions soluble and insoluble in water

To identify the assimilates formed in activated sludge after the addition of glucose and ammonium sulphate, an examination was made to decide whether the synthesized compounds were soluble or insoluble in water. One liter of activated sludge (type 2) was supplied with 1500 mg of glucose and 250 mg of ammonium sulphate; samples were taken after different periods of incubation, the sludge separated by centrifugation, washed and disintegrated (for 25 minutes in a MSI supersonic oscillator). The disintegrated sludge was separated by centrifugation at high speed, so that the supernatant fluid was clear. The dry weight was determined of the centrifuged part as well as that of the supernatant fluid. The results, expressed as percentages of the dry matter, show (table 23) that the compounds formed from glucose in activated sludge are insoluble in water and, after disintegration of the sludge, may be separated by centrifugation as debris. A temporary increase of water-soluble compounds occurred during the uptake phase and also later on during the endogenous phase.

Table 23. Water-soluble and insoluble fractions of assimilated matter in activated sludge after adding glucose and ammonium sulphate.

Time from start	Sludge dry wt (mg/l)	Soluble matter		Insoluble matter	
		(mg/l)	(%)	(mg/l)	(%)
0	1640	795	48.5	845	51.5
0 h. 40′	1986	880	44.3	1106	55.7
1 h. 20′	2202	970	43.0	1233	55.7
2 h. 00′	2464	821	33.3	1643	66.7
3 h. 00′	2470	786	31.8	1684	68.2
8 h. 30′	2482	793	31.9	1689	68.1
19 h. 00′	2472	825	33.4	1647	66.6

6.3.3 Composition of the sludge

From the analyses recorded above (cf table 21, zero time) it can be calculated that the average composition of activated sludge (type 2) grown on glucose and ammonium sulphate was as follows: polysaccharides (23 per cent), proteins (39 per cent), lipids (15 per cent) and ash (11 per cent), accounting for approximately 88 per cent of the dry weight of the sludge. The percentage of proteins was calculated by multiplying the nitrogen content by 6.25.

Though this composition does not tally completely, it is evident that the greater part of the sludge consisted of polysaccharides and nitrogenous compounds.

Twenty-four hours after the last feeding, activated sludge (type 2) grown on glucose and ammonium sulphate, was subjected to an elementary analysis, by which the contents of C, H, N, O and ash were determined. Half of the remaining part of the sludge (suspended in one liter of basal solution of mineral salts) was supplied with 1500 mg of glucose, while the other part (also suspended in one liter of basal solution) was supplied with 1500 mg of glucose and 250 mg of ammonium sulphate. Fifty minutes later, samples were taken of both sludges in which also an elementary analysis was carried out. Besides the elements C, H, N, O and ash, the dry weight of the sludge as well as the COD and the glucose concentration of the culture solution were determined, before and 50 minutes after the addition of the substrates. The analytical results are recorded in table 24.

In view of the results of the elementary analysis the following empiric formula was calculated for the activated sludge used: $C_{18}H_{36}O_9N_2$. Fifty minutes after adding only glucose, during which time 955 mg glucose. H_2O had been taken up and the dry weight of the sludge had increased by 560 mg, the empiric formula according to the analytical results would be: $C_{11}H_{22}O_6N$; moreover it was calculated that the assimilated glucose was transformed into compounds with the following empiric formula: $C_6H_{11}O_4$. Furthermore, it was calculated

Table 24. Contents of C, H, N, O and ash in activated sludge before and after addition of A. 1500 mg of glucose and B. 1500 mg of glucose and 250 mg of ammonium sulphate.

(A) 1500 mg glucose per liter, no ammonium sulphate added.

Time from start (min.)	In medium		Activated sludge					
	COD (mg/l)	glucose. H_2O (mg/l)	dry wt (mg/l)	ash (%)	C (%)	H (%)	N (%)	O (%)
0	71+1451	0+1500	2160	10.25	41.64	6.85	5.40	27.09
50	583	545	2720	7.65	42.49	6.89	4.38	29.81

(B) 1500 mg glucose and 250 mg ammonium sulphate per liter.

0	71+1451	0+1500	1985	9.76	42.21	6.73	5.19	27.66
50	663	636	2660	7.63	42.17	6.82	4.26	30.67

from the carbon balance that about 75 per cent of the absorbed carbon was assimilated, so that 25 per cent must have been respired to carbon dioxide.

In experiment B, when besides glucose also ammonium nitrogen was added, 864 mg of glucose. H_2O was taken up during the first fifty minutes, while the dry weight of the sludge increased by 675 mg. The empiric formula of the sludge after 50 minutes was: $C_{24}H_{44}O_{13}N_2$; that of the combined assimilation products was: $C_{55}H_{110}O_{39}N_2$. Approximately 84 per cent of the carbon absorbed was assimilated by the sludge, so that 16 per cent must have been oxidized to carbon dioxide.

6.4 Discussion

Activated sludge consists of flocculent masses of living, aerobic bacteria held together by a matrix of slimy materials. Types of bacteria, as well as metabolism of the organisms, are in some unknown way responsible for floc formation (cf chapters 4 and 5).

In a short time relatively small amounts of bacterial flocs are able to remove large amounts of organic substrates, present in low concentrations in the waste water. Sludge concentrations approximating 2 g dry weight of sludge (containing about 2×10^{12} living bacteria) per liter waste water are common for normal activated sludge plants. Such concentrations have been used also in the present investigation in which a study was made of the removal of substrates of different character by sludges of different origin, and of the biochemical transformations of these substrates within the flocs.

The ready uptake of the substrates by the activated sludge, well-known from the highly economical way of removing organic matter from waste water

in sewage-purification plants, was shown in several examples in the present paper. It was found that glucose was removed at a rate of 210 to 520 mg per gram activated sludge (dry weight) per hour by sludges grown on this substrate, whereas acetate was taken up at a rate of about 440 mg per gram sludge per hour.

The ready removal of organic substrates by the activated sludge flocs suggests that absorption followed by utilization of the substrate by the living, aerobic bacterial cells are mainly responsible for the activity of the sludge flocs. Evidence concerning this assumption is provided by the results of the following experiments carried out in the present investigation. a. When a substrate-containing suspension of activated sludge was aerated with nitrogen gas, no removal of the substrates by the sludge flocs took place. Replacement of the nitrogen by air (oxygen) gave an immediate resumption of the substrate uptake. b. When in a substrate-containing sludge suspension the substrate had been removed from the solution, the uptake of oxygen used for the oxidation of the substrate fell immediately to the level of endogenous respiration, showing that no accumulation of substrate occurred within the cell or at the cell surface. The results of these experiments generally agree with those of Herb [49] and Wuhrmann *et al.* [97]. In the case of substrates with large molecules, accumulation at the cell surface may take place owing to a strong adsorption, but a poor absorption.

Under the conditions of the Warburg experiments, uptake of substrate and of oxygen proceeded at a constant rate, independently of the substrate concentration, but proportionally to the sludge concentration, until all the substrate had been removed. This shows a. that substrate uptake by the bacterial cells or substrate oxidation within the cells determined the rate of oxygen uptake and b. that oxygen supply was not the limiting factor of the rate of oxygen uptake in the sludge suspensions. That oxygen diffusion into the centre of the flocs was not the limiting factor in oxygen uptake and in substrate removal was also shown by increasing the oxygen tension in the Warburg vessels.

The attack of glucose by the cell enzymes, rather than the uptake mechanism of the cells and the diffusion rate of the substrate molecules from the medium to the cells presumably were rate-limiting in the removal of glucose by activated sludge in the present investigation. This is concluded from the fact that uptake of glucose by sludge, adapted to glucose break-down, proceeded many times more readily than oxygen consumption. The excess of glucose within the cells was not accumulated as such, but was converted for a large percentage to polysaccharides which are more or less resistent to the enzymes affecting dissimilation.

One of the most characteristic metabolic features of activated sludge is the low dissimilation percentage of supplied substrates (cf Placak and Ruchhoft [76]), Wuhrmann *et al* [97], Porges *et al* [78], O'Brien [73], Gaudy and

Engelbrecht [34] and Washington and Symons [93]). In a number of experiments with activated sludge, well-adapted to glucose break-down, dissimilation percentages of about 15 per cent of the amount of glucose taken up have been found in the present investigation. (With Na-acetate as the substrate, dissimilation percentages of about 30–35 were found.) When ammonium sulphate was supplied along with the glucose, the dissimilation percentage was only slightly higher. Since no decomposition products were detected in the culture solution, conversion of the glucose to cell constituents like polysaccharides (intra-cellular, or extra-cellular as capsular material) and perhaps lipids or poly-β-hydroxybutyrate were assumed to occur. Synthesis of such compounds may proceed at the expense of much lower amounts of energy *i.e.* with the consumption of considerably lower amounts of oxygen than are required for synthesis of cellular compounds like amino acids, proteins and nucleic acids. Analysis of the activated sludge shortly after the completion of the uptake period of glucose revealed that nearly 60 per cent of the substrate sugar was recovered as polysaccharides in the sludge. This was true when no ammonium nitrogen was supplied along with the glucose. In the presence of both glucose and ammonium sulphate, approximately 25 per cent of the removed glucose was recovered as polysaccharides and about 50 per cent as a nitrogen-free compound not reacting with anthrone, but having an empiric formula resembling that of a carbohydrate.

The contents of lipids and of poly-β-hydroxybutyrate in activated sludge were found to be relatively low; they did not increase during the absorption of glucose, so that these compounds presumably were not involved in the glucose utilization.

In order to explain the pronounced tendency of the micro-organisms in sludge to synthesize polysaccharides from added sugars, experiments were carried out with a number of bacterial strains isolated from domestic activated sludge. In addition, a number of *Arthrobacter* strains, used by the collaborators of the Laboratory of Microbiology for the study of polysaccharide synthesis, were included in this investigation.

In agreement with the earlier investigations with pure cultures of micro-organisms of Barker [5], Clifton [16], Mulder *et al* [68], it was shown that washed cells of a number of these pure cultures when incubated in a buffer solution without a nitrogen source, but in the presence of glucose, readily synthesized the glucose to polysaccharides. Dissimilation percentages of 25–30 per cent of the added glucose were found to occur and polysaccharide contents of 60–70 per cent were observed under such conditions in certain *Arthrobacter* strains [69]. Dissimilation percentages for glucose of about 30 per cent, although relatively low, were higher than the values of about 15 per cent found in activated sludge.

So far it is unknown what is the cause of the extreme tendency of bacterial cells, occurring in activated sludge, to synthesize polysaccharides from glucose instead of amino acids and nucleic acids to be used for cell multiplication. Lack of available nitrogen is not involved, since in the present investigation the dissimilation percentage of added glucose was only slightly increased by supplying ammonium nitrogen along with the glucose. This is in contrast with the results of Wuhrmann *et al* [97] who found a considerable effect of the addition of ammonium nitrogen on the rate of oxygen consumption, the dissimilation percentage as well as the rate of substrate uptake.

Lack of oxygen within the floc is likewise not the cause of the low dissimilation percentages, since increased oxygen supply was found to have no effect on respiration rate or dissimilation percentage.

Although not examined in detail in the present investigation, organic acids such as acetic and lactic acids are assumed to be assimilated for a considerable part to polysaccharides as well. Washington and Symons [93] estimated about equal percentages of polysaccharides in sludges grown with, either glucose or Na-acetate with ammonium sulphate, or with glycine.

As possible causes of the pronounced tendency to synthesize polysaccharides of the bacterial cells occurring in activated sludge a. age of the cells and b. lack of harmony between uptake of the substrate and oxydative break-down may be mentioned. So far no evidence is available in support of these hypotheses, although the results obtained by Holme and Palmstierna [47] indicate the importance of the former case. These authors found that *E. coli* cells, owing to carbon deficiency occurring in the stationary phase, converted added glucose readily to polysaccharides notwithstanding mineral nutrients and nitrogen were present in the medium. Because of the extra-cellular character and the observation made in the present investigation, that the stored polysaccharides did not serve as a source of energy during endogenous respiration, it may be supposed that they were produced directly as external protection of the aged cells as defense against protozoal attacks.

One of the most important questions concerning the metabolism of activated sludge is the utilization in endogenous respiration of the stored compounds *viz.* polysaccharides and unknown compounds, found in activated sludge supplied with glucose and ammonium sulphate. After the completion of the glucose uptake, oxygen consumption dropped immediately to a value slightly higher than the endogenous respiration of control cells, which had had no glucose. The higher the amount of glucose supplied, the higher the endogenous respiration after the complete removal of the substrate.

When ammonium sulphate was supplied in addition to the glucose, the endogenous respiration rate after the uptake phase was again higher. In the latter case, a considerable amount of the unknown compounds, assimilated

during the period of glucose uptake, was converted to nitrogenous compounds, presumably amino acids. The latter were presumably partly used for the synthesis of proteins and nucleic acids giving rise to cell multiplication, and partly served as the substrate for endogenous respiration. It is a well-known fact, that in many bacteria amino acids represent the main source of energy during endogenous respiration [19]. Evidence that amino acids or organic acids served as the substrate for endogenous respiration in domestic activated sludge as well as in glucose-grown sludges, was derived from the fact that the respiration quotient of the sludge without added glucose equalled 0.7–0.8.

Only part of the stored polysaccharides, as estimated by means of the anthrone method, disappeared during the endogenous phase. So the endogenous metabolism of the activated sludges used in the present investigation resembled somewhat that of *Sarcina lutea*, as examined by Ribbons and Dawes [80]. That at least part of the polysaccharides stored in *Arthrobacter* cells after the addition of ammonium nitrogen may be used for the synthesis of amino acids, was shown by the investigations of Zevenhuizen (personal communication). In the case of lipids, synthesized in the presence of large amounts of glucose, Deinema [20] found a ready utilization of the stored lipids for synthesis of cellular material followed by cell multiplication, when ammonium nitrogen was supplied to lipid-containing cells.

In sludge types grown without regular removal of surplus sludge, *i.e.* aged sludge, the dying off of the bacteria covers the increase, while the sludge mass increases gradually due to the accumulation of cell remnants including the unimpaired polysaccharides stored during the removal of substrate. It was shown that these polysaccharides were insoluble in water and thus belong to the constituents of the slimy substances that probably are the cause of the flocculation of the bacteria to sludge.

Adaptation to the utilization of the substrate by activated sludge, as well as by washed cells of pure cultures of bacterial strains isolated from the sludge, appeared to be an important phenomenon in the removal of such substrates as glucose and glycerol.

Substrates like succinate, malate, ethanol, lactate and particularly acetate were consumed by domestic activated sludge almost without a period of adaptation. This may have been due a. to the presence of small amounts of these substrates in the domestic waste water or b. to the fact that these substrates, in contrast to sugars, mannitol, glycerol and several other compounds, could be consumed by the sludges without a period of adaptation. Although most sewages undoubtedly contain considerable amounts of organic acids, particularly acetic acid, the second explanation at least for acetic acid, was also found to be true. This was shown by comparing sludges grown on glucose, lactate and acetate, respectively, as the carbon source and ammonium sulphate

as the nitrogen source. When these three sludges were supplied with glucose or Na-acetate in Warburg vessels, it appeared that glucose was consumed only if the sludge grown on glucose was used (Q_{O_2}' value: 35.9 as contrasted to values of 4.2 and 3.3 for sludges grown on lactate and acetate, respectively). With acetate as the substrate, however, the Q_{O_2}' values found for sludges grown on glucose, lactate and acetate were as follows: 28.4, 26.8 and 47.3, respectively.

Adaptation of the bacteria in domestic sludges or in sludge grown in synthetic waste water devoid of sugars, to break down glucose did not occur within a few hours, even not in the presence of ammonium nitrogen. In the presence of amino acids, however, the adaptation proceeded more readily. This effect of amino acids on adaptation was observed most clearly during the period of endogenous respiration, following complete removal of added substrate. Gaudy and Engelbrecht [35] also found adaptation only in the presence of a suitable nitrogen source.

Adaptation of activated sludges to the uptake and consumption of certain substrates may be due to a physiological adaptation of bacterial cells present already in large numbers in the sludge, followed by the multiplication of these bacteria. However, it may be due also to the selective promotion within the sludge of the multiplication of certain bacterial strains, particularly able to utilize the substrates in question, and hitherto present only in relatively small numbers in the sludge. Both types of adaptation presumably play a part when activated sludges consisting of mixtures of different bacterial strains are used. The second type of adaptation is presumably, at least partly, responsible for the poor uptake and utilization of glucose by some of the domestic sludges, studied in the present investigation. A considerable part of the bacteria isolated from these sludge types was not adaptable to glucose utilization, *i.e.* belonged to the genera *Alcaligenes* and *Lophomonas*, the members of which are unable to utilize glucose (cf table 10). On the other hand, the first type of adaptation undoubtedly has also occurred in the experiments with domestic sludges. This may be concluded from the experiments with pure cultures of bacteria isolated from Zeist activated sludge and from those with pure-culture flocs of *Zoogloea*. When grown in a medium devoid of glucose, these bacteria displayed practically no glucose uptake. Similar to the sludges, adaptation of the bacterial cultures or of the pure-culture flocs to glucose utilization during the experimental period in the Warburg respirometers was negligible. Some strains in the presence of glucose and ammonium nitrogen showed a slight adaptation during this period. When grown on glucose, however, a ready uptake of glucose occurred with *Arthrobacter* cells.

NUTRIENT MEDIA AND ANALYTICAL METHODS

Basal solution of mineral salts

$CaCl_2 \cdot 2H_2O$	0.04	g/l
$MgSO_4 \cdot 7H_2O$	0.1	g/l
$FeCl_3 \cdot 6H_2O$	0.01	g/l
Sörensen phosphate buffer 0.33 M, pH 7.2	10	ml/l

Tryptone glucose agar

tryptone	2	g/l
glucose	1	g/l
Davis-agar	9	g/l

Ammonium sulphate agar

$(NH_4)_2SO_4$	1	g/l
carbon source	4	g/l
K_2HPO_4	1	g/l
yeast-extract	0.05	g/l
Davis-agar	9	g/l

Azotobacter agar

glucose	20	g/l
K_2HPO_4	1	g/l
$MgSO_4 \cdot 7H_2O$	0.5	g/l
$CaCO_3$	20	g/l
Davis-agar	9	g/l

Oxidation-fermentation medium

tryptone	2	g/l
sugar	10	g/l
K_2HPO_4	0.3	g/l
NaCl	5	g/l
bromo-thymol blue	0.05	g/l
Davis-agar	1.5	g/l

about 4 ml in 10 × 120 mm tubes.

$MnSO_4 \cdot 5H_2O$	1.0	mg/l
$CuSO_4 \cdot 5H_2O$	0.1	mg/l
$ZnSO_4 \cdot 7H_2O$	0.1	mg/l
$CoCl_2 \cdot 6H_2O$	0.01	mg/l
H_3BO_3	0.01	mg/l

Tryptone agar

tryptone	2	g/l
Davis-agar	10	g/l

Yeast-extract glucose agar

yeast-extract	7	g/l
glucose	2	g/l
Davis-agar	9	g/l

Eosin methylene blue agar

peptone	10	g/l
lactose	10	g/l
K_2HPO_4	2	g/l
eosin Y	0.4	g/l
methylene blue	0.065	g/l
Oxoid-agar	15	g/l

Nutrient broth

beef-extract	0.5	g/l
peptone	2.5	g/l
yeast-extract	1.0	g/l
NaCl	2.5	g/l

about 10 ml in 16 × 150 mm tubes.

McConkey broth

peptone	20	g/l	
lactose	10	g/l	
bile salts	5	g/l	
NaCl	5	g/l	about 10 ml in 16×150 mm tubes
bromo-cresol purple	0.01	g/l	with Durham's tubes.

Allen's broth (modified)

tryptone	1.5	g/l	Sörensen phosphate buffer		
yeast-extract	0.5	g/l	0.33 M, pH 7.2	5	ml/l
glucose or lactose	5	g/l	skim milk	40	ml/l
$(NH_4)_2SO_4$	0.2	g/l	bromo-thymol blue	0.05	g/l
$CaCl_2 \cdot 2H_2O$	0.04	g/l			
$MgSO_4 \cdot 7H_2O$	0.1	g/l	about 10 ml in 16×150 mm tubes		
$FeCl_3 \cdot 6H_2O$	0.01	g/l	with Durham's tubes.		

Chemical oxygen demand (COD) [3]

A 5 ml sample, or an aliquot diluted to 5 ml with distilled water, was placed in a 250 ml round-bottom flask and 10 ml (approximately 0.125 N) potassium bichromate solution, 15 ml concentrated sulphuric acid, 100 mg silver sulphate and some pumice granules were added. The flask was attached to a Dimroth's condenser and the mixture refluxed for 15 minutes. Then the mixture was diluted with 30 ml distilled water and after cooling to about 45 °C the excess bichromate was titrated with ferrous ammonium sulphate (about 0.067 N), using two or three drops of ferroin indicator (1.5 g 1,10 phenantroline and 0.7 g ferrous sulphate in 100 ml water).

A blank consisting of 5 ml distilled water instead of the sample together with the reagents, was refluxed in the same manner. No chloride correction was applied.

Micro-determination of reducing sugars [21]

To 2 ml samples in 20×150 mm tubes with a mark at 25 ml level, was 2 ml copper reagent [82] added. Samples, blanks and standard sugar solutions were heated for 10 minutes in boiling water and then cooled in tap water. Two ml of the arseno-molybdate reagent [68] was added and mixed. After all the cuprous dioxide was dissolved, the solution was diluted with distilled water to the 25 ml mark on the tube. The optical density of the mixture was measured after 15 minutes at 660 mμ (or 520 mμ when too much sugar was present) against the blank. The sugar content was calculated on the basis that the optical densities were directly proportional to the concentration of reducing sugars.

Micro-determination of carbohydrates [21]

One ml carbohydrate solution or suspension of bacteria, contained in a tube immersed in cool water, was mixed with fresh anthrone reagent (200 mg anthrone in 100 ml concentrated sulphuric acid). The mixture was placed in boiling water for exactly 3 minutes and then cooled in tap water to room temperature. The optical density was measured within an hour at 620 mμ against a blank. A standard of 60 μg glucose was run along and the calculations were made on the basis that the optical densities were directly proportional to the carbohydrate concentrations.

Micro-determination of total nitrogen by the Kjeldahl method [51]

The samples of activated sludge or bacteria contained in Pyrex tubes (16 × 160 mm) or in 30 ml Kjeldahl flasks were digested with 2–10 ml 10 N sulphuric acid (containing 0.2 g/l $CuSeO_3$ or an equivalent mixture of $CuSO_4$ and Na_2SeO_3). After one hour a few drops of a 30 per cent solution of hydrogen peroxide were added until the solution remained clear. Ammonia was distilled into 2 per cent boric acid and estimated by titration with dilute sulphuric acid.

Determination of the aeration capacity [18]

In the aerated liquid 126 g/l $Na_2SO_3 \cdot 7H_2O$ and 0.16 g/l $CuSO_4 \cdot 5H_2O$ was dissolved. With regular intervals 5 ml was pipetted in an iodine solution (12.79 g I_2 and 25 g KI per 10 ml distilled water). The excess iodine was titrated with Na-thiosulphate solution. The amount of oxygen, which was bound to the sulphite could be calculated from the difference between the used amounts of thiosulphate. The oxygen solution rate or aeration capacity was expressed in mg oxygen per 100 ml per hour.

Wet negative staining of bacterial capsules and slime [25]

One drop of not too washable Indian ink, free from coarse particles, was brought on to an object glass. A heavy suspension of bacteria was mixed with the ink, whereafter a cover-glass was laid down over the suspension and pressed firmly.

Staining bacterial polysaccharides with Alcian blue [70]

On a clean object glass a thin bacterial smear was made which was allowed to dry and fixed over a flame. A 0.1 per cent solution of Alcian blue (Gurr) in dilute ethanol was applied for one minute. After washing with water the smear was counterstained for 10 seconds with an aqueous solution of 0.5 per cent safranine, then washed with water and dried. The bacterial polysaccharides stained blue against the red bacterial cells.

Cytochrome-oxidase test [32]

A few drops of a 3 : 2 mixture of a 1 per cent solution of *p*-aminodimethyl-aniline-2HCl in water and a 1 per cent solution of α-naphtol in 96 per cent ethanol were added to a young slant culture. Most of the positive cultures coloured dark blue within 30 seconds; a reaction within one minute indicated a *Pseudomonas* species.

SUMMARY

An investigation was made into the bacteriology and biochemistry of different types of activated sludge grown in the sewage purification of the village of Zeist and in various types of laboratory installations fed with waste water from Wageningen or with synthetic media of different composition.

Numbers of bacteria were counted by the dilution technique, using agar plates of different composition, including waste water agar, and liquid culture media.

Before diluting the bacterial suspensions, the sludge samples were washed to remove those bacteria presumably not contributing to the formation of flocs and thereafter homogenized to disperse the bacteria.

The washing of the sludge samples effectuated a reduction of the bacterial counts to approximately 30 per cent of the numbers counted in untreated samples. Bacterial counts varied from 10^{11}–10^{12} per gram of washed sludge (dry wt).

The nature of the bacterial flocs of the different types of activated sludge was investigated by determining morphological and physiological characteristics of approximately 100 strains per sludge sample, isolated from the high dilution count plates.

In activated sludge grown in domestic waste water in the purification plant or in the laboratory apparatus, aerobic, Gram-negative, non-spore-forming rods of the family *Achromobacteraceae* (genera *Alcaligenes*, *Flavobacterium* and *Achromobacter*) were found to be the predominant micro-organisms. Members of the genus *Pseudomonas* and coryneform organisms occurred in these types of sludge in relatively low numbers.

In activated sludge grown with synthetic waste water (mineral salts, glucose and ammonium sulphate) about one half of the isolated bacterial strains belonged to the *Achromobacteraceae*, the other half consisting of representatives of the *Micrococcaceae* and of large egg-shaped encapsulated coccoid bacteria not yet defined.

A number of strains isolated from activated sludge and strains obtained from laboratory collections was tested for floc formation under laboratory conditions. These strains included representatives of the genera *Pseudomonas*, *Alcaligenes*, *Achromobacter*, *Zoogloea* and two strains of the above-mentioned large coccoid bacteria.

Bacterial flocs were not formed readily by most of the strains tested in pure

culture. Several strains (including members of *Alcaligenes*, *Achromobacter* and *Zoogloea*) flocculated in media enriched with calcium chloride in amounts of 50–100 mg per liter. Additions of 40 mg per liter ferric chloride to suspensions of the large coccoid bacteria, brought about flocculation, whereas no such effect was obtained with additions of calcium chloride up to 160 mg per liter.

No effect of variation in C/N ratio of the nutrient solution was observed with regard to the production of flocs starting with dilute suspensions of homogenized domestic activated sludge or with pure cultures of *Alcaligenes* and *Zoogloea*.

For the metabolic investigations of activated sludge, the same types of sludge were used which had been analysed bacteriologically (domestic sludge grown in a purification plant and sludges grown in the laboratory in sewage and in synthetic waste waters containing glucose, Na-lactate, or Na-acetate as the carbon source and ammonium sulphate as the nitrogen source).

Removal of various substrates from the medium by the sludge and oxygen consumption during the period of substrate uptake and thereafter were measured in Warburg respirometers. From the data obtained, the rates of uptake of substrate and of oxygen (mg per gram of sludge (dry wt) per hour) and the dissimilation percentages were calculated.

From a number of substrates tested, domestic activated sludge readily absorbed organic acids (acetate, lactate, malate and succinate) and ethanol, whereas sugars and polyalcohols were taken up slowly (approximately 20 mg glucose was absorbed per hour per gram dry weight by these sludges as compared with 400 mg by sludge grown with glucose as the only substrate).

Washed suspensions of pure cultures of bacteria isolated from these domestic sludge flocs (mainly representatives of the genus *Achromobacter*) and a number of strains obtained from different laboratory collections, including strains of the genera *Pseudomonas*, *Flavobacterium* and *Arthrobacter*, grown in a nutrient medium with Na-acetate, were almost unable to absorb sugars, although with a number of strains the rate of glucose consumption slightly increased during the experimental period. Washed flocs of a *Zoogloea* strain, grown in a peptone-containing medium, were also unable to utilize glucose and glycerol but readily absorbed and consumed acetate and lactate.

The reason for the poor utilization of sugars by sludges and washed cultures of bacteria grown in sewage or media devoid of sugars was that no adaptation to sugar break-down had taken place. When nitrogen compounds, particularly amino acids, had been supplied along with the glucose to non-adapted sludge or bacterial suspensions, a more ready adaptation to glucose break-down occurred. Physiological adaptation of existing cells as well as selection of bacterial strains capable of consuming glucose were found to be responsible for substrate adaptation when the sludge had been grown on sugar.

The uptake of oxygen by sludges or bacterial cultures not adapted to acetate

break-down after addition of acetate proceeded almost without any adaptation period. A value of 28 for net-oxygen uptake rate (Q_{O_2}') with acetate as the substrate was found for sludge grown with glucose, and of 47 for sludge grown with acetate; for glucose Q_{O_2}' values of 3 were found for unadapted sludge and of 36 for glucose-adapted sludge.

Dissimilation percentages of 10–20 for glucose and of 30–40 for acetate were found in activated sludge supplied with these substrates. Washed suspensions of bacterial strains behaved more or less similarly, though dissimilation percentages for glucose were higher.

Rates of uptake for glucose as well as for oxygen, and dissimilation percentages were found to be independent of substrate concentration, but, under the conditions of the experiment, uptake rates for glucose and oxygen increased proportionally with the sludge concentration. From these results, it was concluded that glucose consumption by the bacterial cells of the sludge flocs rather than diffusion of the glucose molecules to the sludge is the rate-limiting factor in substrate removal.

From the fact that the rate of glucose uptake by sludge flocs was considerably higher than the oxygen consumption, while increased oxygen supply did not affect oxygen uptake, it is concluded that the enzymatical decomposition of glucose in the bacterial cells rather than glucose uptake is rate-limiting in the removal of glucose and presumably of other substrates by activated sludge.

From the analyses of assimilation products in activated sludge flocs, formed during the period of glucose removal by glucose-adapted sludge, it was found that almost 60 per cent of the added glucose was stored as polysaccharides, when no nitrogen was supplied along with the glucose. Addition of ammonium sulphate had no effect on the rate of oxygen consumption during the uptake of glucose and on the dissimilation percentage, but resulted in a conversion of about 25 per cent of the glucose into polysaccharides (consisting of reducing sugars) and about 50 per cent into unknown compounds. The former fraction remained for the greater part unimpaired during the period of endogenous respiration, whereas the unknown fraction apparently was converted into amino acids, which served as the substrate for endogenous respiration as well as building blocks for cell synthesis.

Compounds extractable with ether (lipids) or chloroform (poly-β-hydroxybutyrate) although occurring in small amounts in activated sludge, were not formed in appreciable amounts during glucose consumption.

Endogenous respiration during the period following the substrate removal was higher, the higher the sludge load (mg substrate (glucose) per mg dry weight of sludge) had been. When ammonium sulphate had been supplied along with the glucose, respiration during the endogenous phase was again considerably higher, presumably as a result of amino acid synthesis.

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SAMENVATTING

Een onderzoek werd verricht over de bacteriologie en de biochemie van verschillende soorten actief slib. Deze waren afkomstig van de rioolwaterzuiveringsinstallatie in Zeist en van laboratoriuminstallaties gevoed met rioolwater van de stad Wageningen, of met voedingsoplossingen van verschillende samenstelling.

Met behulp van de verdunningstechniek werden bacterietellingen verricht. Alvorens de verdunningsseries te maken, werden de slibmonsters gewassen om de bacteriën die niet tot de werkelijke vlokflora behoorden te verwijderen. Daarna werden de vlokken in een keukenmixer behandeld teneinde zoveel mogelijk bacteriën in gedispergeerde toestand te brengen. De verdunde suspensies werden uitgezaaid op agarplaten van verschillende samenstelling w.o. platen van rioolwateragar, en in buizen met vloeibare voedingsoplossing.

In gewassen slibmonsters werden ongeveer 70% minder bacteriën geteld dan in niet gewassen slib. De aantallen varieerden van 10^{11}–10^{12} levende bacteriën per gram slib (droog gewicht).

De aard van de bacterieflora van actief slib van verschillende herkomst werd nagegaan door van ongeveer 100 stammen van iedere slibsoort, geïsoleerd van telplaten van sterk verdunde slibsuspensies, een aantal morfologische en fysiologische kenmerken te bepalen.

Aerobe, Gram-negatieve, niet-sporevormende, staafvormige bacteriën van de familie der *Achromobacteraceae* (geslachten *Alcaligenes*, *Achromobacter* en *Flavobacterium*) bleken in actief slib gegroeid op rioolwater het meest voor te komen. Vertegenwoordigers van het geslacht *Pseudomonas* zowel als van de familie der *Corynebacteriaceae* werden in betrekkelijk geringe aantallen in deze slibsoorten aangetroffen.

In actief slib gegroeid in voedingsoplossingen met minerale zouten, glucose en ammoniumsulfaat, bleek ongeveer de helft van de geïsoleerde stammen te behoren tot de familie der *Achromobacteraceae*, terwijl de rest bestond uit *Micrococcaceae* en grote ei-vormige bacteriën met kapsels. Deze laatsten konden niet worden gedetermineerd.

Van een aantal stammen, geïsoleerd uit actief slib, respectievelijk afkomstig van verschillende collecties, werd het vermogen om onder laboratoriumomstandigheden vlokken te vormen nagegaan. Tot deze stammen behoorden vertegenwoordigers van de geslachten *Alcaligenes*, *Achromobacter* en *Zoogloea*;

verder twee stammen van de eerder genoemde ei-vormige kapselvormende bacteriën.

Het bleek, dat de meeste bacteriën in reincultuur niet op eenvoudige wijze tot vlokvorming konden worden gebracht. Enkele stammen, waaronder soorten behorende tot de geslachten *Alcaligenes*, *Flavobacterium* en *Zoogloea*, werden tot vlokvorming gebracht door ze te laten groeien in voedingsoplossingen met verhoogde concentraties aan calciumchloride (50 à 100 mg/l). Toevoeging van 40 mg ferrichloride per liter aan gewassen suspensies van de ei-vormige bacteriën had een uitvlokking van de bacteriemassa tot gevolg, terwijl toevoeging van calciumchloride tot 160 mg/l zonder resultaat bleef.

Wijziging van de C/N verhouding van de voedingsoplossing had geen invloed op de vorming van bacterievlokken, uitgaande van verdunde gehomogeniseerde suspensies van actief slib zowel als van reincultures van een *Alcaligenes*- en een *Zoogloea*-stam.

Voor het onderzoek van de stofwisseling van actief slib werd gebruik gemaakt van dezelfde slibsoorten die ook bacteriologisch waren onderzocht, afkomstig van de rioolwaterinstallatie te Zeist en van laboratoriuminstallaties gevoed met afvalwater van Wageningen of met voedingsoplossingen bevattende glucose, Na-acetaat of Na-lactaat als koolstofbron en ammoniumsulfaat als stikstofbron.

De opname van verschillende substraten en de zuurstofopname tijdens en na de substraatopname door actief slib werden gemeten met behulp van Warburgrespirometers. Uit de hierbij verkregen gegevens werden de snelheden berekend waarmee het substraat en de zuurstof werden opgenomen (mg per gram slib (dr. gew.) per uur); tevens werd berekend welk deel van het substraat door de opgenomen zuurstof werd verademd (dissimilatiepercentage).

Het bleek, dat rioolwaterslib organische zuren (azijnzuur, melkzuur, appelzuur en barnsteenzuur) en aethanol snel opnam, terwijl daarentegen suikers en polyalcoholen slechts langzaam werden opgenomen (per uur ca 20 mg glucose per gram van deze soorten slib (dr. gew.) tegen ca 400 mg door slib dat op glucose als enige koolstofbron was gegroeid).

Gewassen suspensies van bacteriën die uit rioolwaterslib waren geïsoleerd, (voornamelijk *Achromobacters*) en een aantal stammen afkomstig van verschillende laboratoriumcollecties (w.o. stammen van de geslachten *Pseudomonas*, *Flavobacterium* en *Arthrobacter*), gekweekt in een voedingsoplossing met Na-acetaat als enige koolstofbron, bleken niet of in geringe mate in staat glucose op te nemen, alhoewel bij sommige stammen tijdens het experiment de zuurstofopnamesnelheid na toevoeging van glucose geleidelijk steeg.

Gewassen vlokken van een *Zoogloea*-stam, gegroeid in een voedingsoplossing met pepton, waren eveneens niet in staat glucose en glycerol te verademen, maar wel acetaat en lactaat.

De reden van de slechte opname van suikers door actief slib en gewassen bacteriesuspensies, gegroeid in rioolwater of suikervrije voedingsoplossingen, was dat geen adaptatie aan suikerafbraak had plaats gehad. Werden stikstofverbindingen, speciaal aminozuren, tegelijk met glucose toegevoegd aan niet geadapteerd slib of bacteriemateriaal, dan kon een geleidelijke adaptatie tot afbraak van glucose worden waargenomen. Gevonden werd, dat fysiologische adaptatie van bestaande cellen, zowel als een selectie van glucose-gebruikende bacteriestammen deze substraatadaptatie teweegbracht.

Opname van zuurstof na toediening van Na-acetaat, door actief slib of door reincultures van bacteriën die niet in een acetaathoudend medium waren gegroeid, verliep vrijwel zonder een periode van adaptatie aan de afbraak van acetaat. Na toevoeging van acetaat aan slib, dat op glucose was gegroeid werd een netto-zuurstofopnamesnelheid (Q_{O_2}') van 28 mg O_2 per gram droog slib per uur gevonden, voor slib gegroeid met Na-acetaat was dit 47; na toevoeging van glucose aan niet geadapteerd slib werd een nettozuurstofopnamesnelheid van 3 gevonden, en van 36 met een aan glucose-afbraak geadapteerd slib.

Glucose werd door actief slib voor 10 à 20% verademd; Na-acetaat voor 30 à 40%. Ongeveer hetzelfde beeld werd waargenomen met gewassen suspensies van de eerder genoemde bacteriestammen, alhoewel met glucose een hoger dissimilatiepercentage werd gevonden.

Gevonden werd dat zowel de opnamesnelheden voor glucose en zuurstof als de dissimilatiepercentages onafhankelijk waren van de concentratie van het substraat, maar dat onder de omstandigheden van de proef, de opnamesnelheden voor glucose en zuurstof evenredig met de slibconcentratie toenamen. Hieruit werd geconcludeerd dat de snelheid van het glucoseverbruik door de cellen en niet de snelheid van diffusie van de glucosemoleculen vanuit de vloeistof naar de vlokken de beperkende factor vormt bij het verwijderen van substraat uit de oplossing.

De enzymatische omzetting van de glucose in de bacteriecellen is waarschijnlijk de snelheidsbeperkende factor bij het verdwijnen van glucose en waarschijnlijk ook van de andere substraten uit de oplossing onder invloed van actief slib. Dit werd geconcludeerd uit het feit, dat de snelheid waarmee glucose door de slibvlokken werd opgenomen aanzienlijk groter was dan de zuurstofopnamesnelheid, terwijl een verhoogde zuurstoftoevoer de opname niet beïnvloedde.

Uit de analyses van de assimilatieproducten, die tijdens de opname van glucose door geadapteerd slib werden gevormd bij afwezigheid van een stikstofbron, bleek, dat bijna 60% van de toegevoegde glucose werd opgeslagen in de vorm van polysacchariden. Toevoeging van ammoniumsulfaat tegelijk met de glucose had tijdens de glucoseopname geen invloed op de zuurstofopname-

snelheid, noch op het dissimilatiepercentage, maar had tot gevolg dat slechts ca 25% van de glucose werd omgezet in polysacchariden (bestaande uit reducerende suikers) en ca 50% in onbekende verbindingen. De polysaccharidefractie bleek gedurende de endogene fase nagenoeg niet af te nemen, terwijl de onbekende fractie daarentegen blijkbaar werd omgezet in aminozuren, die zowel als substraat voor de endogene ademhaling dienden als voor de synthese van nieuwe cellen.

Met aether of chloroform extraheerbare stoffen zoals vetten of poly-β-hydroxyboterzuur kwamen in geringe hoeveelheden in actief slib voor. Gedurende de opname van glucose door het slib namen deze extraheerbare stoffen niet in hoeveelheid toe.

Naarmate het slib zwaarder werd belast, nam de endogene ademhaling van het slib nadat het substraat was opgenomen toe. Werd behalve glucose ook ammoniumsulfaat toegevoegd dan werd de endogene ademhaling nog hoger, vermoedelijk tengevolge van de dan optredende vorming van aminozuren.

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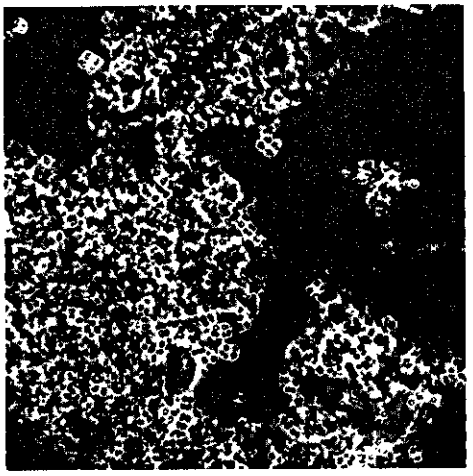
PLATE I



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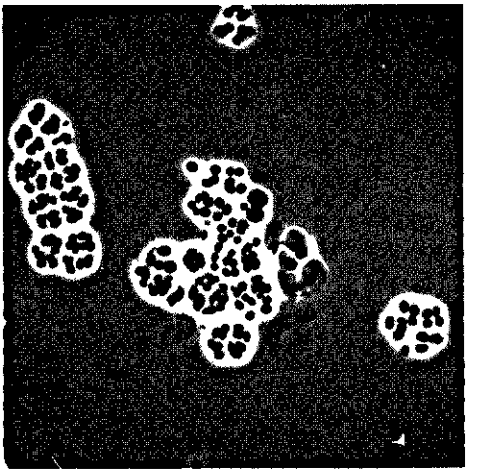

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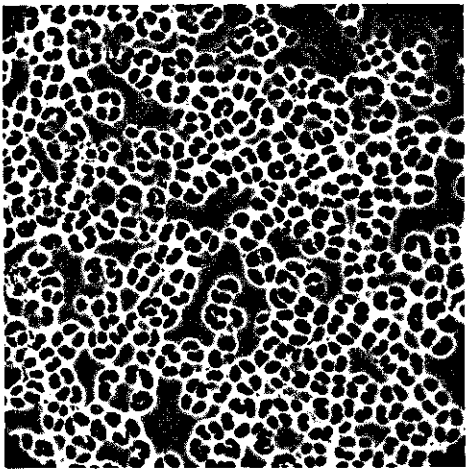
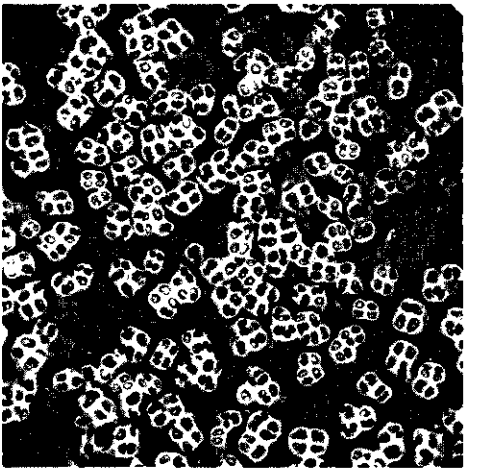


c. 609×



d. 609×

Large encapsulated bacteria occurring in large numbers in activated sludge grown in the laboratory, fed daily with mineral salts, glucose and ammonium sulphate: a. in sludge flocs, mounted in Indian ink; b. pure culture grown on beef-extract; c. pure culture grown on yeast-extract glucose agar; and d. do, mounted in Indian ink.