THE PHYSIOLOGY OF THE FILAMENTOUS BACTERIUM *MICROTHRIX PARVICELLA*

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Herman Slijkhuis

The Physiology of the filamentous bacterium *Microthrix parvicella*

Proefschrift

ter verkrijging van de graad van
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op gezag van de rector magnificus,
dr. C.C. Oosterlee,
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Abstract.

A study has been made of the physiology of *Microthrix parvicella*. This filamentous bacterium often causes poor settleability of activated sludge in oxidation ditches supplied with domestic sewage. The organism was found to utilize only long chain fatty acids (preferably in esterified form) as carbon and energy source. Short chain fatty acids and glycerol are only utilized together with long chain fatty acids; other organic substrates are not consumed. The organism requires ammonium salts and reduced-sulphur compounds for growth. Thiamin and cyanocobalamin were found to be essential vitamins. Optimum temperature is 25°C and the pH of the medium has to be above 7. During the early growth phase in batch culture with an oleic acid ester (Tween 80) as the limiting substrate, *M. parvicella* stores large amount of lipids, mainly oleic acid in esterified form in its cells. In a subsequent period the stored lipids are used for energy supply and synthesis of cell protein.

Growth kinetics were studied by cultivating the organism in a chemostat. The maximum specific growth rate was found to be 0.06 h^{-1}; the K_S value for Tween 80 was too low to be determined. The maximum yield coefficient and the maintenance on Tween 80 were 0.34 g cell dry weight/g Tween 80 consumed and 6.3 mg Tween 80/g cell dry weight.h, respectively. As only the oleic acid moiety of Tween 80 is consumed, these values must be corrected for the non-fatty acid moiety (76%) of the Tween 80 molecule.

Although oleic acid is an excellent substrate for *M. parvicella*, the organism was found to be inhibited at higher concentrations of this compound. Decreasing pH and decreasing biomass concentration promote the inhibiting action of oleic acid in washed cell suspensions. Various surface-active compounds which are not utilized as carbon source enhance the oxygen uptake rate of washed cells. The increase of the oxygen uptake due to added Tween 80 or oleic acid is probably caused by substrate respiration as well as a non-substrate-specific stimulation caused by surface-active properties of these compounds. The affinity for oxygen of *M. parvicella* is high (K_S value < 0.016 mg/l).

Abundant growth of *M. parvicella* was obtained in activated sludge in a bench-scale plant fed with waste water containing oleic acid and supplied with a limited amount of oxygen. These results explain the excessive growth of *M. parvicella* in activated sludge supplied with raw domestic sewage. This type of sewage contains a considerable amount of fatty matter and the activated-sludge process is usually operated at a low oxygen concentration in the mixed liquor.

Keywords. *Microthrix parvicella* - filamentous bacterium - bulking sludge - activated sludge - growth kinetics - reduced-sulphur compounds - fatty acids - oleic acid - Tween 80 - surfactants - grease - respiration - oxygen.

STELLINGEN

1. Licht slib ten gevolge van sterke groei van *Microthrix parvicella* wordt veroorzaakt door de aanwezigheid van vet(zuren) in het afvalwater en een lage zuurstofconcentratie in de aeratietank.

   Dit proefschrift.

2. Opslag van vetten door *Microthrix parvicella* gedurende de eerste groeifase bij het kweken op oliezuurbevattende media berust op de ongunstige invloed van vrij oliezuur op het metabolisme van dit organisme.

   Dit proefschrift.

3. Bepaling van de K_S waarde van oppervlakteactieve verbindingen voor microorganismen zoals uitgevoerd door Novak en Kraus is onjuist.

   J.T. Novak and D.L. Kraus (1973). Water Research,*7*, 843-851.

4. Biologische afbraak van organische verbindingen bepaald door middel van zuurstofopname kan tot foutieve conclusies leiden.

   R.C. Loehr and J.C. Roth (1968). J.Wat.Poll.Contr.Fed.,*40*, R385-R403.

5. Het metabolisme van azijnzuur in de blauw-groene alg *Anabaena variabilis* zoals is opgesteld door Pearce e.a., is niet in overeenstemming met hun resultaten.

   J.Pearce and N.G. Carr (1967). J.gen.Microbiol.,*49*, 301-313.
   J. Pearce, C.K. Leach and N.G. Carr (1969). J.gen.Microbiol.,*55*, 371-378.

6. Het fenomeen van de fosfaatafgifte in een zuurstofvrije zone door actief slib dat in staat is tot biologische fosfaatverwijdering wordt niet verklaard door de hypothese opgesteld door Hall e.a., noch door die opgesteld door Marais e.a.

   E. Hall, H.A. Nicholls and D.W. Osborn (1978). 11th Essen Conference, March, 1978.
   G.v.R. Marais, R.E. Loewenthal and I. Siebritz (1982). 11th Conference of I.A.W.P.R., Pretoria. Post conference seminar on phosphate removal in biological treatment processes, April, 1982.

7. Het is merkwaardig, dat bij de bestudering van fytoalexinen zo weinig aandacht wordt besteed aan fotochemische aspecten.

Phytoalexins (1982). J.A. Bailey and J.W. Mansfield (eds), Blackie, Glasgow.

8. Het overheidsbeleid inzake discriminatie van bevolkingsgroepen is hypocriet.

9. Deeltijdarbeid: gedeelde smart is dubbele vreugd.

10. Een laatste stelling is maar zelden het lezen waard.

Herman Slijkhuis
The physiology of the filamentous bacterium *Microthrix parvicella*.
Wageningen, 7 oktober, 1983.

Aan mijn ouders

Aan Karin

Voorwoord.

Het is een goede gewoonte om een proefschrift te beginnen met een dankwoord. Zonder de hulp van anderen zou dit proefschrift nooit in deze vorm zijn verschenen; daarom wil ik allen die op enigerwijze hebben bijgedragen tot de voltooiing van dit proefschrift hartelijk danken.

Mijn ouders voor de wijze waarop zij mij hebben gestimuleerd om te gaan studeren.

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Contents.

1. Introduction

1.1 ACTIVATED SLUDGE

The activated-sludge process is the most extensively used process in the aerobic treatment of domestic sewage as well as industrial waste water containing biodegradable material. This is due to the flexibility and high efficiency of the process which depends on the mixing of activated sludge with waste water (influent), intensive agitation and aeration of the mixture for some hours, and subsequently the separation of sludge and purified waste water (effluent) by sedimentation. Activated-sludge flocs consist of active organisms, mainly bacteria, dead biomass and undefined organic and inorganic compounds, including waste-water components adsorbed by the flocs. The agitation of the mixture of sludge and waste water in the aeration tank provides the active organisms with oxygen which is required for the aerobic breakdown of the substrates in the influent. In addition it keeps the sludge flocs in suspension, ensuring a good contact between flocs and compounds in the waste water. In the sedimentation tank the sludge flocs settle and the clarified waste water can be disposed off to surface water without disturbing the ecology of the environment to a great extent. The settled sludge is partly returned to the aeration tank whereas the excess (surplus) sludge is withdrawn from the process (Fig. 1).

The settleability of activated sludge greatly affects the operational capacity of the treatment plant, as a poor settling may result in loss of sludge flocs from the final sedimentation tank with the effluent. Settleability of sludge depends on the floc structure and problems may occur when the sludge flocs are not compact and dense.

Pipes (1967) has reviewed the various types of settling problems and distinguishes three major groups:

a. problems in floc formation.
b. problems due to low floc density.
c. problems due to low sludge compaction.

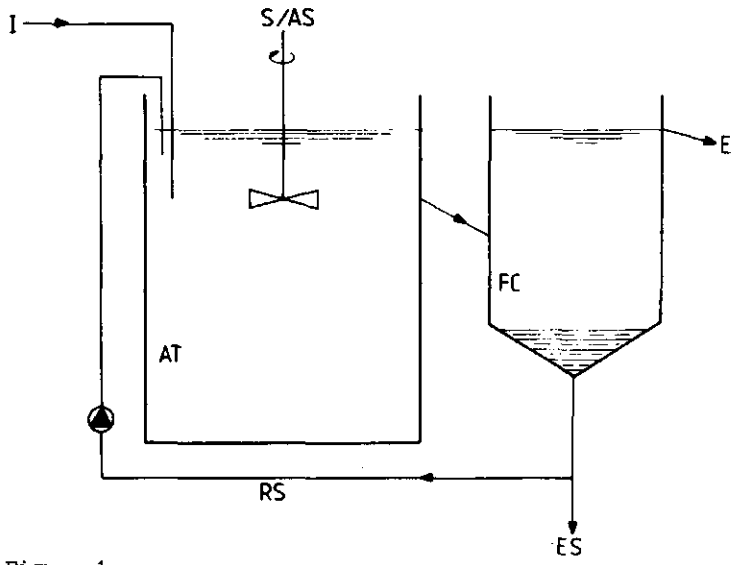

Figure 1.
Outline of a continuously operating activated-sludge plant.
I = influent; AT = aeration tank; S = stirrer; AS = air supply; FC = final clarifier; E = effluent; RS = recycled sludge; ES = excess sludge.

Sludge which compacts poorly, resulting in a slow settling, is called bulking sludge. Two types of bulking sludge can be distinguished.

1. Flocculated sludge with poor settling properties due to slime-forming bacteria (zoogloeal bulking);
2. Sludge with poor settling properties due to abundant growth of filamentous microorganisms.

The excessive growth of filamentous bacteria is the principal cause of poor settleability of activated sludge.

1.2 FILAMENTOUS MICROORGANISMS IN ACTIVATED SLUDGE

Filamentous microorganisms may contribute to a good structure of activated-sludge flocs, provided that the amount of these organisms is relatively low (Sezgin et al., 1978). However, abundant gorwth of filamentous microorganisms usually results in poorly compacting sludge.

Already since the introduction of the activated-sludge process, about 70 years ago, problems in the operation of the plants occurred due to poor settling properties of the sludge and many investigations into the phenom-

enon of bulking have been undertaken. Initially the investigators, usually engineers and chemists, attempted to improve settleability of the sludge by variation of the operation conditions. The activated sludge itself, the ecosystem in which many types of microorganisms and higher organisms participate, was beyond their discipline and therefore usually considered as a black box.

In several of the early reports dealing with bulking sludge the authors attributed the poor settling properties to the presence of the sheathed bacterium *Sphaerotilus natans*. This organism, known as the "water fungus" has been taken for a long time as the main cause of poor settling sludge. Only during the past 20 years applied microbiologists extensively studied the activated-sludge process, in particular the problem of filamentous microorganisms occurring in activated sludge (Mulder and van Veen, 1963; Adamse, 1968b; Cyrus and Sladká, 1970; Hünerberg et al., 1970; Farquhar and Boyle, 1971a,b; Mulder et al., 1971; van Veen, 1973; Eikelboom, 1975; Eikelboom and Buijsen, 1981; Salcher et al., 1982). At least 25 types of filamentous microorganisms, mainly bacteria, have been observed in activated sludge (Eikelboom, 1975) and several of them may cause bulking sludge.

1.3 MICROBIOLOGICAL APPROACH TO THE BULKING PROBLEM

1.3.1 Ecological aspects

Activated sludge is an open ecosystem of mainly microorganisms which grow on the substrates present in the waste water; the excess biomass is removed from the system. The composition of the population and the functioning of activated sludge are determined by:

a. The characteristics of the waste water, including flow and strength, chemical composition and physical characteristics such as temperature, pH and nature of the substrate (suspended, soluble).
b. Plant operation, including oxygen supply, sludge loading (and the retention time of biomass), mixing characteristics, pattern of supplying the waste water and recycled sludge to the aeration tank and final clarifier characteristics.

The common activated-sludge plants are systems with complete mixing and continuous feeding. In these plants the conditions do not vary during the treatment, in other words, the system tends to a steady-state process.

On the other hand many types of activated-sludge plants have been developed which operate differently. Among the types of this category are systems with a reduced degree of longitudinal mixing, including the "plug-flow" process, systems with intermittent feeding, such as the batch system ("fill-and-draw"), systems fed with a premixed flow of waste water and recycled sludge (contact tanks) and systems with an anoxic zone. All types of this category have in common, that the ratio biodegradable compounds to activated-sludge biomass varies during the treatment process, either with or without a variation in oxygen supply.

The population of microorganisms that develops in activated sludge is the optimally functioning ecosystem under the limitations of the nature of the waste water and the conditions of plant operation. The composition of the biomass population is determined by the selection of microorganisms as a result of competition between various types for available substrates. The outcome of this selection is mainly determined by physiological characteristics of the organisms although the influence of morphological characteristics can not be ignored.

1.3.2 Morphological aspects

Microorganisms generally may display three different types of morphological structures, viz. single cells, filaments and clumps (Fig. 2). Suppose a specific organism is able to grow in these three forms which would have identical physiological properties. Without other selection criteria, the single-cell type will be selected independently of cultivation method. In the competition for the growth-limiting substrate, the single cells have an advantage over cells growing in filaments or in clumps owing to their higher cell surface to cell volume ratio (Fig. 2). This hypothesis will be true whatever the growth-limiting substrate is. However, in a cultivation process in which the biomass is partly recycled to the cultivation vessel (external feedback of biomass) after sedimentation in a clarifier, another selection criterium is introduced. The single cells will be largely removed with the used medium, whereas the cells growing in filaments or in clumps will settle and will be recycled and thus retained in the system. In this process filamentous organisms will successfully compete with the organisms in clumps because their surface to volume ratio is more favourable for uptake of nutrients and thus for growth than that of the clumps.

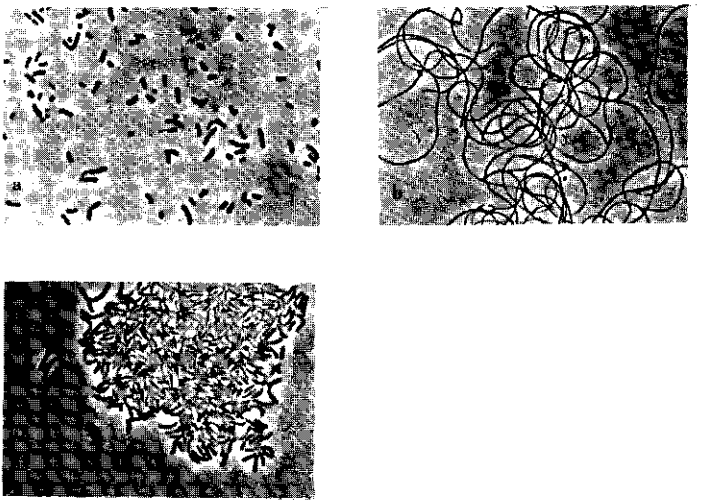

Figure 2.
Morphological structures of microorganisms. Single cells (a); filamentous growth (b) and growth in clumps (c).

The activated-sludge process resembles the system with external feedback of biomass and if the result of the selection of organisms in an activated-sludge plant would be determined only by morphological characteristics, the filamentous organisms would always prevail over organisms growing as single cells or in clumps.

The surface area to volume (A/V) hypothesis has been used to explain the abundant growth of filamentous bacteria in activated sludge under the conditions of oxygen limitation (Heukelekian, 1941) or at a high amount of soluble organic matter in the waste water (Littman, 1940). However, the latter hypothesis can only be confirmed with physiologically identical microorganisms as otherwise differences in physiological characteristics may interfere.

The method of controlling bulking sludge with toxic agents is partly based on the above-mentioned hypothesis. Filaments partly protruding from flocs are more susceptible to toxic compounds like chlorine and hydrogen peroxide than cells growing in clumps within flocs due to the higher A/V ratio. Cells in the centre of the clump will be protected by the cells on the outside. This method has been succesfully applied for the control of

various filamentous bacteria (Jenkins et al., 1982; Cole et al., 1973). However, it will not give satisfactory results with all filamentous bacteria as sensitivity to toxic compounds is a physiological characteristic of microorganisms which may vary with species.

1.3.3 *Physiological aspects*

The physiological characteristics of a microorganism determine to a great extent the position of the organism in an ecosystem like activated sludge. The microorganism should be able to utilize the nutrient compounds supplied with the waste water. Essential nutrient elements such as carbon, nitrogen, phosphorus, sulphur, potassium, magnesium, calcium and some trace elements should be present in adequate amounts and in available form. Compounds toxic to the organism should be absent. The growth of the organism should proceed at a sufficient rate to maintain the organism in the ecosystem. The growth rate of a sludge organism is determined by its physiological capacities as well as by plant operation. As regards the latter, particularly the way in which the sludge is supplied with the sewage (substrates) is of major importance. Two main types of substrate supply can be distinguished and will be discussed in connection with the occurrence of filamentous bacteria.

Activated-sludge plants with complete mixing of sludge and waste water and with continuous feeding. In activated-sludge plants of this type, the continuously supplied substrates are mixed (i.e. diluted) with the entire volume of agitated sludge suspension in the aeration tank. This means that the microorganisms have to compete for the available substrates which are usually present at very low concentrations.

Considering the overall purification process, the growth of activated-sludge organisms may be limited by the inadequate supply of one of the main nutrients (C, N, P, S) or by the inadequate supply of oxygen. However, it should be stressed that activated sludge consists of many microorganisms with widely varying nutritional requirements. These organisms may compete for many organic and inorganic compounds in the waste water supplied. A survey of a number of such competitions is given in Table 1.

In activated-sludge plants with complete mixing of sludge and waste water and with continuous feeding, the growth rate of the microorganisms is low, because the limiting-substrate concentration is low. The specific

Table 1.

Possible competitions for a number of compounds present in waste water between various sludge organisms.

Competitive substrate	Possible function of a compound					
	carbon	energy	Source of nitrogen	electron acceptor	phosphorus	sulphur
Various carbohydrates and polymers	+	+				
Amino acids and proteins	+	+	+			
Fatty acids and fats	+	+				
Organic acids	+	+				
Organic sulphur compounds	+	+				+
Organic phosphorus compounds	+	+			+	
Ammonium salts		+	+			
Nitrates			+	+		
Reduced inorganic sulphur compounds		+				+
Oxygen				+		

growth rate of a microorganism depends on the concentration of the limiting substrate as given in the following equation (Monod, 1949):

$$\mu = \mu_{max} \frac{S}{K_S + S}$$

in which μ = the specific growth rate, μ_{max} = the maximum specific growth rate, S = the substrate concentration and K_S = the rate constant; the latter equals the substrate concentration at which the organism can grow at half the maximum value. In this equation S may be a nutrient compound as well as oxygen.

The actual specific growth rate of an organism is determined by the physiological characteristics of the organism, viz. μ_{max} and K_S as well as an environmental parameter, viz. S. In a homogeneous suspension of a

mixed culture of microorganisms and a low limiting-substrate concentration ($S < K_S$ or S and K_S are of the same size), an organism with a high affinity for the limiting substrate (i.e. with a low K_S value) has a competitive advantage over organisms with a lower affinity. This has been shown by Jannasch (1967, 1968) for various mixtures of pure cultures growing in continuous culture. On the other hand at a high substrate concentration ($S \gg K_S$), the maximum growth rate of an organism is the most important physiological characteristic determining the result of the competition between various species.

Chudoba et al. (1973b) suppose that the growth of organisms in activated sludge is also determined by the parameters as given in the equation of Monod. However, in activated sludge the circumstances are more complicated because the limiting-substrate concentration in the mixed system varies. In the liquor of the completely mixed, continuously fed activated sludge, the limiting-substrate concentration is usually low, but in the centre of the sludge flocs the concentration is even lower. The latter concentration is determined by diffusion of the substrate from the liquor into the centre of the floc, which depends on substrate properties (molecular size, particle size, charge), substrate gradient ($S_1 - S_2$ in Fig. 3) and compaction of the floc. The actual specific growth rate of an organism in activated sludge is determined by the actual substrate concentration and the physiological characteristics of the organism. Suppose that substrate S (a nutrient or oxygen) is the growth-limiting substrate for the filamentous organism (A) as well as for the floc-forming organism (B) and that this concentration is S_1 and S_2, respectively. The filamentous organism will grow at the specific growth rate of

$$\mu_A = (\mu_{max})_A \frac{S_1}{(K_S)_A + S_1}$$

and the floc-forming organism at the specific growth rate of

$$\mu_B = (\mu_{max})_B \frac{S_2}{(K_S)_B + S_2}$$

Because the activated sludge is completely mixed and continuously fed, the substrate concentration S_1 is low, but S_2 is even lower. The result of the competition between A and B is determined by three parameters: μ_{max}, S and K_S.

The physiological characteristics of various activated sludge organisms may be determined with pure cultures. However, the specific growth

rate of an organism in activated sludge can not be estimated using these K_S and μ_{max} values as the type of limiting substrate and the actual concentration in the proximity of the organism usually are unknown. A high μ_{max} and a low K_S value for various substrates undoubtedly favour organisms occurring in activated sludge, especially filamentous organisms as they are protruding from the flocs in the liquor in which the substrate concentration has its maximum value. On the other hand, a low μ_{max} and a high K_S value do not mean that filamentous organisms cannot successfully compete with floc-forming organisms as the difference in substrate concentration in the surrounding liquid may be a conclusive factor.

Considering the model of growth kinetics according to Monod, an interesting hypothesis can be set up concerning the expected diversity of microorganisms in the population in relation to the limiting substrate. Suppose that oxygen is the limiting factor in the overall process. The organism with the highest specific growth rate under the conditions prevailing in the activated sludge/waste water system will compete successfully with all other species. If this organism is a filamentous bacterium, abundant growth, and consequently settling problems of the activated sludge, may be expected. On the other hand, if the carbon nutrient is the overall growth-limiting substrate, it will depend on the diversity of the carbon compounds in the waste water what kind of population develops. Various microorganisms will compete for every type of carbon compound but it is unlikely that filamentous bacteria would have a competitive advantage over floc-forming microorganisms for all types of carbon compounds available in the waste water. However, in some industrial wastes and synthetic waste water used in laboratory scale plants, the carbon nutrient is mainly present in the form of one type of compound. In this case, the circumstances are comparable to those of the oxygen-limited process and filamentous bulking may occur, assuming that filamentous organisms would exhibit a relatively high specific growth rate. Similar hypotheses may be formulated for processes in which nitrogen, phosphate or sulphur is the limiting nutrient element.

Summarizing, it may be stated that in a completely mixed system the specific growth rate of a microorganism as it is determined by the maximum growth rate, the affinity for the limiting substrate (K_S) and the actual substrate concentration is the conclusive factor in the competition between various organisms.

Problems due to abundant growth of a filamentous microorganism may occur when the following requirements are met.

a. The growth-limiting factor is oxygen or a nutrient like a carbon, nitrogen, phosphorus or sulphur compound, which should mainly be present in one type of such a compound.
b. The filamentous organism, under the conditions prevailing in activated sludge, has the highest specific growth rate, considering the fact that the actual concentration of the limiting substrate in the liquor is higher than within the floc.

That low oxygen and low substrate concentrations may induce filamentous bulking is supported by several reports. Abundant growth of filamentous organisms in activated-sludge plants operating under limited-oxygen supply has often been reported (Pipes, 1967; Adamse, 1968b; Metcalf and Eddy, 1972; Palm et al., 1980). Filamentous bulking is also observed in plants treating industrial wastes such as petrochemical waste water (Poffé et al., 1979) and food-processing industries (Mulder et al., 1971; Eikelboom, 1982b).

On the other hand, the above-mentioned hypothesis is not generally supported by reports concerning the effects of sludge loading on the operation of the waste-water treatment plant. The substrate concentration in the mixed liquor rises with increasing sludge loading and this effect should not explicitly favour the filamentous organisms. However, Genetelli and Heukelekian (1964) and Ford and Eckenfelder (1967) reported a stimulated growth of filamentous organisms as a result of an increased loading. Rensink (1974) reported abundant growth of *Sphaerotilus natans* in bench-scale activated-sludge plants, continuously fed with synthetic sewage, in a broad range of sludge-loading rates. In a survey of 65 full-scale plants, Tomlinson (1976) was unable to demonstrate a significant difference between bulking and non-bulking plants in relation to sludge-loading rate. These results of investigations on the effect of sludge-loading rates on the operation of activated-sludge plants are not in agreement with the hypothesis that an improved substrate supply would favour floc-forming organisms. However, under the conditions of an improved substrate concentration, bulking may still occur due to filamentous organisms which are able to grow with a high growth rate (high μ_{max} value). The adverse effect of high loading rates on sludge settling may also be due to short experimental periods or to a limited oxygen supply at high loading rates. The dissolved-

oxygen concentration in mixed liquor is considerably affected by the loading of the sludge (Adamse, 1968a).

The most important factor that affects the specific growth rate of a microorganism in an activated-sludge system with complete mixing and continuous feeding is hardly to determine. Values of μ_{max} and K_S for oxygen and various substrates of organisms occurring in activated sludge are hardly available and the actual concentration of the limiting substrate cannot be determined except that for the organisms on the surface of the flocs or protruding from the flocs in the liquor (S_1 in Figure 3).

Sphaerotilus natans has probably a low K_S value for oxygen as indicated by investigations of Mulder et al. (1971). Good growth of *S. natans* was obtained in aerated as well as non-aerated cultures, whereas an *Arthrobacter* species only showed good growth in the aerated culture. The results of competition experiments with *S. natans* and an *Arthrobacter* strain in mixed cultures indicated that *S. natans* has also a high affinity for glucose (Houtmeyers, 1978). On the other hand, the K_S value for glucose of another filamentous bacterium, *Haliscomenobacter hydrossis*, appeared to be approximately 5 mg/l (Krul, 1977b) and this value is relatively high compared to values reported for a *Pseudomonas* species (2 mg/l; Eagon and Phibbs, 1971) and *Zoogloea ramigera* (1.8 mg/l; Krul, 1977a). The maximum growth rates of two strains of *H. hydrossis* have recently been reported. The $_{max}$ values for strains AN and AZ were 0.05 h^{-1} and 0.09 h^{-1}, respectively (van Veen et al., 1982).

Activated-sludge systems deviating from the type with complete mixing and continuous feeding. In these systems, the affinity of an organism for the limiting substrate is not of dominating importance in the competition with other organisms because in the treatment processes included in this category the substrate concentration is temporarily high. Consequently, the substrate to biomass ratio or the less exactly defined food to mass ratio (F/M) is temporarily high and decreases gradually ("plug-flow" system, "fill and draw" system). In a second type the F/M ratio decreases abruptly to a low level during the treatment process (system with a contact tank, in which the recycled sludge is mixed with the waste water, prior to the complete mixing of pretreated sludge and liquor in the aeration tank). Under the conditions of a relatively high substrate concentration, the substrate-uptake rate as well as the storage capacity of an organism are important physiological characteristics. A high substrate uptake of an

organism often corresponds with a high maximum growth rate but in principle this does not need to be always true. Organisms which can resist stress situations as may occur at temporarily high loadings with organic substrates and temporarily insufficient oxygen supply may also be in a more favourable position than sensitive organisms.

The floc-forming organisms in such systems are in a less unfavourable position as compared to filamentous organisms than in systems with complete mixing and low substrate concentration. In the former type the diffusion rate of the substrates from the liquor into the centre of the floc is considerably higher than in systems with complete mixing, due to the higher substrate concentration in the liquor. In order to be able to compete successfully with the filamentous organisms, the floc-forming organisms should be more adapted to the non-steady state conditions than the filamentous organisms.

Many authors reported good results in preventing and curing filamentous bulking in these types of treatment systems (Chudoba et al., 1973 a,b; Rensink, 1974; Heide and Pasveer, 1974; Tomlinson and Chambers, 1979). Recently, Rensink et al. (1982) described a modified system of mixing activated sludge and waste water giving good settling properties of the sludge using contact tanks. The best results were obtained by using a rectangular contact tank.

Pasveer (1969) reported an improvement of sludge characteristics of an oxidation ditch after changing from a continuous to an intermittent feeding pattern. Houtmeyers et al. (1980) and Verachtert et al. (1980) clearly showed that intermittent feeding of activated sludge with synthetic waste water prevented abundant growth of filamentous bacteria. Van den Eynde et al. (1982) also proved the beneficial effect of this feeding pattern on sludge settleability in the treatment of various industrial wastes. Improvement of sludge characteristics by introducing an anoxic zone has been reported by Chambers (1982). The recycled sludge and sewage were mixed in an anoxic tank prior to the treatment in the aeration tank with complete mixing. Settling of the sludge from a "plug-flow" plant also improved after cutting off the air supply in the first compartment. So far the effect of an anoxic zone on the growth of filamentous organisms in sludge has not been studied.

The reports on the control of filamentous bulking by treating the waste water in systems different from those with complete mixing and con-

tinuous feeding may lead to the thesis that the substrate-uptake rate and storage capacity of floc-forming organisms exceed those of filamentous organisms so that the former organisms will outgrow the filamentous types. This thesis is strongly supported by the investigations on biosorption capacities of sludges (Eikelboom, 1981, 1982a). The biosorption capacity is defined as the amount of organic material which can be taken up per unit of time; it equals the substrate removal per unit of time. Activated sludge samples containing a huge amount of filamentous microorganisms showed a considerably lower biosorption capacity than samples of well settling sludge. The above-mentioned thesis was confirmed by experiments with pure cultures of *Sphaerotilus natans* and of an *Arthrobacter* species (Houtmeyers, 1978). The glucose-removal rate of *S. natans* grown in a system with intermittent feeding was only slightly higher than that of cells grown in a system with continuous feeding, whereas the glucose-removal rate of the *Arthrobacter* sp. considerably increased after changing to the former system. In competition experiments *S. natans* outgrew the *Arthrobacter* sp. in a system with continuous feeding in which the substrate concentration was very low, but in the system with intermittent feeding and consequently, temporarily a high substrate concentration the latter organism prevailed. This result might be due to a difference in substrate-uptake rate or utilization rate of stored substrates between these types of organisms. It is likely that the substrates were partly stored in the biomass during the peaks of substrate concentration in the intermittently fed system. *S. natans* readily looses its reserve material when shaken in a medium without substrate unlike *Arthrobacter globiformis* which under similar conditions utilizes stored glycogen for a much longer period (Mulder, 1964). This property undoubtedly favours the latter organism.

On the other hand, the postulated thesis has no absolute validity as data reported by Chudoba et al. (1974), Rensink (1974) and Tomlinson (1976) have shown that filamentous-bulking problems in "plug-flow" systems may still occur, especially at higher sludge-loading rates. However, the settling properties of the sludge in these systems were clearly superior to those of the systems with complete mixing of sludge and waste water and continuous feeding.

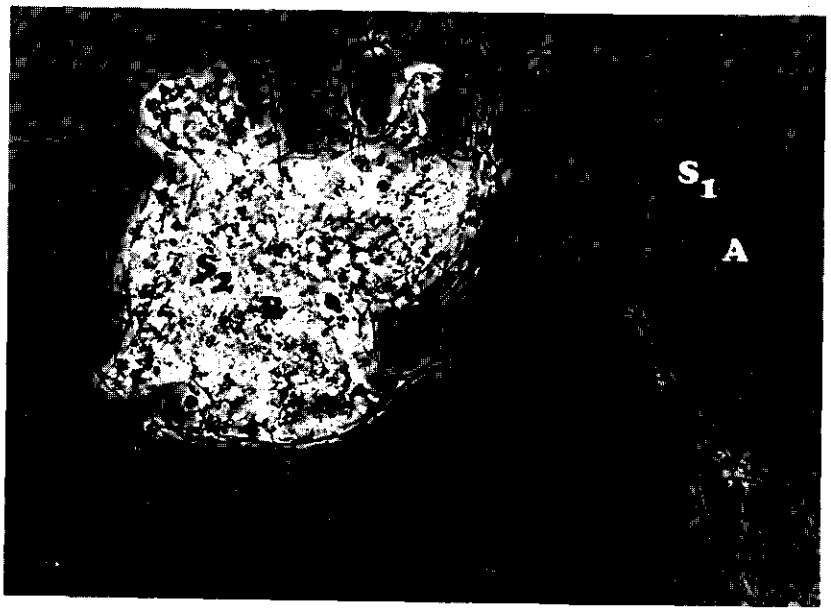

Figure 3.
Filaments protruding from an activated-sludge floc. Filamentous organism (A) and organism within the floc (B); substrate concentration in the liquor (S_1) and substrate concentration within the floc (S_2).

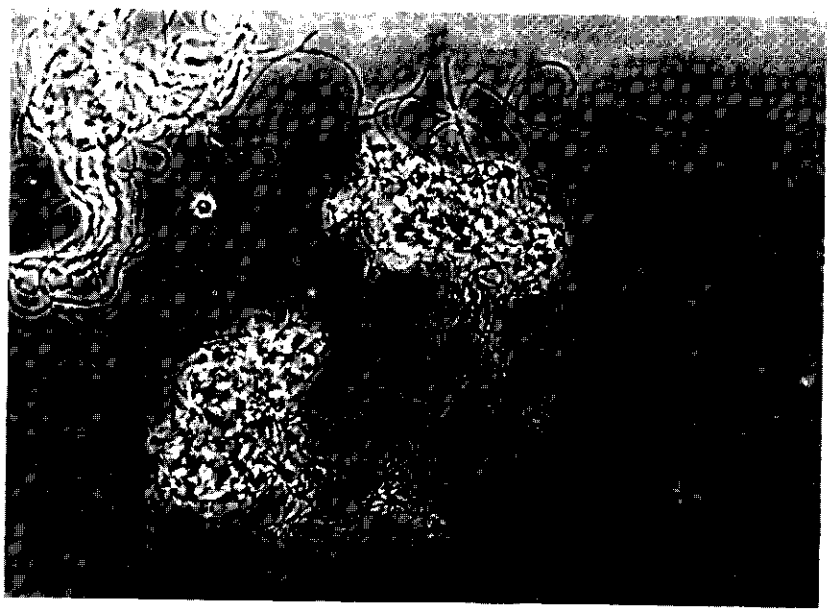

Figure 4.
Microthrix parvicella filaments protruding from activated-sludge flocs (625x).

1.3.4 Conclusions

1. Filamentous microorganisms, protruding from flocs of activated sludge, have in principle a competitive advantage over floc-forming (clump-forming) microorganisms owing to their easier access to substrates in the liquor.
2. The physiological characteristics of a microorganism determine to a great extent the result of the competition for the limiting substrate between various species of microorganisms in activated sludge.
3. The problem of filamentous bulking is very complicated due to a great diversity of filamentous microorganisms causing poor settling of sludge.
4. Fundamental investigations on the physiology of the predominant filamentous microorganisms are required as the results of such investigations may contribute to the development of methods to control abundant growth of a particular microorganism.

1.4 OUTLINE OF THE PRESENT INVESTIGATION

Approximately 50% of all waste-water treatment plants in the Netherlands are oxidation ditches, i.e. low-loaded activated-sludge plants fed with unsettled sewage. The oxidation ditches operated to entire satisfaction for nearly 20 years after the introduction of this process in 1954. However, during the past 10 years poor settleability of the activated sludge due to excessive growth of filamentous microorganisms has frequently been observed (Pasveer, 1969; Heide and Pasveer, 1974; Eikelboom, 1982b). Extensive investigations have shown that the filamentous bacterium *Microthrix parvicella* is the causative organism in nearly all plants treating domestic sewage (Eikelboom, 1982b). Some characteristics of this organism, isolated by van Veen (1973) and Eikelboom (1975) are given in Table 2 and a phase-contrast micrograph is shown in Fig. 4.

The aim of the present investigation was to elucidate some physiological characteristics of *M. parvicella*. The results of the experiments might be applied for preventative or remedial methods based on scientific principles to control filamentous bulking in oxidation ditches.

Table 2.
Characteristics of *Microthrix parvicella*.

Habitat	low-loaded activated sludge plants and oxidation ditches fed with domestic sewage.
Appearance	thin, coiled and unbranched filaments, cell septa not visible by phase-contrast microscopy.
Filament length	300-500 µm
Cell diameter	0.4-0.6 µm
Cell length	0.7-1.8 µm
Gram stain	positive
Neisser stain	positive

Chapter 2 contains data on the nutritional requirements of *M. parvicella* and on the utilization of various nutrient sources by this organism.

Chapters 3 and 4 report on the growth of *M. parvicella* in batch and in continuous culture, respectively.

M. parvicella utilizes only long chain fatty acids as carbon and energy source (Chapter 2). In view of this result it was interesting to study the effect of short chain fatty acids on the growth yield (Chapter 5).

The affinity of *M. parvicella* for various carbon substrates and for oxygen has been determined by respiration experiments. Because long chain fatty acids have surface-active properties, the effect of various surface-active compounds on growth and respiration of *M. parvicella* were determined. Chapter 6 contains the results of these experiments.

The results of the investigations with pure cultures of *M. parvicella* have been used to study the effect of the composition of domestic sewage and of synthetic waste water on the growth of the bacterium in low-loaded activated-sludge systems. These experiments were carried out in a pilot plant as well as in laboratory scale activated-sludge plants. In the latter system the effect of oxygen supply on the growth of *M. parvicella* was also studied. The results of the experiments are recorded in Chapter 7.

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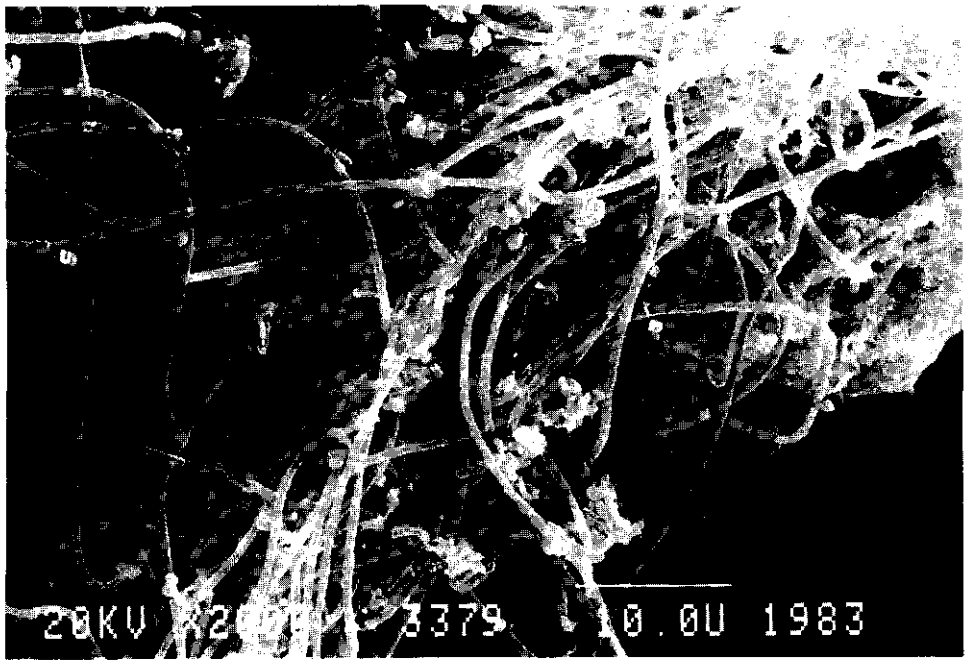
Microthrix parvicella in activated sludge.
Scanning-electron micrograph



2. *Microthrix parvicella*, a filamentous bacterium isolated from activated sludge; cultivation in a chemically defined medium containing Tween 80 as sole carbon source.

INTRODUCTION

Filamentous bacteria, if present in large amounts in activated-sludge flocs may be responsible for sludge bulking. Van Veen (25) and Eikelboom (10, 11) have studied the dominant filamentous bacteria found in various sludges. Pipes (21, 22) reviewed the several types of bulking observed in activated-sludge plants.

The growth of a microorganism in activated sludge is determined by the chemical and physical nature of the influent and the type of the purification process. The abundant growth of filamentous bacteria in activated sludge plants should be correlated with their physiological properties but even with *Sphaerotilus* and *Haliscomenobacter* spp. this is still difficult to decide (18, 26, 27, 28).

In the Netherlands about 50% of the waste water treatment plants are oxidation ditches and sludge bulking in these plants is mainly caused by *Microthrix parvicella* (Eikelboom, paper presented at the international conference: Oxidation Ditch Technology, 1982). This non-sheathed, Gram-positive bacterium has been isolated by Van Veen (25) and Eikelboom (10) and it may be cultivated in a complex medium as described by Slijkhuis and Deinema (24).

To study the physiological characteristics of *M. parvicella* a less complex medium had to be developed and the present paper describes the experiments leading to a chemically defined medium.

MATERIALS AND METHODS

Organisms: Microthrix parvicella, strain B, was isolated from activated sludge of the oxidation ditch of Bennekom, the Netherlands; five other isolates were obtained from various other waste water treatment plants. Pure cultures were kept on slants containing medium A agar, supplemented

with 3-5 ml sterile tap water and 0.1 ml vitamin stock solution. Pure slant cultures were stored at 4°C.

Media: Medium A, modified Tween 80/peptone medium (24) contained per 1 of distilled water: Tween 80 (Sigma), 4 g; Bacteriological peptone (Oxoid), 4 g; $(NH_4)_2SO_4$, 0.8 g; K_2HPO_4, 8.2 g; KH_2PO_4, 0.35 g; $MgSO_4.7H_2O$, 0.075 g; $CaCl_2$, 0.05 g; $FeCl_3.6H_2O$, 5 mg; $MnSO_4.H_2O$, 3 mg; $ZnSO_4.7H_2O$, 0.1 mg; $CuSO_4$, $5H_2O$, 0.1 mg; H_3BO_3, 0.1 mg; $Na_2MoO_4.2H_2O$, 0.05 mg; $CoCl_2.6H_2O$, 0.05 mg and vitamin stock solution, 10 ml; final pH of the medium: pH = 8. The vitamin stock solution (adjusted to pH = 3 with HCl) contained (mg/l): pantothenic acid, 10; nicotinamide, 10; pyridoxine, 10; *p*-aminobenzoic acid, 10; inositol, 10; thiamin, 10; riboflavin, 10; choline, 10; biotin, 10; cyanocobalamin, 0.5; folic acid, 0.5.

Medium A agar: A supplemented with agar, 7.5 g/l.

Medium B : A without Tween 80.

Medium C : A without Bacteriological peptone.

Medium D : A, the Bacteriological peptone has been replaced by Casamino acids (Merck), 2 g/l.

Medium D_1 : A, the Bacteriological peptone has been replaced by Casamino acids (Merck), 0.5 g/l.

Medium E : A without Bacteriological peptone and the vitamin stock solution.

Medium E_1 : E supplemented with Casein hydrolysate vitamin free (Merck), 2 g/l.

Medium E_2 : E supplemented with Vitamin assay Casamino acids (Difco), 2 g/l.

Medium F : E supplemented with $Na_2S_2O_3.5H_2O$, 0.12 g/l, thiamin, 100 µg/l and cyanocobalamin, 5 µg/l.

Media were sterilized by autoclaving (15 minutes, 121°C), phosphate buffer (sterilized separately) and vitamin stock solution (sterilized by Seitz filtration) were added to the cooled media.

Inoculation procedure. The cell material from one slant culture was suspended and precultured in 10 ml medium A. In general, 5 ml of bacterial suspension was transferred to 50 ml of fresh medium (inoculation percentage = 10%). To test a medium of deviating composition initially 10 ml

culture was brought into 50 ml. To exclude a positive effect of growth factors from the original medium, the inoculation procedure (with 5 ml of bacterial suspension in 50 ml) was repeated at least three times.

Tested media were considered to be suitable for *M. parvicella* if growth in the 1st and 4th culture was identical. Good growth could be obtained in shake cultures at 25°C after 6-10 days.

Determination of the yield. Cell yield was determined by measuring the optical density at 620 nm using a Bausch and Lomb Spectronic 20. Before measuring, 5 ml diluted suspension (treated with Ultra Turrax type 18/10 homogenizer during 5 seconds) was supplied with 0.5 ml of a mixture of EDTA (ethylenediaminetetraacetic acid, 50 mM) and Triton-X-100 (50 g/l) at pH = 8. The mixture of EDTA and Triton-X-100 had to be added to exclude scattering due to insoluble fatty acids in various media. Absorption values at 620 nm of the uninoculated media treated in the same way were subtracted. Cell yield was determined from the relation between optical density and dry weight.

Temperature. Growth temperature was 25°C unless otherwise stated.

^{15}N incorporation. $(^{15}NH_4)_2SO_4$ was added to the culture medium to an enrichment of 7.93% ^{15}N. After various incubation periods, cells were harvested by membrane filtration (pore size 0.45 µm) and washed twice with NaCl (2.5 g/l). Washed samples were digested (Kjeldahl) and ammonia was distilled. NH_4^+-concentration was determined with Nessler's reagent and ^{15}N was analysed by emission spectrometry (A.D.L. Akkermans, Thesis, University of Leiden, the Netherlands, 1971). Used medium was treated in the same way to determine changes in ammonium concentration due to ammonification.

Nitrite and nitrate. Nitrite and nitrate in used medium were determined photometrically as described in Standard Methods (1) and NEN (Nederlands Normalisatie Instituut, methods for analysis of waste water, No. 3235, 6.3; 1969), respectively.

Amino acid analysis. Cultures of *M. parvicella* (medium + biomass) or used medium were hydrolyzed (24 hr in 6 N HCL at 110°C) and filtered (membrane filtration, pore size 0.2 µm). The filtrate was evaporated under reduced

pressure and the residue taken up in distilled water. The liquid was evaporated under reduced pressure and the residue dissolved in Li-citrate buffer (0.06 M, pH = 1.9). Amino acid concentrations of these samples were determined in a Biotronik L.C.6000E amino acid analyzer. The concentration of amino acids in biomass was calculated by subtracting the values of used medium from the values of the total culture.

Chemicals. Tween 60, Tween 40 and Tween 20 were obtained from Lamers and Indemans, the Netherlands, other carbon sources were pure grade chemicals. Amino acids (pure grade) were obtained from Merck.

RESULTS

Carbon sources.

Tween 80 (polyoxyethylenesorbitan mono-oleate) as well as oleic acid serve as sources of carbon and energy for *Microthrix parvicella* (24) and an excellent dose response can be demonstrated (Fig. 1). From the data of this graph it can be calculated that the yield on 0.5 g Tween 80 corresponds with the yield on approx. 0.12 g oleic acid. It may be assumed that only the oleic acid part of Tween 80 is utilized by the organism as theoretically 1 g Tween 80 contains 0.22 g oleic acid. A quantitative determination of oleic acid by the method described by Zevenhuizen (29) in the chloroform extract of a saponified solution of Tween 80 (after acidification), resulted in 0.26 g oleic acid per g Tween 80. The assumption that only the oleic-acid moiety of Tween 80 was utilized by *M. parvicella* was proved to be correct by testing polyoxyethylene (2 g/l) and sorbitol (1 g/l) added to medium B with oleic acid (0.1 g/l). The cell yield of *M. parvicella* in this medium was not higher than in medium B with oleic acid (0.1 g/l) as the only carbon source.

Compared to oleic acid the use of Tween 80 as carbon source has two advantages. Tween 80 is soluble in water and is not growth-inhibiting in the concentration range tested (up to 10 g/l), whereas oleic acid may inhibit growth at concentrations exceeding 0.15 g/l (Fig. 1). The deviating results obtained for 0.2 g/l oleic acid must be considered in relation to the previous history of the inoculum. The number of cells in the inoculum precultured in medium B supplemented with oleic acid (0.1 g/l) was only 10% of those present in the inoculum precultured in medium A.

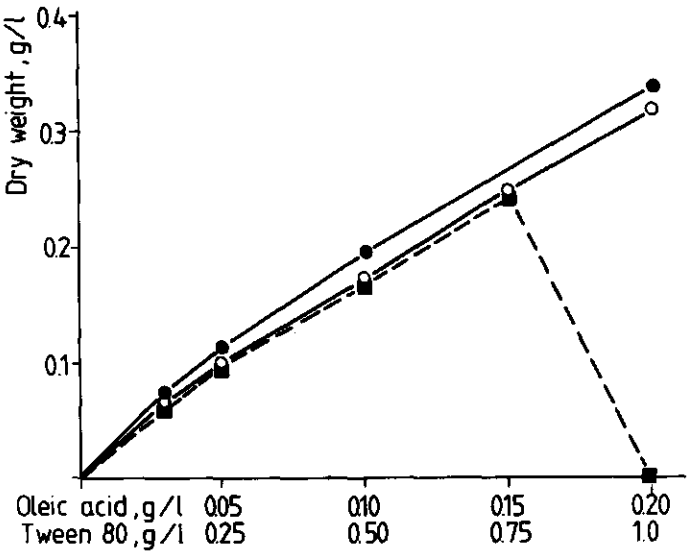


Figure 1.
Yield of *M. parvicella* grown for 6 days in medium B with different concentrations of Tween 80 (●——●) and of oleic acid (○——○) and (■– –■). *M. parvicella* was precultured in medium A (●——●) and (○——○) and in medium B supplemented with oleic acid, 0.10 g/l (■– –■).

Various carbon sources have been tested in medium B. The data presented in Table 1 show that Tween 80 can be replaced by the polyoxyethylene-sorbitan derivates of stearic, palmitic and lauric acids but the free fatty acids with a long chain length are hardly used by *M. parvicella*. The saturated fatty acids with medium chain length (caproic, caprylic and lauric acids) as well as the volatile fatty acids can not replace Tween 80 but do increase cell yield if supplemented to a medium also containing a small amount of Tween 80. Fructose, glucose, citric acid, succinic acid and lactic acid, which are in general excellent microbial substrates, are not utilized.

Nitrogen sources. Ammonium sulphate is the main nitrogen source for *M. parvicella* as was shown by an experiment with $(^{15}NH_4)_2SO_4$ (Table 2). If grown in medium A at least 75% of the nitrogen incorporated into cell material originated from $(^{15}NH_4)_2SO_4$. In this medium ammonium sulphate can not be omitted, it neither can be replaced by potassium nitrate. Oxidation of ammonium to nitrite or nitrate does not occur.

Table 1.

Utilization of a number of carbon compounds by *M. parvicella*.

Carbon source	Yield in medium B[1]	Yield in medium B containing Tween 80[2]	
		Tween 80-oleic acid + additional C-source	Increment due to additional C-source[3]
	dry weight, g/g C	dry weight, g/g C	dry weight, g/g C
---	0.0	1.9	---
Tween 80-oleic acid	1.8	1.9	1.9
Tween 60-stearic acid	1.6	1.9	1.9
Tween 40-palmitic acid	1.6	1.9	1.9
Tween 20-lauric acid	1.0	1.6	1.3
Oleic acid	0.0 (1.9[4])	1.9	1.9
Stearic acid	0.2	1.1	0.3
Palmitic acid	0.3	1.1	0.3
Lauric acid	0.3	1.6	1.3
Caprylic acid	0.3	1.8	1.7
Caproic acid	0.4	1.9	1.9
Butyric acid	0.5	1.7	1.5
Acetic acid	0.4	1.5	1.1
Fructose	0.0	0.9	0.0
Glucose	0.0	0.9	0.0
Citric acid	0.0	0.9	0.0
Succinic acid	0.0	0.9	0.0
Lactic acid	0.0	0.9	0.0

1) Determined after incubation for 10 days in medium B supplemented with one of the carbon sources tested at 0.25 g C/l. Inoculum (5 ml in 50 ml) was precultured in medium A.

2) Determined after incubation for 10 days in medium B supplemented with Tween 80-oleic acid, 0.1 g C/l, and one of the carbon sources tested at 0.1 g C/l. Inoculum (5 ml in 50 ml) was precultured in medium A.

3) Derived from the data of column 2 by subtracting the yield of the Tween 80-oleic acid part of the mixture of two carbon compounds (0.95 g/g of C present in the two compounds) and adjusting the values obtained for comparison with the yield values on single C-compounds by multiplying them by 2.

4) Result obtained in medium B supplemented with 0.1 g oleic acid-C/l.

Table 2.
$^{15}NH_4^+$-N incorporation by *M. parvicella* during growth in medium A.

Days after inoculation[1)]	0	2	3	7
Dry weight, g/l	0.11	1.20	1.40	1.45
Total N in biomass, mg N/l	9.7	65.5	123	154
^{15}N enrichment in biomass, % of total N	0.20	5.29	5.32	5.07
^{15}N enrichment in biomass, after correction for inoculated biomass, % of total N	--	6.17	5.76	5.40
NH_4^+ in medium, mg N/l	171	120	91	84
^{15}N enrichment in NH_4^+ in medium, % of total NH_4^+-N	7.93	7.88	--[2)]	6.90
Ratio ^{15}N enrichment in biomass to ^{15}N enrichment in NH_4^+ in medium	--	0.78	--	0.78

1) $^{15}NH_4^+$ enriched media were inoculated (5 ml in 50 ml) from a culture grown in medium A.

2) Not determined.

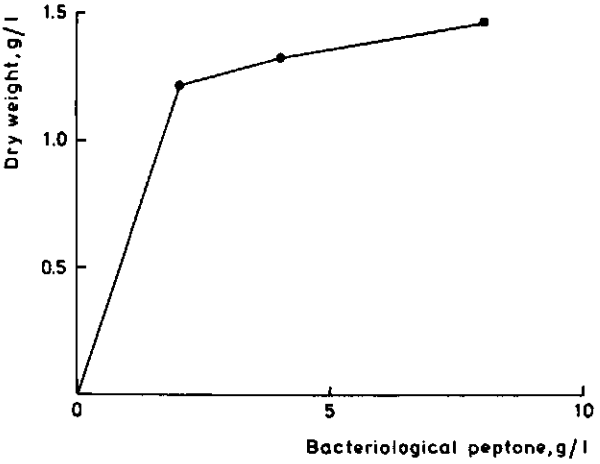


Figure 2.
Yield of *M. parvicella* grown for 6 days on various amounts of Bacteriological peptone supplemented to medium C. Inoculum (5 ml in 50 ml) was precultured in medium A.

Peptone is hardly used as a carbon source (Fig. 2) as in the range 2-8 g/l peptone the yield increment was only 0.25 g/l. The peptone cannot be omitted from the medium (no growth in medium C), suggesting that Bacteriological peptone contains an essential unknown growth factor. The unknown factor is growth-limiting in the range 0-2 g peptone/l, whereas increasing amounts of peptone had little effect on the yield (Fig. 2). Bacteriological peptone could be replaced by Casamino acids (medium D) and growth in medium D and medium D_1, inoculated from a culture grown in medium A, was obtained after an incubation period of 20 days.

The amino acid composition of *M. parvicella* cultures was investigated during growth in medium D_1. The results of this experiment, reported in Table 3, show that *M. parvicella* when growing in this medium, synthesizes all amino acids except L-methionine. After incubation in medium C, a medium without an organic N-source, this result was even more pronounced. From this result it is concluded that *M. parvicella* requires L-methionine. The requirement for L-methionine was confirmed by growth experiments, carried out in medium C supplemented with various mixtures of amino acids. The amino acid composition of Casamino acids was analysed (Table 4) and

Table 3.

Amino acids (µmols/l) in cultures (medium + biomass) and biomass of *M. parvicella* during growth.

Amino acid	Concentration at zero time and after 20 days.							
	Medium D_1 [1]				Medium C [2]			
	culture		biomass		culture		biomass	
	0	20	0	20	0	20	0	20
Aspartic acid	219	513	40	255	49	210	25	160
Threonine	122	330	16	244	25	198	18	159
Serine	157	248	15	178	25	137	17	109
Glutamic acid	563	1300	108	410	170	700	72	235
Proline	304	374	27	201	28	140	28	122
Glycine	126	386	39	292	35	200	23	152
Alanine	191	753	75	477	71	420	50	256
Valine	185	545	34	340	49	289	30	218
Methionine	20	18	--	16	--	--	--	--[3]
Isoleucine	99	227	7	115	21	124	9	74
Leucine	155	342	30	271	29	178	23	150
Tyrosine	14	41	7	42	3	30	3	26
Phenylalanine	70	144	19	106	15	71	11	60
Lysine	175	207	25	149	20	70	12	83
Histidine	45	79	3	54	7	32	5	32
Arginine	72	230	13	199	17	100	15	113
Σ	2517	5737	458	3349	564	2899	341	1949

1) Medium D_1 (100 ml) was inoculated with 10 ml of a culture grown for 8 days in medium D.

2) Medium C (100 ml) was inoculated with 20 ml of a culture grown for 8 days in medium D_1.

3) Not detectable.

Table 4.
Amino acid composition of Casamino acids.

Amino acid	mg/g Casamino acids
Aspartic acid	47
Threonine	21
Serine	32
Glutamic acid	96
Proline	69
Glycine	15
Alanine	20
Valine	22
Methionine	9
Isoleucine	11
Leucine	25
Tyrosine	7
Phenylalanine	14
Lysine	35
Histidine	10
Arginine	15
Σ	439

mixtures of amino acids of identical composition omitting one amino acid were prepared. Growth experiments carried out in medium C supplemented with the various mixtures resulted in the requirement for methionine (Table 5). Growth in medium C supplemented with L-methionine as the sole organic nitrogen source, inoculated from a culture grown in medium D, was obtained after a long incubation period (20 days).

Table 5.

Amino acid requirement for *M. parvicella*.

Amino acid mixture	Omitted amino acid	Yield, dry weight, g/l[1)]	
		1st culture	4th culture
--		0.6	0.0
1[2)]	---	1.5	1.4
2	Aspartic acid	1.4	1.4
3	Threonine	1.4	1.3
4	Serine	1.3	1.4
5	Glutamic acid	1.4	1.3
6	Proline	1.3	1.3
7	Glycine	1.3	1.5
8	Alanine	1.3	1.4
9	Valine	1.4	1.4
10	Methionine	0.6	0.0
11	Isoleucine	1.3	1.3
12	Leucine	1.4	1.4
13	Tyrosine	1.4	1.3
14	Phenylalanine	1.3	1.3
15	Lysine	1.4	1.3
16	Histidine	1.4	1.5
17	Arginine	1.3	1.3

1) Determined after incubation for 10 days in medium C supplemented with amino acid mixtures. The yield was determined in cultures after 1 and 4 transfers.

2) Complete amino acid mixture resembling Casamino acids, 2 g/l; composition is given in Table 4.

Sulphur sources. *M. parvicella* does not require L-methionine as an essential amino acid, but as a source of reduced sulphur (Table 6). The amino acid can be replaced by various other reduced-sulphur compounds; sulphate is not utilized.

Table 6.
Yield of *M. parvicella* after growth for 6 days in medium C with various additional sulphur sources.

Additional sulphur source	Concentration (mM)	Yield, dry weight, g/l
-----[1]	--	0.0
L-methionine	0.25	1.3
L-cysteine[2]	0.25	1.3
Sodium sulphide[2]	0.25	1.3
Sulphur	0.25	1.3
Sodium thiosulphate	0.25	1.3

[1] Medium C contains $(NH_4)_2SO_4$ at a concentration of 6.4 mM.

[2] Sterilized by membrane filtration.

Vitamin requirement. Various vitamin mixtures have been tested in medium E supplemented with Caseine hydrolysate vitamin-free (medium E_1) or Vitamin assay Casamino acids (medium E_2). These experiments showed that *M. parvicella* requires only thiamin and cyanocobalamin (Table 7). Good growth was obtained in medium E supplemented with thiamin, cyanocobalamin and one of the reduced sulphur compounds mentioned in Table 6.

Table 7.

Vitamin requirement of *M. parvicella*.

Vitamin mixture	Omitted vitamin	Yield[1], dry weight, g/l, in cultures after 1 and 4 transfers in			
		medium E_1		medium E_2	
		1st	4th	1st	4th
-		0.5	0.0	0.4	0.0
1[2]	---	1.3	1.4	1.3	1.3
2	pantothenic acid	1.3	1.5	1.3	1.5
3	nicotinamide	1.3	1.3	1.3	1.3
4	pyridoxine	1.4	1.3	1.3	1.5
5	*p*-aminobenzoic acid	1.3	1.3	1.3	1.4
6	inositol	1.3	1.4	1.3	1.5
7	thiamin	0.4	0.0	0.4	0.0
8	riboflavin	1.4	1.3	1.4	1.4
9	cholin	1.3	1.3	1.4	1.3
10	biotin	1.3	1.4	1.3	1.4
11	cyanocobalamin	0.7	0.0	0.6	0.0
12	folic acid	1.3	1.4	1.3	1.4

1) Determined after incubation for 10 days.

2) Complete vitamin mixture, composition is given in medium A.

Effect of pH on growth. Medium A is buffered at pH = 8 but *M. parvicella* acidifies this medium to pH = 7.6 during growth. The growth yield is strongly affected by the pH as shown in Table 8. The yield drops sharply to zero after lowering the initial pH of medium A to values below 7.1.

Temperature. An investigation has been made into the growth of *M. parvicella* in medium A at four different temperatures (Fig. 3). It resulted in an optimum growth temperature approximating 25°C.

Table 8.

Effect of pH on growth of *M. parvicella* in medium A.

Culture	1	2	3	4	5	6	7
Initial pH[1)]	5.7	6.4	6.7	7.1	7.5	7.7	8.0
Final pH[2)]	5.7	6.4	6.7	7.1	7.1	7.4	7.6
Cell yield, dry weight, g/l[2)]	0	0	0	0	1.2	1.6	1.6

1) pH values are adjusted to initial values after sterilization by adding H_2SO_4 to medium A.

2) After 6 days.

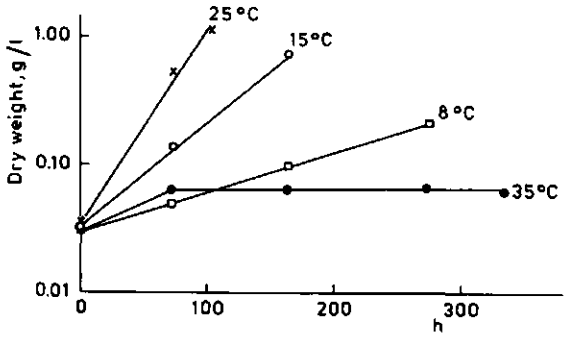


Figure 3.
Growth of *Microthrix parvicella* in medium A at 4 different temperatures.

Chemically defined medium. The composition of a chemically defined, minimal medium for cultivating *M. parvicella* is given in Table 9. The yield on medium F (= medium E supplemented with sodium thiosulphate, thiamin and cyanocobalamin) is about 1.3 g/l, dry weight.

Table 9.
Chemically defined medium for cultivating *M. parvicella.*

Tween 80	4 g/l
$(NH_4)_2SO_4$	0.8 g/l
$Na_2S_2O_3.5H_2O$	0.12 g/l
K_2HPO_4 [1)]	8.2 g/l
KH_2PO_4 [1)]	0.35 g/l
Solution I [2)] ($MgSO_4$ and trace elements)	10 ml/l
Solution II [3)] ($CaCl_2$ and trace elements)	10 ml/l
Vitamin solution [4)]	10 ml/l

1) Potassium phosphate is sterilized separately.

2) Solution I contains (g/l): $MgSO_4.7H_2O$, 7.5; $MnSO_4.H_2O$, 0.3; $ZnSO_4.7H_2O$, 0.01; $CuSO_4.5H_2O$, 0.01.

3) Solution II contains (g/l): $CaCl_2$, 5.0; $FeCl_3.6H_2O$, 0.5; H_3BO_3, 0.01; $Na_2MoO_4.2H_2O$, 0.005; $CoCl_2.6H_2O$, 0.005.

4) Vitamin stock solution contains (mg/l): thiamin, 10 and cyanocobalamin, 0.5. The solution (final pH = 3 by adding HCl) is sterilized by Seitz filtration and added aseptically to the cooled medium.

Amount of inoculum. A large amount of inoculum is required to obtain growth of *M. parvicella* in a medium of a deviating composition. Depending on the composition of the medium, the amount of inoculum into fresh medium could be reduced: from 5-10% in medium F to 0.1% in medium A. The required minimum amount of inoculum is also affected by the concentration

of the carbon source. In the modified medium F with only 0.2 g/l Tween 80 or 0.02 g/l oleic acid, growth was obtained after inoculation with 0.1% from a culture grown in medium F.

Test of other strains. Five other strains of *M. parvicella* showed good growth in media A, D and F but no growth was observed in medium C.

DISCUSSION

Long chain fatty acids supplied as esters of polyoxyethylenesorbitan (e.g. Tween 80) proved to be good sources of carbon and energy for *M. parvicella* (Table 1). Oleic acid, if supplemented to medium B is finely dispersed and an initial concentration of 0.15 g/l is readily utilized. However, oleic acid concentrations exceeding 0.15 g/l may be growth-inhibiting. Toxicity of long chain fatty acids to Gram-positive bacteria has often been reported (4, 12, 14, 17). It is probably caused by the surface activity of the fatty acids, which are able to function as anionic detergents. The ratio oleic acid to the inoculated biomass rather than the concentration of oleic acid in the nutrient medium seems to determine the maximum amount of oleic acid which can be utilized by *M. parvicella* as shown for 0.2 g/l oleic acid in Fig. 1. It has been indicated that the toxicity of oleic acid to *Mycobacterium* spp. depends on the ratio fatty acid to biomass rather than on the oleic acid concentration in the medium (2, 3). However, this suggestion was not confirmed by experimental data (17). The saturated long chain fatty acids (stearic and palmitic acids) in unesterified form are hardly utilized. These acids are insoluble in water and may be very difficult of access for *M. parvicella*. Toxicity of stearic and palmitic acids may not be excluded, but the bactericidal action of these acids to *Bacillus megaterium* is 6-8 times lower than that determined for oleic acid (12).

The general biochemical pathway of the long chain fatty acid degradation is the ß-oxidation (15). This oxidation results in acetyl-CoA which can be used for energy-yielding reactions and biosynthesis. Many organisms growing on long chain fatty acids also grow on fatty acids of medium chain length and volatile fatty acids. However, *M. parvicella* does not grow on these substrates when supplied as the sole carbon source (Table 1). In combination with Tween 80, fatty acids of medium- and short

chain length do increase the cell yield. These results suggest that *M. parvicella* is missing the enzymes for the long chain fatty acid synthesis (e.g. required for membranes).

A remarkable property of *M. parvicella* is the lack of growing on carbohydrates, simple organic acids and protein hydrolysates. Yield increase on media with Tween 80 due to addition of these substrates does not occur (Table 1). *M. parvicella* apparently does not possess the enzyme systems for the uptake or degradation of these substrates.

Ammonium sulphate is the sole (medium F) or main N-source (medium A and D). In the beginning of this study when the function of peptone had not been elucidated, a complex nitrogen source had to be added to the medium to supply *M. parvicella* with reduced-sulphur compounds. The inability to reduce sulphates for providing the cell with reduced sulphur is a remarkable property of *M. parvicella* and is almost unique amongst aerobic bacteria. A similar requirement was found with a *Rhizobium leguminosarum* strain (9).

M. parvicella requires cyanocobalamin and thiamin. The requirement for these vitamins is quite common among water microorganisms and has been reported for the filamentous bacteria *Haliscomenobacter* (26), *Sphaerotilus natans* (for cyanocobalamin, 27) and a *Leucothrix* type bacterium (for thiamin, 23).

The requirement of *M. parvicella* for a large amount of inoculum may be caused by the inhibiting effect of Tween 80 at a high ratio Tween 80 to the amount of inoculated biomass as has been suggested for the growth-inhibiting effect of oleic acid. The difference between the results obtained in inoculation experiments in medium A and medium F must be correlated with the presence of Bacteriological peptone in medium A. Certain compounds present in Bacteriological peptone may protect the inoculated cells against bactericidal activity of Tween 80. Similar results have been reported for cultivation of *Mycobacterium* spp. in Tween 80-containing media: a high inoculum and addition of serum albumin were required to obtain growth (6, 8, 16). The growth-inhibiting action of Tween 80 is caused by oleic acid wich may be present in commercial Tween 80 or may be liberated from Tween 80 by bacterial action (5, 7). Albumin acts as a protective growth-factor, binding traces of unesterified fatty acids (6).

It may be concluded that bulking in activated-sludge plants due to *M. parvicella* only occurs if the sewage contains sufficient fatty material and if nitrogen and sulphur are present in the reduced form. In general waste water treatment plants are supplied with domestic sewage in combination with industrial waste water. Fats contribute for up to 40% to the chemical oxygen demand of domestic sewage (13, 19, 20) and this value increases if waste water from agro-industries (e.g. slaughterhouses, fish-processing industries) or laundries is included. The ammonium concentration in oxydation ditches is usually very low, due to nitrification and sulphur compounds will be readily oxidized to sulphates in these plants. As *M. parvicella* requires reduced nitrogen and reduced sulphur compounds for growth, the organism apparently is very effective in the competition for these compounds. *M. parvicella* requires a pH value above 7.1 when grown under the conditions of the experiment reported in Table 7.

Other *M. parvicella* strains tested in the various media showed the same nutritional requirements as determined for strain B. Microscopical observations of the various strains showed a strong similarity in morphology, so it may be concluded that all isolated strains are closely related.

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3. *Microthrix parvicella*, a filamentous bacterium from activated sludge: growth on Tween 80 as carbon and energy source.

INTRODUCTION

Waste water treatment by the activated sludge process does not always operate satisfactorily because of poor settling properties of the sludge. Abundant growth of filamentous bacteria usually causes the poor settling ("bulking sludge"). As many as 25 types of filamentous bacteria have been observed microscopically in activated sludge (Eikelboom, 1975; Eikelboom and Buijsen, 1981). However, only a limited number of these types are able to grow so abundantly in activated sludge that bulking sludge develops. *Microthrix parvicella*, a thin non-sheathed filamentous bacterium often causes bulking in oxidation ditches and low-loaded activated sludge plants (Eikelboom, 1982).

The physiological characteristics of an organism determine to a great extent its occurrence in a mixed culture like activated sludge. A detailed study of the physiology of *M. parvicella* may contribute to the development and application of selective methods for controlling this organism in the activated sludge process.

The nutritional requirements of *M. parvicella* have been elucidated. The organism utilizes long chain fatty acids, preferably the oleic acid moiety of Tween 80 as carbon and energy source and requires reduced nitrogen and reduced sulphur compounds for growth (Slijkhuis, 1983). The growth in a complex medium showed remarkable shifts of the cell components protein and lipids as percentage of cell dry weight during growth (Slijkhuis and Deinema, 1982).

This report deals with the growth characteristics of *M. parvicella* in batch culture.

MATERIALS AND METHODS

Organism and growth conditions. Microthrix parvicella, strain B, was isolated from activated sludge of the oxidation ditch of Bennekom, the Netherlands. Stock cultures were kept on slants of medium A agar. Medium A (Slijkhuis, 1983) contains (g/l): Tween 80 (polyoxyethylenesorbitan monooleate; Sigma), 4; Bacteriological peptone (Oxoid), 4; $(NH_4)_2SO_4$, 0.8; K_2HPO_4, 8.2; KH_2PO_4, 0.35; $MgSO_4.7H_2O$, 0.075; $CaCl_2$, 0.05; trace elements (mg/l): $FeCl_3.6H_2O$, 5; $MnSO_4.H_2O$, 3; $ZnSO_4.7H_2O$, 0.1; $CuSO_4.5H_2O$, 0.1; H_3BO_3, 0.1; $Na_2MoO_4.2H_2O$, 0.05; $CoCl_2.6H_2O$, 0.05; vitamin stock solution, 10 ml; pH = 8. The vitamin stock solution (final pH adjusted to 3 with HCl) contains (mg/l): pantothenic acid, 10; nicotinamide, 10; pyridoxine, 10; *p*-aminobenzoic acid, 10; inositol, 10; thiamin, 10; riboflavin, 10; choline, 10; biotin, 10; cyanocobalamin, 0.5; folic acid, 0.5. Medium C: A without Bacteriological peptone. Medium D: A with Bacteriological peptone replaced by Casamino acids (Merck), 2 g/l. Medium D_{60}: D with Tween 80 replaced by 4 g/l Tween 60 (polyoxyethylenesorbitan mono-stearate; Lamers and Indemans, 's Hertogenbosch, The Netherlands).

Media were sterilized by autoclaving (15 min, 121°C); phosphate buffer (sterilized separately) and vitamin stock solution (sterilized by Seitz filtration) were added to the cooled media.

Media were inoculated (10 ml into 100 ml of fresh medium) from a culture grown for 10 days in a medium of identical composition. Cultures of *M. parvicella*, shaken at the rate of 260 rev/min, were incubated at 25°C.

Culture treatment. Cultures (75 ml) were centrifuged (20 min, 4000 g in a swing-out rotor) and the supernatant partly removed by decanting (used medium). The loosely packed pellet was filtered by membrane filtration (cellulose acetate membrane filters, pore size 0.45 µm) and washed twice with NaCl solution (2.5 g/l). The residue was taken up in 75 ml of this solution. Washed cells were disintegrated by ultrasonic treatment (Branson sonifier B-12). All filaments were broken after 2 x 1 min treatment at 35 W as determined by microscopic observation.

Analyses. Dry weight was determined in duplicate using 20 ml washed cell suspension. These suspensions and blanks containing NaCl, 2.5 g/l, were dried for 20 h at 103°C.

Protein content of disintegrated cell suspensions was determined by the method of Lowry as modified by Herbert et al. (1971). Blanks with the disintegrated cell suspension but without reagents had to be included because of light scattering due to the lipids present in cells. Bovine albumin serum was used as standard.

Lipids in washed cell suspensions were determined as oleic acid. Suspensions were digested with 1 M HCl at 100°C for 2 h and extracted with chloroform. The oleic acid concentration was determined spectrophotometrically after reaction with concentrated H_2SO_4 (Zevenhuizen, 1974; Zevenhuizen and Ebbink, 1974).

The total amount of carbohydrates in washed cell suspensions were determined as glucose with the anthrone reagent in H_2SO_4 (Herbert et al., 1971).

RNA in washed cell suspensions were determined as ribose by the orcinol method (Herbert et al., 1971). RNA from yeast (Koch-Light) was used as standard.

Total organic carbon content of disintegrated cell suspensions and of used medium was determined in a Beckman model 915A Total Organic Carbon Analyzer by subtracting the amount of inorganic carbon from the amount of total carbon. A solution of $NaHCO_3$ and an acidified solution (0.1 M H_2SO_4) of phthalic acid were used as standards.

To determine total nitrogen, samples of washed cell suspensions and of used medium were digested (Kjeldahl). Ammonia of these samples and that of non-digested samples of used medium was distilled off and determined with Nessler's reagent (APHA, 1975; 421).

Total phosphate was determined by digesting a suitable volume of a washed cell suspension by heating with persulphate. The liberated orthophosphate was determined by the ascorbic acid method (APHA, 1975; 425C and 425F, respectively).

A rapid method to determine Tween 80 - oleic acid concentration in medium has not been reported in the literature. Therefore the following method was developed. Tween 80 in samples of used medium (5 ml) was saponified by adding 0.5 ml of 10 M NaOH and heating the samples in loosely glass-stoppered tubes for 10 min in a boiling water bath. The samples were acidified by adding 1 ml 5 M H_2SO_4, after cooling in tap water, and thoroughly mixed for 1 s. The scattering due to the colloidal suspension

of oleic acid was measured after 10 min at 620 nm. The sample cell of the spectrophotometer had to be cleaned frequently with ethanol because of oleic acid adsorption to the glasswall. Tween in non-inoculated medium was used as a standard.

Infra-red spectroscopy. Cells from 200 ml culture were harvested by membrane filtration, washed with a mixture of EDTA (50 mM) and Triton X-100 (50 g/l) in NaCl solution (2.5 g/l) at pH 8 and then washed twice with NaCl solution. The residue was taken up in 40 ml NaCl solution, disintegrated by ultrasonic treatment and extracted with 40 ml chloroform by shaking for 20 h at 30°C in glass-stoppered Erlenmeyer flasks. The chloroform phase was withdrawn after centrifugation (10 min, 4000 g) in a swing-out rotor and the water phase was re-extracted with chloroform. The combined chloroform phases were brought to dryness in a rotary evaporator at 30°C and the lipid residue was dried over NaOH pellets in a desiccator under reduced pressure. Chloroform was added and infra-red spectra were taken from samples containing 10-20 mg lipids/ml. Spectra were analysed for absorption frequencies of the functional groups (ester bonds, free acids and dissociated acids).

Chromatography. Chloroform extracts of digested washed cell suspensions were saponified and methylated (Brian and Gardner, 1967). Methyl esters of fatty acids were separated in a gas chromatograph equipped with a flame ionization detector at a gas flow rate of 20 ml nitrogen per min.

Column: 2000 x 4 mm stainless steel containing 10% (w/w) of diethylene glycolsuccinate on Chromosorb G - HP (80-100 mesh) and operating at 180°C.

Methyl esters of various pure fatty acids were used as standards.

Carbon dioxide production. Inoculated medium (165 ml) was placed into a sterilized vial (inner diameter, 6 cm; total volume 900 ml), the cotton plug pushed down and the vial closed by a rubber stopper. The rubber stopper was perforated for air inlet and outlet and cultures were stirred by a magnetic stirrer, separated from the vial by an isolating plate (Tempex, 2 cm). Experiments were carried out at 25°C. Inlet and outlet were connected to a gas distributor and an infra red gas analyser (both purchased from the Analytical Development Company Ltd., England). Differences between

CO_2 concentrations of air stream through samples and that through fresh medium (reference) were intermittently recorded.

Sudan Black staining. Microscopic slides with fixed *M. parvicella* cells incubated for various periods of time were stained with Sudan Black as described by Gurr (1965).

Electron microscopy. Washed cell suspensions of *M. parvicella* cultures were centrifuged and the cells suspended in a 1% solution of OsO_4 in acetate/Veronal buffer, pH 6.5, containing 1 M $CaCl_2$. After fixation for 2 h at 4°C, the suspended bacteria were washed twice in the same buffer and embedded in agar. The cells were dehydrated rapidly by several changes in acetone of increasing concentrations (10 to 100%, v/v) and were then embedded in Spurr A in Beem capsules (LKB). Ultrathin sections were made, by use of a LKB 8800 ultramicrotome. They were poststained with a 2% (w/v) solution of uranyl acetate, washed in distilled water, stained with Reynolds Pb citrate (7 min) and then washed with 0.02 M NaOH and distilled water respectively. The preparations were examined in a Philips EM-400T electron microscope.

RESULTS

Cell yield, cell protein and Tween 80 - oleic acid concentration in used medium during growth of *M. parvicella* in medium D, in which Tween 80, the carbon and energy source, was the growth-limiting substrate, are given in Fig. 1a. The pH of the strongly buffered culture dropped from pH 7.8 (inoculated medium) to pH 7.5 (after 20 d incubation). The amounts of total organic carbon and nitrogen and the C/N percent ratio of the biomass (Fig. 1b) and the carbon dioxide production of a growing culture were also determined (Fig. 2). The carbon and nitrogen values as shown in Figs 1b and 2, supplemented with the organic carbon and nitrogen values of used medium, enabled the calculation of C- and N-balances of growing cultures (Fig. 3a,b).

In order to explain the unexpected arithmetic growth and the unusual change in the C/N ratio of the cell material, the bacteria were analysed for various polymers (Fig. 4). The initial internal lipid content of the biomass was extremely high (about 25% of the dry weight after an incuba-

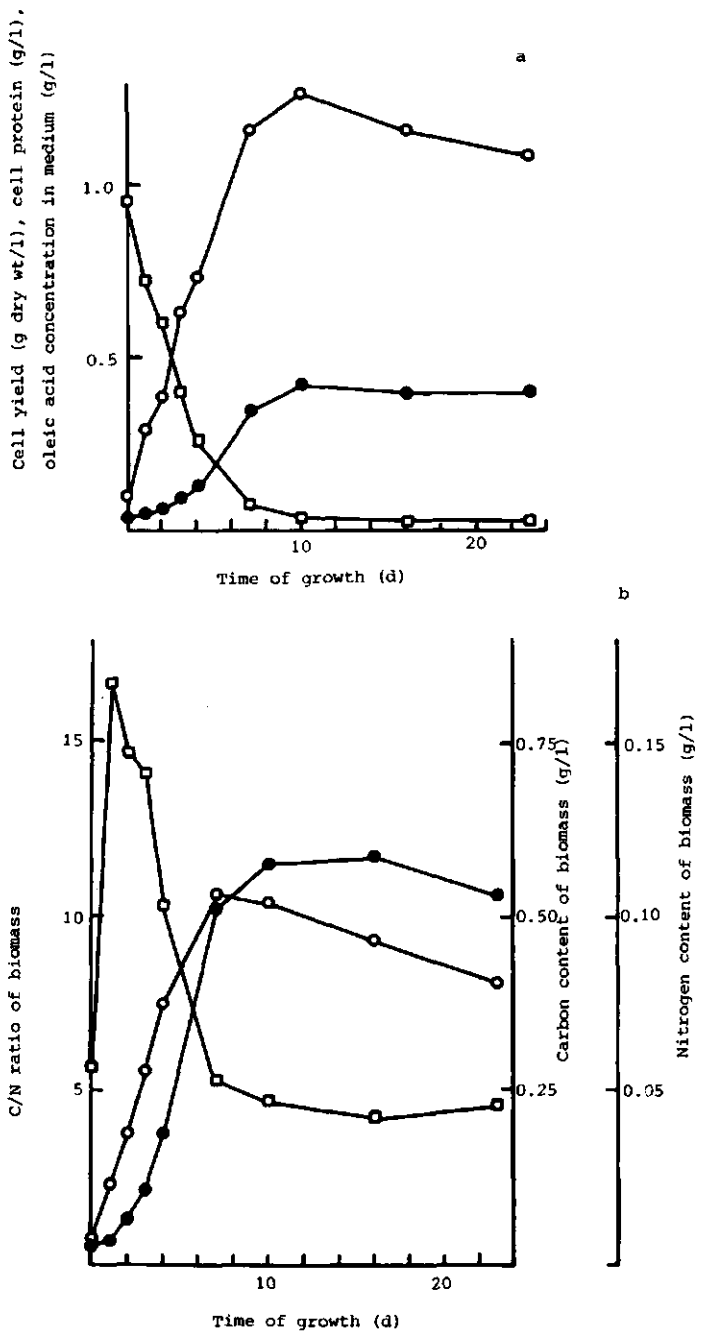

Figure 1.
Growth of *Microthrix parvicella* in medium D. (a) Cell dry weight (0); cell protein (●) and oleic acid (supplied as Tween 80) in used medium (□). (b) Total organic carbon (0); total nitrogen (●) and C/N ratio of bacteria (□).

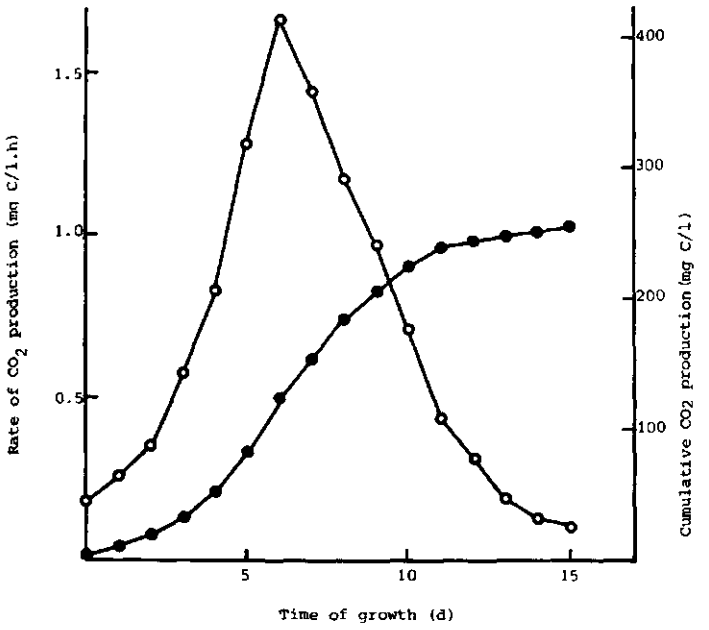

Figure 2.
Carbon dioxide production of *Microthrix parvicella* during growth in medium D. Production rate (O) and cumulative amounts (●).

tion period of 1 day and more than 35% after 4 days; Fig. 4). These lipids appeared as large globules of low electron density in the cells (Fig.5). The globules became also clearly visible by staining with Sudan Black. To obtain some information concerning the chemical composition of the lipid granules, a qualitative analysis of the fatty acids of washed cells of *M. parvicella* during the early growth phase (1 and 2 days after inoculation) was performed. Oleic acid was the main fatty acid when the organism was grown in medium D, whereas stearic and palmitic acids were present in large amounts in cells grown in medium D_{60} (Table 1). The nature of the lipids in cells grown for 30 h in medium D and cells grown for 12 days in medium D were studied in more detail using infra-red spectroscopy. The fatty acids in the lipids were mainly present in esterified form.

The changes in polymer concentration during growth in medium D were similar to the results shown for growth in medium A (Slijkhuis and Deinema, 1982). However, the maximum value of the C/N ratio of the biomass grown in medium A was lower (Table 2), owing to the more rapid protein synthesis in the peptone-containing medium A than in Casamino acids-containing medium D. The phosphorus content of the biomass grown in medium A did not

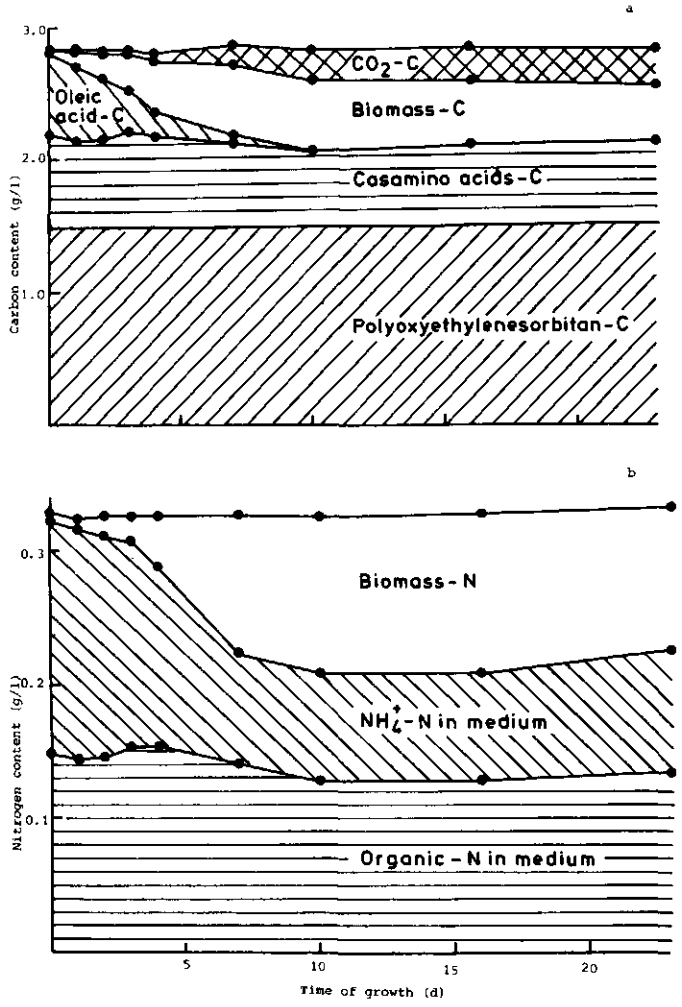

Figure 3.
Distribution of carbon (a) and nitrogen (b) in a culture of *M. parvicella* during growth in medium D.

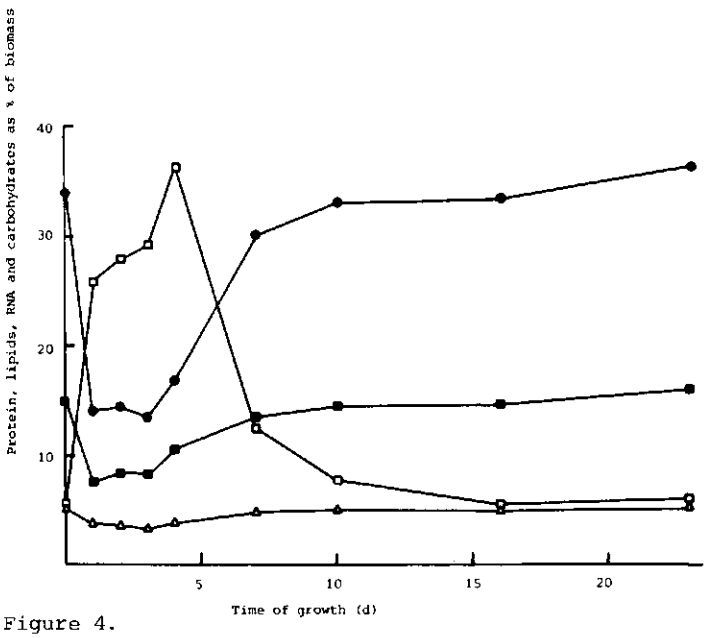

Figure 4.
Polymer composition of *M. parvicella* as % of cell yield during growth in medium D. Protein (●), lipids, determined and plotted as oleic acid (□), RNA (■) and carbohydrates (△).

vary during growth. The P content was about 3.3% of the dry weight and C/P ratio varies between 32 and 38 (Table 2).

Casamino acids were found to supply *M. parvicella* with a reduced-sulphur compound; sulphates were not utilized by this organism. The sulphur requirement was fulfilled by the addition of 0.5 g/l Casamino acids to medium C (Slijkhuis, 1983). Increasing amounts of Casamino acids supplemented to medium C stimulated the rate of protein synthesis and reduced the maximum value of the internal oleic acid as percentage of biomass. The subsequent consumption of the internal substrate showed a pronounced rise with an increasing supply of Casamino acids (Table 3). The amount of protein of the culture in medium C (without Casamino acids) doubled only once during an incubation period of 7 days and cells were still completely filled with lipid globules after that period.

Figure 5.
Inclusions of *M. parvicella*. Electron micrographs of a section of cells, grown for 30 h in medium D, showing large lipid globules (a) and of cells, grown for 12 d in medium D (b).
The bar marker represents 100 nm.

Table 1.

Distribution of palmitic, stearic and oleic acids in culture medium D (containing Tween 80) and D_{60} (containing Tween 60) and in *M. parvicella* cells growing in these media.

	% of total fatty acids[1]		
	Palmitic acid	Stearic acid	Oleic acid
Medium D (Tween 80)	7	-[2]	86
Biomass grown in medium D[3]	6	-	72
Medium D_{60} (Tween 60)	41	58	-
Biomass grown in medium D_{60}[3]	29	55	8

1) Saturated and unsaturated fatty acids with a chain length of 12 up to 18 carbon atoms were determined.

2) Not detectable.

3) Averages of samples taken from *M. parvicella* cultures grown for 1 and 2 days (four analyses).

Table 2.

C/N percent ratio and C/P percent ratio of *M. parvicella* during growth in medium A.

Time of growth (d)	C/N ratio of biomass	C/P ratio of biomass
0	4.7	32
2	8.8	38
4	5.0	38
7	4.2	32

Table 3.

Dry weight of cells and percentages of protein and oleic acid in *M. parvicella* during growth in medium C with various concentrations of Casamino acids.

	Dry weight of biomass (g/l)				Protein (% of biomass)				Oleic acid (% of biomass)			
Time after inoculation[1)]	0	3	5	7	0	3	5	7	0	3	5	7
Concentration of Casamino acids in medium (g/l)												
0	0.11	0.32	0.41	0.58	39	19	13	16	3	44	46	50
0.5	0.11	0.41	0.80	1.02	39	17	15	16	3	43	37	32
1.0	0.11	0.47	0.98	1.12	39	17	21	25	3	40	30	24
2.0	0.11	0.68	1.10	1.30	39	17	27	34	3	40	20	10
5.0	0.11	0.91	1.17	1.47	39	20	29	34	3	37	12	8

1) The inoculum (10% v/v) was derived from a culture grown for 10 days in medium D.

DISCUSSION

The yield of *M. parvicella* in medium D containing Tween 80 (4 g/l), the growth-limiting substrate, was about 1.2 g/l (Fig. 1a). Casamino acids were hardly used as carbon source (Fig. 3a). As only the oleic acid moiety of the Tween 80 molecule (oleic acid content is 24%) was utilized by this bacterium (Slijkhuis, 1983), cell yield can be calculated to be about 1.35 g of dry weight per g of oleic acid consumed. The yield from this substrate was high compared to that reported for bacterial growth on hydrocarbons. Wodzinski and Johnson (1968) reported cell yields of 1.0 g biomass/g of substrate consumed for a *Pseudomonas* sp. and a *Nocardia* sp. growing on *n*-octadecane, *n*-octane or *n*-heptane and Raymond and Davis (1960) determined a yield of 0.85 g biomass/g of *n*-octadecane consumed for a *Pseudomonas aeruginosa* strain. *Mycobacterium vaccae* is apparently more efficient in biomass synthesis from alkanes. From the data on this organism (Blevins and Perry, 1971), cell yields (g biomass/g of substrate consumed) of 1.22 and 1.23 from ethane and propane, respectively, can be calculated.

No exponential growth curve of *M. parvicella* on oleic acid was obtained, when the plotted data were derived from cell yield. However, when protein values were plotted a normal exponential growth curve appeared showing a specific growth rate of *M. parvicella* in batch culture in medium D of about 0.016 h^{-1} (Fig. 6). The deviating results, obtained when plotting dry weight data were caused by the large amounts of lipids in the cells during the early growth phase (Figs 4, 5). These lipids accumulated much more readily than normal cell growth proceeded.

The only carbon and energy sources for *M. parvicella* are long chain fatty acids preferably in esterified form (Slijkhuis, 1983). The fatty acids are readily taken up by the organism and stored in lipid globules (Fig. 5). It was proved by infra-red spectroscopy that the fatty acids accumulated in an esterified form but the nature of the alcoholic compound has not been elucidated. The fatty acids present in the globules were shown to be similar to those supplied in the external medium (Table 1).

The property of storing the carbon source taken up in the early growth phase has only rarely been observed in bacteria. A *Mycobacterium* species cultivated with Tween 80 as growth-limiting substrate showed a

similar behaviour (Schaefer and Lewis, 1965). The structure of the lipid globules of that organism, shown in electron micrographs of ultra-thin sections, resembles that of the globules in *M. parvicella*.

The accumulation of the carbon substrate as soon as it is available may be of ecological importance. Mixing of influent (substrates) and activated sludge (the habitat of *M. parvicella*) in waste water treatment plants is rarely ideal so that temporarily high substrate concentrations may occur. Therefore, an organism with a relatively high substrate removal rate may be favoured in the competition for substrates. A further competitive advantage of *M. parvicella* compared to bacteria living within the activated sludge flocs concerns the supply of nutrients. The substrates are accessible more readily to filaments, protruding from the flocs. *M. parvicella* is the cause of bulking activated sludge in most of the oxidation ditches fed with domestic waste water (Eikelboom, 1982). In view of the results with pure cultures reported in this paper, it is concluded that the organism readily utilizes the long chain fatty acids occurring in considerable amounts in this type of waste water (Painter and Viney, 1959). As these fatty acids, probably present in the waste water in an insoluble esterified form, are thought to be adsorbed by the sludge flocs, contact of *M. parvicella* and sludge flocs will be necessary to achieve solubilization and uptake of the substrates.

The ready uptake and early storage of the carbon substrate is reflected in the ready increase of biomass and total organic carbon of the biomass, proceeding arithmetically. As protein synthesis and nitrogen uptake show the normal logarithmic growth, enormous variations occur in the nitrogen content of the biomass (from 2.5 - 10%) and in the C/N ratio (from 5.5 to nearly 17) of a batch culture of *M. parvicella* (Fig. 1b). The phosphorus content of *M. parvicella* (Table 2) is slightly higher than that usually found in bacteria viz. 1-3%. Phosphorous is partly stored as polyphosphate (volutin) which occurs as Neisser-positive granules in the cells (Eikelboom, 1975).

When the biomass has attained a maximum value (after an incubation period of 10 days, Fig. 1a), about 70% of the substrate oleic acid carbon has been converted into biomass and only 28% has been oxidized to carbon dioxide. The high ratio biomass-C/CO_2-C (= 2.6) implicates a high efficiency of *M. parvicella* concerning the utilization of the substrate and consequently a high yield from this substrate. The increase of the carbon

dioxide production rate during the early growth phase parallels the protein production rate (Fig. 6); both phenomena reflect the biomass activity during the exponential growth phase.

The main constituents of the biomass (protein, oleic acid, RNA and carbohydrates) contributed for 55 to 65% to the dry weight (Fig. 4). Cell wall components, DNA, pool of simple organic compounds, fatty acids with the exception of oleic acid, alcoholic components of the lipids and inorganic material, which were not determined may account for the remaining part of the biomass.

Casamino acids in the culture medium have at least two functions. On one hand, they supply *M. parvicella* with a reduced sulphur compound which is essential for growth. This requirement is already fulfilled by 0.5 g Casamino acids/l (Slijkhuis, 1983). On the other hand the growth rate of the organism is greatly affected by higher concentrations of Casamino acids (Table 3). However, this effect is not due to the functioning of Casamino acids as a pool of nutrients, because these acids hardly contribute to the yield of biomass (Figs 3a,b). It is suggested that Casamino

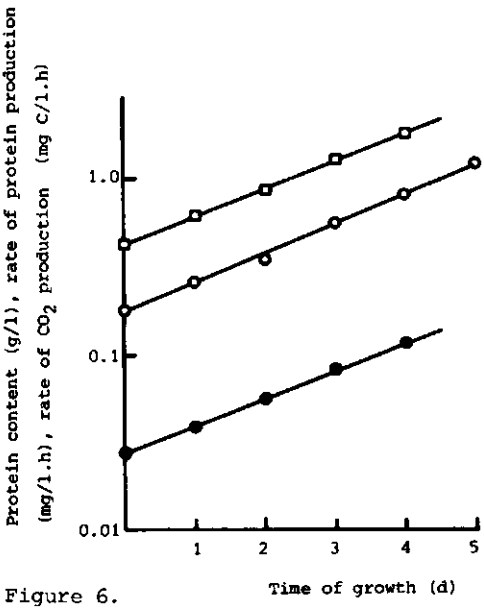

Figure 6.
Semi-logarithmic plot of carbon dioxide production rates (O), amount of protein (●) and protein production rates (□) in cultures of *M. parvicella* during growth in medium D.

acids exert a protective effect against some inhibiting component of the medium which might be free oleic acid. In an earlier paper (Slijkhuis, 1983) it was reported that *M. parvicella* is sensitive to free oleic acid. The free acid might be present in Tween 80 or might be liberated from Tween 80 by bacterial action. Bacteriological peptone which was shown to have a favourable effect on growth in medium A was thought to function as a protective factor against free oleic acid. Casamino acids supplemented to medium C probably have a similar effect in addition to their function of supplying *M. parvicella* with reduced sulphur compounds.

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4. Growth kinetics of *Microthrix parvicella*, a filamentous bacterium occuring in activated sludge.

INTRODUCTION

The filamentous bacterium *Microthrix parvicella* is often present in the sludge of low-loaded activated sludge plants and oxidation ditches treating domestic waste water (Eikelboom, 1982). A small amount of this bacterium in the sludge flocs hardly affects the operation of the purification plant; however, abundant growth results in a poor settling of the flocs ("bulking sludge").

Activated sludge is an ecosystem of mainly bacteria and its microbial composition is the result of the competition for sewage substrates between various species. The physiological characteristics of an organism and the growth kinetics determine to a great extent the ability of the organism to grow in such a mixed culture.

The nutritional requirements of *M. parvicella* have been elucidated; it was shown that this bacterium utilizes as carbon and energy source only long chain fatty acids (preferably in esterified form e.g. Tween 80) and requires reduced nitrogen and reduced sulphur compounds (Slijkhuis, 1983). During the growth in batch culture, the oleic acid moiety of Tween 80 is readily taken up and stored as lipids, resulting in a high lipid content of the cells in the early growth phase (Slijkhuis et al., 1983). In order to study the growth kinetics of *M. parvicella*, the organism was grown in a chemostat.

MATERIALS AND METHODS

Organism. Microthrix parvicella, strain B, as described previously (Slijkhuis, 1983), has been used in this study.

Medium. The compounds of the medium used in continuous cultivation were made up in two solutions to prevent precipitation. Solution I contained per 1 of distilled water: Tween 80 (polyoxyethylenesorbitan mono-oleate, Sigma), 8.0 g; $(NH_4)_2SO_4$, 1.6 g; L-methionine, 0.06 g; $MgSO_4.7H_2O$, 0.15 g; $MnSO_4.H_2O$, 6 mg; $ZnSO_4.7H_2O$, 0.2 mg; $CuSO_4.5H_2O$, 0.2 mg and phosphate solution, 100 ml. Phosphate solution (K_2HPO_4, 82 g/l and KH_2PO_4, 3.5 g/l) was sterilized separately from the rest of solution I.

Solution II contained per 1 of distilled water: $CaCl_2$, 0.10 g; $FeCl_3.6H_2O$, 10 mg; H_3BO_3, 0.2 mg; $Na_2MoO_4.2H_2O$, 0.1 mg; $CoCl_2.6H_2O$, 0.1 mg and vitamin stock solution, 20 ml. Vitamin stock solution (thiamin, 10 mg/l and cyanocobalamin, 0.5 mg/l) was adjusted to pH 3 with HCl and sterilized by Seitz filtration.

Solutions I and II were sterilized by autoclaving (30 min, 121°C); phosphate solution and vitamin stock solution were added to the cooled solutions.

Continuous culture. The bacterium was cultivated in two Eschweiler fermentors (Kiel, W-Germany) of 2 l capacity. The working volume was 1 l and the pressure inside the vessel was kept at an overpressure of a 0.5 m watercolumn to reduce the possibility of infection. The temperature was kept at 25°C and the pH at 7.8. At 300 rev./min, a sterilized air flow of about 10 l/h was led over and not through the culture to prevent foaming. By this procedure the O_2 concentration in the solution did not drop below 50% of its saturation value. To remove wall growth, a teflon-bar magnet was present in the fermentor and by the use of a powerful magnet at the outside, the glass wall was cleaned twice a day. Solutions I and II of the medium, stored in 10 l flasks and placed on a magnetic stirrer, were pumped (LKB VarioPerpex pumps) into the fermentor at equal rates. The flow rate was determined by using burettes placed in the tubes connecting storage vessels and chemostat. Tween 80, the carbon and energy source, was the limiting substrate.

A batch culture (100 ml) of *M. parvicella*, grown for 10 days in a medium of the same composition supplemented with additional K_2HPO_4 (4.1 g/l) and KH_2PO_4 (0.17 g/l), was added to 500 ml medium in the fermentor. When the optical density of the culture had reached its maximum value (absorbancy 0.4 at 620 nm of a 1:5 diluted sample), the supply of fresh medium was started at a constant rate. After 2-3 volume passages at a

fixed flow rate, samples of 75 ml were taken three times a week at regular intervals. The biomass of the cultures in each steady state (3 samples) was analyzed for dry weight, protein, lipids, total organic carbon and total nitrogen and the medium for esterified oleic acid, total organic nitrogen and ammonium nitrogen.

Oxygen uptake rate. A 5 ml sample from the continuous culture was brought into the vial of an oxygen monitor (Yellow Springs Instruments). The oxygen consumption was measured during 5-10 min at 25°C. Time between sampling and recording was only about 3 min and the oxygen concentration in the liquor was estimated from these data.

Cell treatment. Of a chemostat culture sample, 20 ml was centrifuged (20 min at 4000 x g in a swing-out rotor) and the supernatant partly removed by decanting. The loosely packed pellet was filtered by membrane filtration (cellulose acetate membrane filters, pore size 0.45 µm) and washed with NaCl solution (2.5 g/l). The residue was taken up in 50 ml of this solution. Washed cells were disintegrated by ultrasonic treatment (Branson sonifier B-12). All filaments were broken after 2 x 1 min treatment at 35 W as verified by microscopic observation.

Analyses. Cell dry weight was estimated by measuring the optical density at 620 nm, using a Bausch and Lomb Spectronic 20 with the aid of a calibration curve. Before measuring, 5 ml 1:5 diluted suspension (treated with Ultra Turrax type 18/10 homogenizer during 5 seconds) was supplied with 0.5 ml of a mixture of EDTA (ethylenediaminetetraacetic acid, 50 mM) and Triton X-100 (50 g/l) at pH 8. The absorbancy value at 620 nm of the uninoculated medium treated in the same way was subtracted.

The protein content of disintegrated cell suspensions was determined by the method of Lowry as modified by Herbert et al. (1971). Blanks containing the disintegrated cell suspensions but without reagents had to be included because of light scattering due to lipids present in the cells. Bovine albumin serum was used as a standard.

Lipids in washed cell suspensions were determined as oleic acid. Suspensions were digested with 1 N HCl for 2 h at 100°C and extracted with chloroform. The oleic acid concentration was determined spectrophotometrically after reaction with concentrated H_2SO_4 (Zevenhuizen, 1974; Zevenhuizen and Ebbink, 1974).

Total carbon and inorganic carbon in disintegrated-cell suspensions were determined in a Beckman model 915A Total Organic Carbon Analyzer. Organic carbon was obtained by subtracting inorganic from total carbon. A solution of $NaHCO_3$ and an acidified solution (0.2 N H_2SO_4) of phthalic acid were used as standards.

To determine total nitrogen, samples of washed-cell suspensions and of used medium were digested (Kjeldahl). Ammonia of these samples and that of non-digested samples of used medium was distilled off and determined with Nessler's reagent (APHA, 1975: 421).

A rapid method to determine Tween 80 is not reported in the literature. Therefore, the following method was developed. A 5 ml sample of used medium was saponified by adding 0.5 ml of 10 N NaOH and heating in a loosely glass-stoppered tube for 10 min in a boiling water bath. After cooling in tap water, the sample was acidified by adding 1 ml of 10 N H_2SO_4 and thoroughly mixed for 1 s. The scattering due to the colloidal suspension of oleic acid was measured after 10 min at 620 nm. The sample cell of the spectrophotometer had to be cleaned frequently with ethanol because of oleic acid adsorption to the glass wall. Tween 80 in uninoculated medium was used as a standard.

RESULTS

Steady state of growth of *Microthrix parvicella* has been obtained at five dilution rates; cell dry weight is given in Table 1. The Tween 80 concentration in the chemostat culture at dilution rates up to 0.05 h^{-1} did not exceed 20 mg Tween 80/l but these low concentrations could not be properly determined. Consequently, it can be stated that the substrate constant of Tween 80 for *M. parvicella* is very low but an exact value could not be calculated. The maximum specific growth rate (μ_{max}) of *M. parvicella* was estimated by following the biomass wash-out after increasing the dilution rate from 0.050 h^{-1} to the arbitrarily chosen value of 0.069 h^{-1} (Fig. 1) and using the equation of Pirt (1965) for calculation:

$$\ln X = (\mu_{max} - D)\, t + \ln X_0 \qquad (1)$$

In this equation X_t and X_0 are the biomass concentrations (g cell dry weight/l) at times t and t = 0, respectively; t is given in (h), D is the dilution rate (h^{-1}). From these data (Fig. 1) μ_{max} was calculated to be 0.060 h^{-1}.

Table 1.

Cell dry weight, cell yield on Tween 80 and percentage of cell protein and cell lipids in *M. parvicella* during growth in a chemostat supplied with 4 g/l Tween 80 at various dilution rates.

Dilution rate (h^{-1})	Cell dry weight (g/l)	Cell yield g dry weight /g Tween 80	Cell protein (% of dry weight)	Cell lipids (% of dry weight)
0.011	1.15	0.288	29.4	8.3
0.022	1.20	0.300	25.7	11.3
0.033	1.26	0.315	22.2	19.0
0.041	1.31	0.328	19.4	24.3
0.050	1.30	0.325	--[2]	--

1) Determined as oleic acid.

2) Not determined.

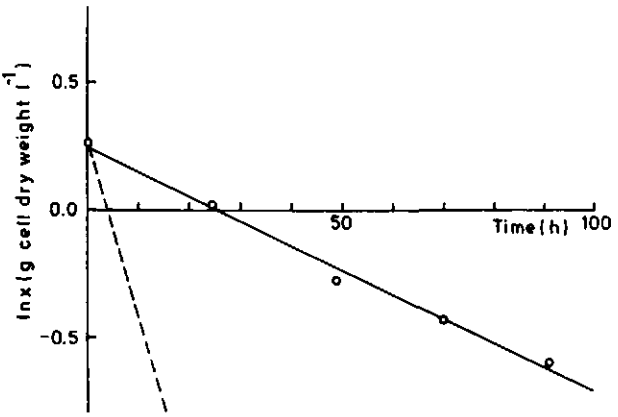

Figure 1.
Wash-out of *M. parvicella* at a dilution rate of 0.069 h^{-1} (0—0). Theoretical wash-out (μ_{max} = 0) is given by the dotted line.

The yield on Tween 80 at various dilution rates were used for determining the maintenance coefficient (m) and the maximum yield (Y_{max}) on Tween 80 (Table 1, Fig. 2). The relation between these parameters and the specific growth rate is given by equation (2).

$$Y^{-1} = Y_{max} + m\ \mu^{-1} \qquad (2)$$

In this equation Y is the observed cell yield, g cell dry weight/g Tween 80, and μ is the specific growth rate which equals the dilution rate D under steady state conditions (h^{-1}). The amount of Tween 80 required by *M. parvicella* for maintenance was found to be 6.1 mg/g cell dry weight . h and the maximum yield on Tween 80 was 0.34 g cell dry weight/g as calculated by linear regression from the data in Table 1.

The activity of *M. parvicella* cells, determined as oxygen uptake rate per unit mass of cell protein, increased proportionally with the dilution rate (Fig. 3). The minimum requirement for oxygen as found by extrapolation was about 0.4 mmoles O_2/g protein . h.

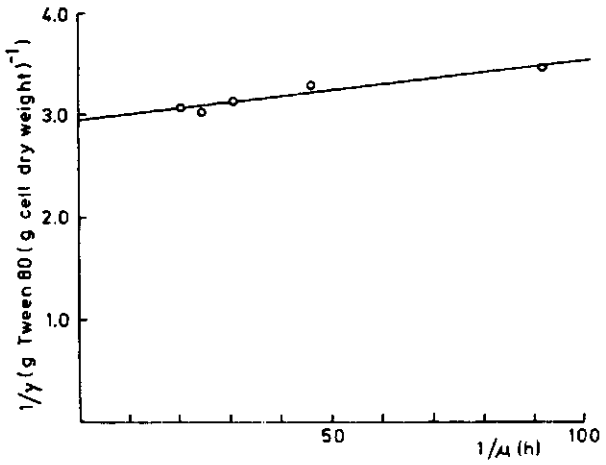

Figure 2.
Relation between the reciprocal of the yield of *M. parvicella* cells on Tween 80 and the reciprocal of the specific growth rate.

The composition of the biomass grown at various dilution rates was not uniform. The protein content of the cells decreased with increasing growth rate, whereas the lipid content increased due to accumulation of the substrate, the oleic acid moiety of Tween 80. The variations in biomass composition were reflected in the nitrogen balance of the cultures (Table 2). The amount of nitrogen in biomass was higher at $D = 0.011\ h^{-1}$ than at $D = 0.041\ h^{-1}$. The amount of carbon in the biomass increased slightly with dilution rate from 485 to 510 mg/l culture, both alterations resulting in an increase of the C/N ratio of *M. parvicella* cells from 4.5 at $D = 0.011\ h^{-1}$ to 6.8 at $D = 0.041\ h^{-1}$.

Table 2.
Distribution of nitrogen in cultures of *M. parvicella* at various dilution rates.

Dilution rate h^{-1}	Total nitrogen in bacteria (mg/l culture)	Total nitrogen in used medium[1)] (mg/l)	Total nitrogen in culture (mg/l)
0.011	108	69	177
0.022	97	79	176
0.033	86	85	171
0.041	75	96	171

[1)] These values were identical with ammonium nitrogen values.

DISCUSSION

The maximum specific growth rate of *M. parvicella* grown in a chemostat is 0.060 h^{-1} (Fig. 1). In batch culture, the specific growth rate of this bacterium is 0.016 h^{-1} when calculated from the data on protein production (Slijkhuis et al., 1983). The difference in the determination procedure (chemostat versus batch culture) as well as the difference in the determination of the yield (cell dry weight versus cell protein) may explain this discrepancy.

The maximum specific growth rate of *M. parvicella* is low compared to those of non-filamentous bacteria usually studied in the chemostat. Comparison with other activated-sludge microorganisms is hardly possible as only few growth parameters of typical activated-sludge bacteria have been reported in the literature. Among the filamentous organisms occurring in activated sludge, only two species have been cultivated in a chemostat, viz. *Haliscomenobacter hydrossis* and *Sphaerotilus natans*. The maximum growth rate of *H. hydrossis* varied considerably between strains of this species and was dependent on growth conditions. Values of 0.05 h^{-1} and 0.09 h^{-1} have been determined, but for two strains the curves showing the relation between biomass concentration and dilution rate differed from the theoretical (Krul, 1977; Van Veen et al., 1982). The maximum growth rate of *S. natans*, grown in a chemostat, has not been determined (Houtmeyers, 1978) but from studies in batch culture a value of 0.13 can be calculated from the data given by Adamse (1968).

The low maximum growth rate of *M. parvicella* hardly limits the growth of this bacterium in activated sludge. The overall growth rate of the biomass in waste water treatment plants varies from 0.010 h^{-1} in high-loaded activated sludge plants to 0.001 h^{-1} in low-loaded plants e.g. oxidation ditches (Metcalf and Eddy, 1979). A μ_{max} of 0.06 h^{-1} would allow a 10^6-fold increase of *M. parvicella* in sludge of oxidation ditches within 10 days when conditions are favourable for the organism.

The maximum yield on Tween 80 and the Tween 80 requirement for maintenance were found to be 0.34 g cell dry weight/g and 6.1 mg/g cell dry weight . h, respectively. *M. parvicella* assimilates only the oleic acid part of Tween 80 (Slijkhuis, 1983). Oleic acid accounts for 24% of Tween 80; therefore the values of the maximum yield and of the maintenance co-

efficient are 1.41 g cell dry weight/g oleic acid and 1.46 mg oleic acid/g cell dry weight . h, respectively. The determination of Y_{max} and m values of this bacterium with Tween 80 as the limiting substrate is not entirely correct. Equation (2) given by Pirt (1965) may only be used if the composition of the biomass does not vary with dilution rate. As it is clearly shown in Tables 1 and 2, the composition of the *M. parvicella* cells changes with growth rate, although the alterations in cell composition during growth in batch culture are even more pronounced (Slijkhuis et al., 1983).

Variations in the composition of the biomass grown at various dilution rates have often been reported e.g. for *Aerobacter aerogenes* (Herbert, 1959; Tempest and Hunter, 1965), *Azotobacter vinelandii* (Dalton and Postgate, 1969), *Escherichia coli* (Forchhammer and Lindahl, 1967) and *Salmonella typhimurium* (Maaløe and Kjeldgaard, 1966). Cell protein usually decreases with increasing dilution rate, whereas ribonucleic acid (RNA) shows a pronounced increase. Although the variations of the composition of the biomass at different growth rates are a general phenomenon, Y_{max} and m values are generally estimated using the equation (2) of Pirt.

The calculated maximum yield on oleic acid is high (1.41 g cell dry weight/g), but this value is in agreement with the data obtained in batch culture (1.35 g/g), and can be explained by the nature of this substrate (Slijkhuis et al., 1983).

The maintenance requirement of *M. parvicella* as estimated from the oxygen requirement m_{O_2} approximates the value of m calculated from data in Table 1 using equation (2) (6.1 mg Tween 80/g cell dry weight . h). The protein content of the biomass at specific growth rate $\mu = 0\ h^{-1}$ is 0.34 g/g dry weight as found by extrapolation of the data on protein content at various dilution rates (Table 1) to specific growth rate $\mu = 0\ h^{-1}$. The m_{O_2} was found to be 0.14 mmoles of O_2/g of cell dry weight . h. The complete oxidation of oleic acid stoichiometrically requires 0.09 mmoles of O_2/mg of oleic acid and an m_{O_2} of 0.14 mmoles of O_2/g of cell dry weight . h corresponds with a maintenance on oleic acid of 1.54 mg/g cell dry weight . h and on Tween 80 of 6.4 mg/g cell dry weight . h which are similar to the values obtained from yield data. The carbon content of the cell dry weight of *M. parvicella* was found to be about 40%, resulting in an m_{O_2} of 0.004 moles of O_2/mole of biomass-C . h. This value is low com-

pared to values reported for various other microorganisms grown on various substrates (0.10 - 0.31; Heijnen and Roels, 1981).

Lipids determined as oleic acid may account for 35% of the cell dry weight during the early growth phase in batch culture (Slijkhuis et al., 1983). A similar phenomenon appears in the chemostat. The supply of substrate increases with dilution rate and all oleic acid (Tween 80) is taken up by the biomass at the various steady states. The conversion of oleic acid into cell components (e.g. protein) at higher dilution rates is not proportional to the oleic acid uptake, resulting in an accumulation of oleic acid as lipids in the biomass and an increase of the C/N ratio of the biomass from 4.5 to 6.8.

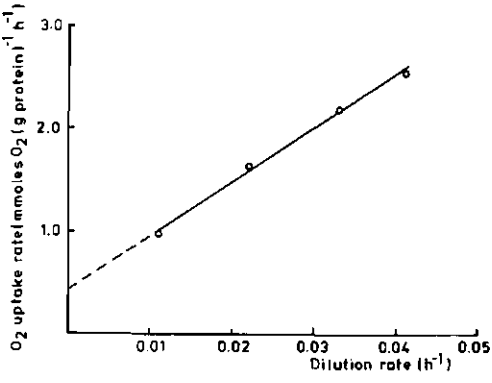

Figure 3.
Oxygen uptake rates of *M. parvicella* cultures grown at various dilution rates.

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5. Some aspects of the acetic acid metabolism in *Microthrix parvicella.*

INTRODUCTION

Microthrix parvicella, a filamentous bacterium occurring in activated sludge, grows readily in media containing long chain fatty acids as the only carbon and energy source. Tween 80 (polyoxyethylenesorbitan monooleate) serves as an excellent substrate; it may be replaced by sodium oleate at a low concentration. Acetic and butyric acids can not be utilized by *M. parvicella* as the only carbon and energy source; however, the addition of these short chain fatty acids to media containing Tween 80 increases the cell yield (Slijkhuis, 1983).

The common pathway of the degradation of long chain fatty acids in bacteria is the β-oxidation (Mahler, 1967) resulting in acetyl-CoA esters. Acetyl-CoA is an important intermediate in most reactions concerning the biosynthesis and energy production and therefore growth on acetic and butyric acids would be expected when the organism can grow on long chain fatty acids.

MATERIALS AND METHODS

Organism and growth conditions. Microthrix parvicella, strain B, was isolated from activated sludge of the oxidation ditch of Bennekom, The Netherlands. Stock cultures were kept on slants of medium A agar (Slijkhuis, 1983). The basic medium used (Cas medium) contains (g/l): Casamino acids (Merck), 2; $(NH_4)_2SO_4$, 0.8; K_2HPO_4, 8.2; KH_2PO_4, 0.35; $MgSO_4.7H_2O$, 0.075; $CaCl_2$, 0.05; trace elements (mg/l): $FeCl_3.6H_2O$, 5; $MnSO_4.2H_2O$, 3; $ZnSO_4.7H_2O$, 0.1; $CuSO_4.5H_2O$, 0.1; H_3BO_3, 0.1; $Na_2MoO_4.2H_2O$, 0.05; $CoCl_2.6H_2O$, 0.05; vitamin stock solution, 10 ml; pH 8. The vitamin stock solution (final pH adjusted to 3 with HCl) contains (mg/l): panthotenic acid, 10; nicotinamide, 10; pyridoxine, 10; *p*-aminobenzoic acid, 10; inositol, 10; thiamin, 10; riboflavin, 10; choline, 10; biotin, 10; cyanocobalamin, 0.5;

folic acid, 0.5. Media were sterilized by autoclaving (15 min, 121°C); phosphate buffer (sterilized separately) and vitamin stock solution (sterilized by Seitz filtration) were added to the cooled media. Short chain fatty acids and other acids were added as sodium salts. The pH of cultures in media containing these acids were determined daily and corrected to pH = 7.5 - 7.8 with 1 M H_2SO_4. Malonic, glycolic and glyoxylic acids were sterilized by membrane filtration. Media were inoculated by transferring 10 ml of a culture grown for 20 days in Cas medium, supplemented with 4 g/l Tween 80 (polyoxyethylenesorbitan mono-oleate; Sigma) containing 0.96 g of oleic acid/l, to 100 ml of fresh medium. Shake cultures (260 rev/min) were incubated at 25°C.

Culture treatment. Culture samples were centrifuged (20 min, 4000 x g in a swing-out rotor) and the supernatant partly removed by decanting (used medium). The loosely packed pellet was filtered by membrane filtration (cellulose acetate membrane filters, pore size 0.45 µm) and washed twice with NaCl solution containing 2.5 g NaCl in 1 l distilled water. The residue was taken up in this solution and cells were disintegrated by ultrasonic treatment (Branson sonifier B-12). All filaments were broken after 2 x 1 min treatment at 35 W as determined by microscopic observation.

Analyses. Dry weight was estimated by measuring the optical density at 620 nm as previously described (Slijkhuis, 1983).

Protein content of disintegrated cell suspensions was determined by the method of Lowry as modified by Herbert et al. (1971). Blanks with the disintegrated cell suspension but without reagents had to be included because of light scattering due to the lipids present in the cells. Bovine albumin serum was used as standard.

A rapid method for the estimation of the concentration of Tween 80 - oleic acid was used (Slijkhuis et al., 1983). A sample of 5 ml used medium was saponified by adding 0.5 ml of 10 M NaOH and heating for 10 min in a loosely glass-stoppered tube in a boiling water bath. The sample was cooled in tap water, acidified with 1 ml of 5 M H_2SO_4 and thoroughly mixed for 1 s. The scattering due to the colloidal suspension of oleic acid was measured after 10 min at 620 nm. The sample cell of the spectrophotometer had to be cleaned frequently with ethanol because of oleic acid adsorption to the glass wall. Tween 80 in Cas medium was used as standard.

Short chain fatty acids (acetic, propionic and butyric acids) in used medium were determined chromatographically using a Packard-Becker model 417 gaschromatograph equipped with a flame ionization detector at a gas flow rate of 18 ml per min of nitrogen, saturated with formic acid. Column: 2000 x 2 mm glass, containing chromosorb 101 (60-80 mesh) and operating at 190°C.

RESULTS AND DISCUSSION

Excellent growth of *M. parvicella* was obtained in Cas medium supplemented with Tween 80 - oleic acid. The growth in Cas medium supplemented with only a short chain fatty acid or glycerol was poor and addition of glucose or succinic acid did not at all stimulate the growth of *M. parvicella*. However, supplied together with Tween 80, the short chain fatty acids and glycerol gave considerably higher cell yields than were obtained without these short chain compounds. Such a response was not obtained with additional glucose or succinic acid (Table 1).

The effect of acetic acid on the growth of *M. parvicella* has been studied in more detail. From Table 2 it follows that the amount of cell protein in cultures grown on Cas medium with Tween 80 - oleic acid (0.48 g/l) had increased considerably when an additional amount of acetic acid had been given, whereas the protein production on acetic acid only was poor. The consumption of Tween 80 - oleic acid corresponded with the increment of cell protein produced on this substrate (Slijkhuis, 1983), but acetic acid consumption in the culture supplied with acetic acid only was relatively high in relation to protein production. It is assumed that acetic acid is utilized for maintenance requirements. Similar results were obtained when acetic acid had been added to an 8 days old culture of *M. parvicella* grown in Cas medium with Tween 80 - oleic acid. In this case (Fig. 1) the addition of acetic acid was carried out in the absence of Tween 80 - oleic acid, as this carbon source had readily been taken up by the cells within 6 days. The addition of acetic acid gave a distinct increase in growth but it is assumed that certain cell components which are indispensable for the growth of *M. parvicella* can be synthesized only from oleic acid. Consequently in this experiment, growth of the bacterium on acetic acid as the carbon and energy source was possible because these components, originating from the preceding growth on Tween 80 - oleic acid,

Table 1.

Yield of *M. parvicella* in Cas medium supplemented with various carbon sources.

Carbon source	Concentration in medium (g/l)	Cell yield[1] (g dry weight/l in Cas medium	Cell yield[1] (g dry weight/l in Cas medium supplemented with Tween 80 - oleic acid (0.48 g/l)
---		0.0	0.65
Tween 80 - oleic acid	0.48	0.65	1.25
	0.96	1.25	--[2]
Acetic acid	1.06	0.15	0.98
	2.12	0.15	--
Propionic acid	0.76	0.14	0.95
	1.52	0.14	--
Butyric acid	0.67	0.16	0.96
	1.34	0.16	--
Glycerol	1.0	0.14	1.0
	2.0	0.14	--
Glucose	1.0	0.0	0.63
	2.0	0.0	--
Succinic acid	2.0	0.0	0.63
	2.0	0.0	--

1) Determined after incubation for 10 days at 25°C. Media (100 ml) were inoculated with 10 ml of a culture grown for 10 days in Cas medium supplemented with Tween 80 - oleic acid (0.96 g/l). Data were corrected for inoculum.

2) Not determined.

were still present in the cells in sufficient amounts. Protein production and substrate consumption in Cas medium supplemented with acetic acid (Table 2) and in a full-grown culture supplemented with acetic acid (Fig. 1) clearly show that acetic acid can be metabolized by *M. parvicella*; in both experiments the amount of cell protein in the cultures had increased with 70% due to added acetic acid.

Table 2.
Protein production and substrate consumption of *M. parvicella* grown in Cas medium supplemented with Tween 80 - oleic acid or/and acetic acid.

		Protein production (g/l)		Substrate consumption (g/l)	
Time of incubation (d)[1]		8	20	8	20
Carbon source	Concentration in medium (g/l)				
Tween 80-oleic acid	0.96	0.500	0.480	0.870	0.870
Acetic acid	2.20	0.033	0.035	0.215	0.290
Tween 80-cleic acid + acetic acid	0.48	0.427	0.400	0.435	0.435
	1.10			0.715	0.990

1) Media (100 ml) were inoculated with 10 ml of a culture grown for 10 days at 25°C in Cas medium supplemented with Tween 80 - oleic acid (0.96 g/l). Data were corrected for inoculum (0.050 mg protein/l).

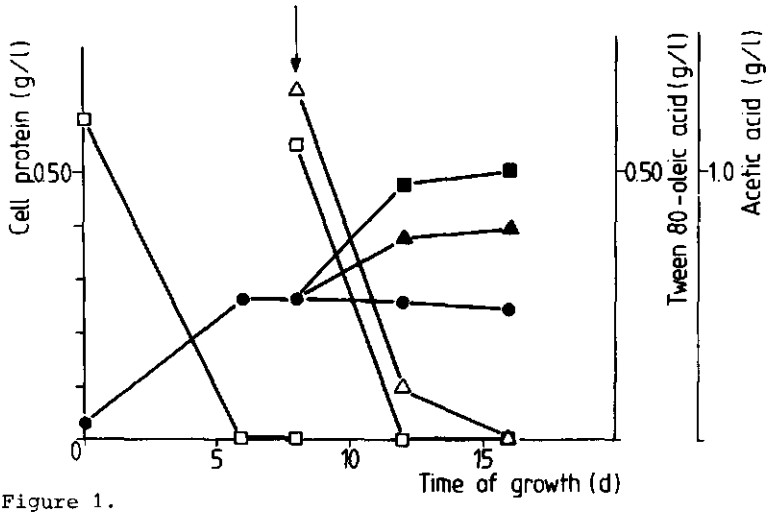

Figure 1.
Effect of substrate on protein content of *M. parvicella* grown in Cas medium supplemented with Tween 80 - oleic acid (0.66 g/l). After 8 days the following substrates were added (at arrow): none (●—●); Tween 80 - oleic acid (■—■) and acetic acid (▲—▲). Substrate concentration in medium: Tween 80 - oleic acid (□—□) and acetic acid (△—△). Media (100 ml) were inoculated with 10 ml of a culture grown for 10 days at 25°C in Cas medium supplemented with Tween 80 - oleic acid (0.96 g/l).

To test the hypothesis that certain cell components can be synthesized from oleic acid only, the increment of cell protein was determined after adding various amounts of Tween 80 - oleic acid to Cas medium containing acetic acid (2.3 g/l). Fig. 2 shows that the amount of cell protein increased with increasing amounts of added Tween 80 - oleic acid, whereas the acetic acid concentration decreased proportionally (Fig. 3). However, the data of Figs 2 and 3 do not allow an exact calculation of the cell yield derived from acetic acid because of the relatively high consumption of acetic acid in the culture without an additional amount of Tween 80. The amounts of added Tween 80 - oleic acid (Figs 2 and 3) were completely taken up by the cells of *M. parvicella* within 8 days; this uptake preceded the uptake of acetic acid (Fig. 4). The results given in Figs 2 - 4 are in agreement with earlier investigations which showed that Tween 80 - oleic acid was readily consumed by *M. parvicella*. This resulted in a pronounced accumulation of cell lipids (containing mainly oleic acid) in *M. parvicella* during the early growth phase. The stored lipids were subsequently converted into protein and other cell constituents so that cells of cultures grown for 10 days only contained a small amount of oleic acid (Slijkhuis et al., 1983).

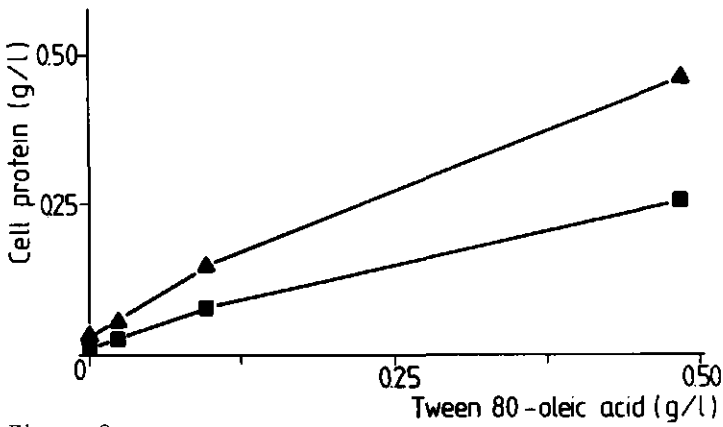

Figure 2.
Effect of Tween 80 - oleic acid on the protein content of *M. parvicella* grown for 8 days in Cas medium (■—■) and Cas medium supplemented with 2.3 g acetic acid/l (▲—▲). Inoculation as described under Fig. 1.

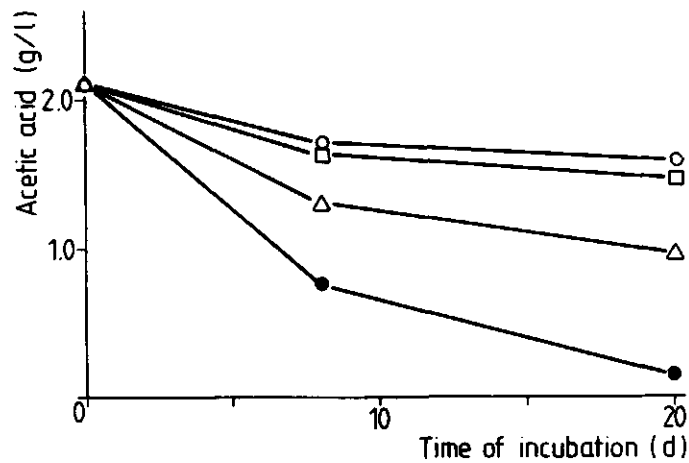

Figure 3.
Consumption of acetic acid by *M. parvicella* grown in Cas medium supplemented with acetic acid (2.3 g/l) and various amounts of Tween 80 - oleic acid viz. 0 (○—○); 0.1 g/l (□—□); 0.4 g/l (△—△) and 2.0 g/l (●—●). Inoculation as described under Fig. 1.

The above-described experiments clearly demonstrate the essential role of oleic acid in the physiology of *M. parvicella*. This long chain fatty acid provides the cells with a number of indispensable components whose presence enables growth on acetic acid and other short chain fatty acids.

The role of acetic acid in the biosynthesis and energy supply of *M. parvicella* has not been elucidated and the structures of the indispensable cell components are still unknown; it is suggested that acetic acid cannot be applied for the synthesis of long chain fatty acids.

The usual pathway of the degradation of long chain fatty acids depends on the β-oxidation, the formed acetyl-CoA being the intermediate in many biosynthetic pathways. As acetic acid is converted to acetyl-CoA, these reactions will also proceed in cultures with acetic acid as substrate. On the other hand the long chain fatty acids required for lipid synthesis are directly available for organisms growing on Tween 80 - oleic acid, whereas they must be synthesized from acetyl-CoA by growth on acetic acid.

The synthesis of long chain fatty acids is initiated by the condensation reaction of acetyl-CoA and malonly-CoA. After the reduction of the keto group the chain is elongated through condensation of the developing fatty acid with additional molecules of malonyl-CoA which is an important intermediate of the fatty acid-synthesizing system. A deficit of the enzym acetyl-CoA carboxylase, which catalyzes the condensation of bicarbonate and acetyl-CoA into malonyl-CoA, excludes the formation of long chain fatty acids and consequently the growth on short chain fatty acids.

The growth of *M. parvicella* on acetic acid was not stimulated by the addition of malonic acid to the culture medium (Table 3). The cell yield in a medium containing Tween 80 - oleic acid was also not increased by added malonic acid, suggesting that this acid was not taken up by the organism.

Glyoxylic acid plays an important role in many biosynthetic reactions involving acetyl-CoA. The addition of glyoxylic acid and its precursor glycolic acid had no stimulatory effect on the growth of *M. parvicella* in media with acetic acid nor in media with Tween 80 - oleic acid (Table 3). The latter results suggest that these acids too are not taken up by *M. parvicella*.

Table 3.

Cell dry weight of *M. parvicella* grown in Cas medium supplemented with various carbon substrates.

C sources in medium			Concentration of C sources in medium (g"C"/l)			Cell yield[1] (g dry weight/l)
1	2	3	1	2	3	
Tween 80			0.74[3]			1.20
Tween 80 + Ma[2]			0.74	0.35		1.10
Tween 80 + Gca			0.74	0.48		1.10
Tween 80 + Goa			0.74	0.48		1.15
Acetic acid			0.88			0.14
Acetic acid + Ma			0.88	0.35		0.15
Acetic acid + Gca			0.88	0.48		0.13
Acetic acid + Goa			0.88	0.48		0.06
Acetic acid+Ma+Gca			0.88	0.35	0.48	0.13
Acetic acid+Ma+Goa			0.88	0.35	0.48	0.06
Malonic acid			0.35			0.02
Glycolic acid			0.48			0.01
Glyoxylic acid			0.48			0.01

1) After 15 days at 25°C. Data were corrected for inoculum. Media (100 ml) were inoculated with 10 ml of a culture grown for 10 days in Cas medium supplemented with Tween 80 - oleic acid (0.96 g/l).

2) Ma = Malonic acid; Gca = Glycolic acid; Goa = Glyoxylic acid.

3) The oleic acid part of Tween 80 is reported.

Tween 80 or oleic acid has a favourable effect on the growth of various bacteria viz. species of *Mycobacterium* (Dubos and Davis, 1947), of *Corynebacterium* (Silliker et al., 1963), of *Streptococcus* (Deibel and Niven, 1955) and of *Lactobacillus* (Kitay and Snell, 1950). Oleic acid and related compounds act as a growth factor for these organisms but in most investigations their special role has not been elucidated. Tween 80 or oleic acid can replace biotin in media for *Lactobacillus casei* and various other bacteria (Williams and Fieger, 1946, 1947; Williams et al., 1947). *M. parvicella* requires Tween 80 - oleic acid at a high concentration to enable growth on short chain fatty acids or glycerol. Tween 80 does not replace biotin as this vitamin was added to the medium and the required amount of Tween 80 is too high to suppose a role as vitamin.

Tween 80 can be utilized by *M. parvicella* as the only source of carbon and energy and a clear dose response to Tween 80 was demonstrated. For the utilization of acetic acid as carbon and energy source Tween 80 - oleic acid is essential. Optimum growth on this short chain fatty acid requires an amount of at least 0.35-0.40 g of Tween 80 - oleic acid per g of acetic acid supplied.

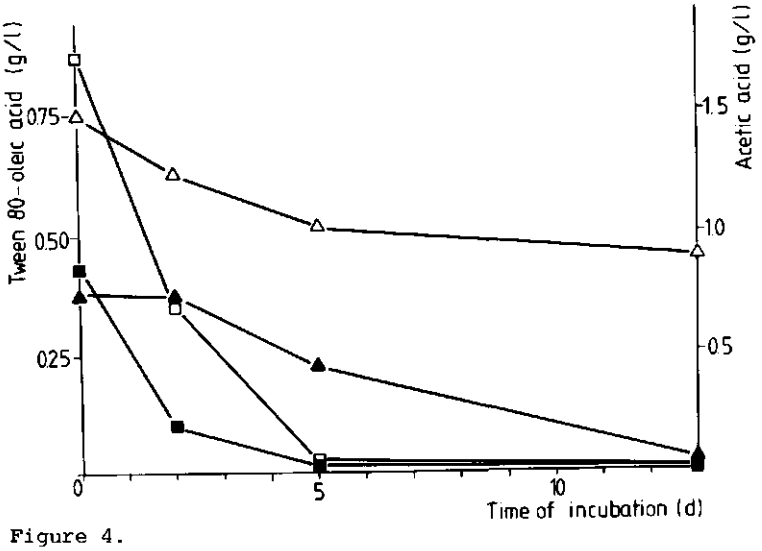

Figure 4.
Substrate concentration in media during growth of *M. parvicella* in Cas medium supplemented with: 0.96 g Tween 80 - oleic acid/l (□—□); 1.65 g acetic acid/l (△—△) and 0.48 g Tween 80 - oleic acid/l + 0.84 g acetic acid/l (oleic acid■—■; acetic acid ▲—▲). Inoculum as described under Fig. 1.

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6. *Microthrix parvicella*, a filamentous bacterium occuring in activated sludge; substrate respiration and effects of surface-active compounds.

INTRODUCTION

Abundant growth of the filamentous bacterium *Microthrix parvicella* in waste water purification plants of the activated-sludge type treating domestic waste water, often prevents the proper operation of the process (Eikelboom, 1982). Activated sludge is a mixed culture of mainly bacteria and the biological composition of the sludge is determined by the result of the competition between organisms for various substrates. The chemical composition of the waste water, the plant operation and the physiological and morphological characteristics of organisms are the most important factors affecting the result of the competition. In most purification plants the activated sludge is completely mixed with waste water and continuously fed with the waste water substrates. Therefore the concentration of substrates in the liquor is usually very low. Under such conditions, physiological characteristics, like the affinity for substrates and oxygen, affect the position of an organism in the competition to a great extent.

The variety of substrates which can be utilized by organisms is also of ecological importance. The range of carbon substrates utilized by *M. parvicella* is restricted. It comprises only long chain fatty acids, preferably in esterified form. Short chain fatty acids and glycerol can be utilized when added together with Tween 80 (soluble ester of oleic acid), whereas carbohydrates, amino acids, lactic, succinic and citric acids are not utilized at all. *M. parvicella* requires reduced-nitrogen and reduced-sulphur compounds for growth (Slijkhuis, 1983; Slijkhuis and Deinema, 1982).

Long chain fatty acids and Tween esters of fatty acids have surface-active properties. Such agents may have several effects on bacteria, usually including an inhibitory action on growth and respiration (Baker et al., 1941 a,b; Hugo, 1967). Inhibition of growth of *M. parvicella*, a Gram-positive bacterium, was observed with oleic acid at a concentration of 0.25 g

of oleic acid - C/1 (Slijkhuis, 1983) and in a preliminary investigation it was shown that the respiration of washed *M. parvicella* cells was inhibited by oleic acid at a concentration of 0.1 g of this compound/l (Slijkhuis and Deinema, 1982).

This report describes the results of experiments on substrate respiration of *M. parvicella* under various conditions. In additional experiments the effect of physical-chemical analogues on the growth and respiration of this bacterium was studied.

MATERIALS AND METHODS

Organism and culture conditions. Microthrix parvicella, strain B, and an *Acinetobacter* sp., strain 28, have been isolated from activated sludge of the oxidation ditch of Bennekom, The Netherlands.

M. parvicella was cultured at 25°C in Tween 80/Casamino acids medium (Medium D; Slijkhuis, 1983). This medium contains (g/l): Tween 80 (Sigma), 4; Casamino acids (Merck), 2; $(NH_4)_2SO_4$, 0.8; K_2HPO_4, 8.2; KH_2PO_4, 0.35; $MgSO_4.7H_2O$, 0.075; $CaCl_2$, 0.05; trace elements (mg/l): $FeCl_3.6H_2O$, 5; $MnSO_4.H_2O$, 3; $ZnSO_4.7H_2O$, 0.1; $CuSO_4.5H_2O$, 0.1; H_3BO_3, 0.1; $Na_2MoO_4.2H_2O$, 0.05; $CoCl_2.6H_2O$, 0.05; vitamin stock solution, 10 ml; pH 8. The vitamin stock solution (final pH adjusted to 3 with HCl) contains (mg/l): panthotenic acid, 10; nicotinamide, 10; pyridoxine, 10; *p*-aminobenzoic acid, 10; inositol, 10; thiamin, 10; riboflavin, 10; choline, 10; biotin, 10; cyanocobalamin, 0.5; folic acid, 0.5.

The *Acinetobacter* sp. was cultured at 25°C in butyrate medium (Deinema et al., 1980). This medium contains (g/l): Na butyrate, 2.3; yeast extract (Difco), 0.35; NH_4Cl, 0.5; $MgSO_4.7H_2O$, 0.05; KH_2PO_4, 0.15; tap water, 1000 ml.

Media were sterilized by autoclaving (15 min, 121°C); phosphates (sterilized separately) and vitamin stock solution (sterilized by Seitz filtration) were added to the cooled media.

Washed-cell suspensions. Cultures were centrifuged (10 min, 4,000 x g in a swing-out rotor) and the supernatant removed by decanting. The loosely packed pellet was filtered by membrane filtration and washed twice with NaCl solution (9 g/l). The residue was taken up in this solution to a final concentration of 2.5 g cell dry weight/l, unless otherwise stated. The

respiration activity at 25°C of this suspension, stored in ice water, did not decrease within 10 h.

Oxygen uptake. The oxygen uptake rate was determined in a Yellow Springs Instruments oxygen monitor; the vessel contained 5 ml culture or 4 ml washed-cell suspension and 1 ml 125 mM buffer solution (phosphate buffer in the range pH 5-8 and Tris-HCl buffer at higher pH values). The respiration experiments with washed-cell suspensions were carried out at 25°C and at pH 7.8, unless otherwise stated. Substrate respiration was determined by subtracting the endogenous oxygen uptake from the oxygen uptake measured after addition of 100 µl of a freshly prepared concentrated solution of substrate. Long chain fatty acids were dissolved in 1 M NaOH and heated in a water bath, if required. The affinity for oxygen was determined by switching the recorder to the appropriate range (full scale: 0-2% of oxygen saturation).

Analyses. Cell dry weight in cultures was estimated by determining the optical density at 620 nm with the aid of a calibration curve (Slijkhuis, 1983).

Washed-cell suspensions were disintegrated by ultrasonic treatment (Branson sonifier B-12; 2 x 1 min at 35 W) and protein determined by the method of Lowry as modified by Herbert et al. (1971) using bovine albumin serum as a standard. Blanks with the disintegrated cell suspensions but without reagents had to be included because of light scattering due to lipids present in the cells.

RESULTS

The oxygen uptake rate of a culture of *M. parvicella* incubated in medium D increased with time to a maximum value of 0.35 µmol O_2/ml of culture.h, followed by a slow decrease at a prolonged incubation period (Fig. 1). Addition of the growth-limiting substrate (Tween 80) during the early growth phase hardly stimulated the oxygen consumption rate. The maximum response to Tween 80 added to the culture sample was obtained with a culture incubated for 8 to 11 days. Similar results were obtained with washed-cell suspensions prepared from these cultures (Table 1). From these results it was decided to determine the oxygen uptake rates with

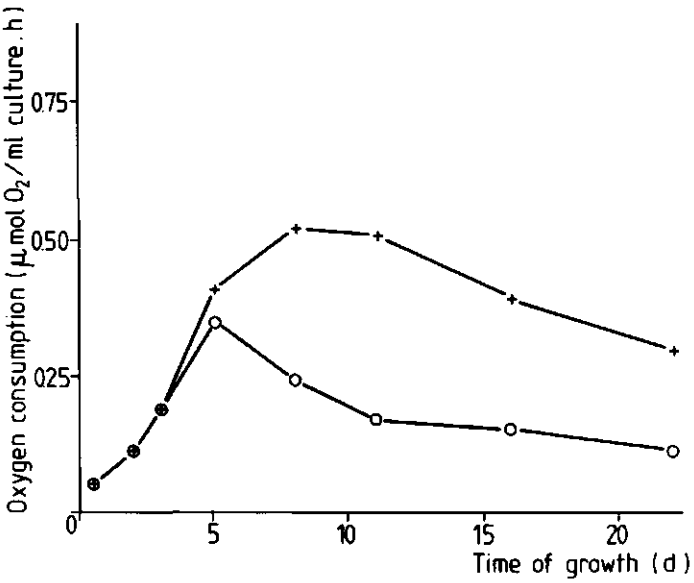

Figure 1.
Oxygen consumption rate of samples (5 ml) taken from a culture of *Microthrix parvicella* grown in medium D after various incubation periods. Respiration rate of culture, 0——0; respiration rate after addition of the growth-limiting substrate, 4 g Tween 80/l, +——+. Medium (200 ml) had been inoculated with 20 ml of a culture grown for 10 days in medium D.

washed-cell suspensions of *M. parvicella* grown for 10 days in medium D. The maximum Tween 80 respiration rate was not equal with all washed-cell suspensions but varied from 1.0 to 1.6 µmol O_2/mg protein.h. As the cultivation conditions were identical, this phenomenon could not be explained; therefore the respiration of Tween 80 (4 g/l) was determined as a control in most experiments.

The oxygen consumption rate depends on the concentration of the added substrate. The maximum value of a washed-cell suspension containing 2 g of cell dry weight/l resulting from added substrate was obtained at concentrations of 0.5 g Tween 80/l and above. The oxygen uptake rate (V_{O_2}) depends on the substrate concentration (S) as given by equation (1):

$$V_{O_2} = (V_{O_2})_{max} \frac{S}{K_S + S} \qquad (1)$$

in which K_S is the substrate constant, the substrate concentration at which the respiration rate is half the maximum value. Equation (1) may be written as:

$$\frac{1}{V_{O_2}} = \frac{K_S}{(V_{O_2})_{max}} \cdot \frac{1}{S} + \frac{1}{(V_{O_2})_{max}} \qquad (2)$$

Table 1.

Oxygen consumption rates of samples taken from a *Microthrix parvicella* culture grown for various periods in medium D.

Incubation period (h) of culture	Cell protein in culture (mg/l)	Oxygen consumption rate (µmol O_2/mg protein.h)			
		Culture		Washed cells	
		R[a)]	S[b)]	E[c)]	S
8	31	1.64	1.64	1.25	1.25
40	52	2.23	2.23	1.63	1.71
65	90	2.20	2.22	1.58	2.07
115	172	2.05	2.38	1.45	1.97
190	296	0.83	1.75	0.41	1.38
260	309	0.55	1.64	0.27	1.25
370	286	0.56	1.37	0.26	0.95
520	294	0.37	1.04	0.20	0.91

a) Respiration rate of culture.

b) Substrate respiration rate, determined after the addition of 4 g Tween 80/l.

c) Endogenous respiration rate.

The oxygen uptake rates measured at various Tween 80 concentrations are plotted (Fig. 2) according to equation (2). The apparent K_S value for Tween 80 was about 15 mg/l. As only the oleic acid moiety of Tween 80 was found to be utilized by *M. parvicella* (Slijkhuis, 1983), a similar response might be expected when oleic acid was used as the substrate. However, oleic acid concentrations exceeding 50 ml/l inhibited the respiration to some extent and washed cells were completely inhibited at an oleic acid concentration of 80 mg/l. *M. parvicella* respired oleic acid at a maximum rate when it was added at a concentration of 40 mg/l; its apparent K_S value is approximately 8 mg/l (Fig. 2).

The respiration of Tween 80 and oleic acid was pH-dependent (Fig.3). Washed cells respired these substrates at maximum rates in suspensions buffered at pH 8-9.

Oleic acid (80 mg/l) inhibited the respiration of *M. parvicella* but the concentration of oleic acid causing inhibition depended on the concentration of washed cells in the vial. The inhibitory concentration of oleic acid was proportional to the cell density (Table 2).

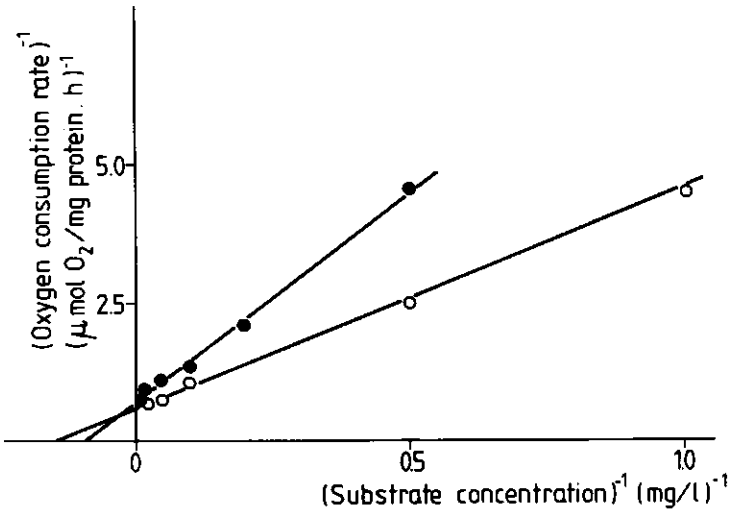

Figure 2.
Substrate respiration rates of washed cells of *Microthrix parvicella* at various substrate concentrations. Substrates: oleic acid, 0——0 and Tween 80, ●——●. *M. parvicella* had been grown for 10 days in medium D at 25°C.

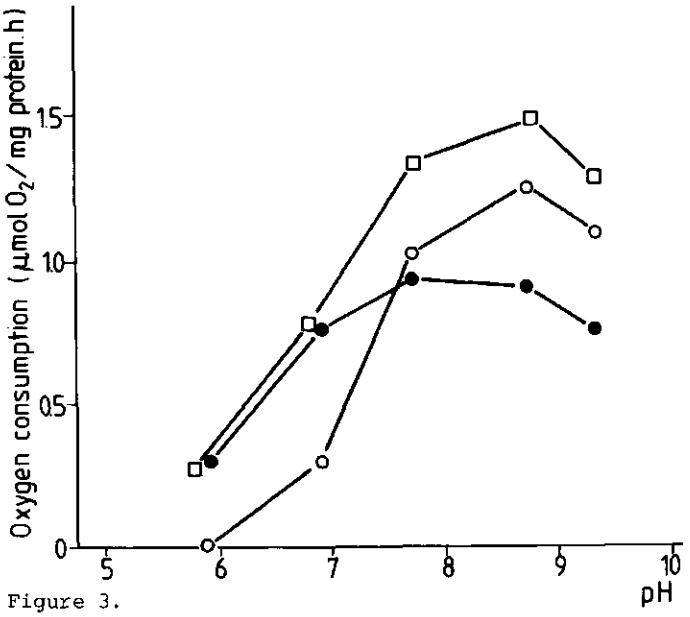

Figure 3.
Substrate respiration rates of washed-cell suspensions of *Microthrix parvicella* at various pH values. Substrates: 50 mg oleic acid/l, O—O; 5 mg oleic acid/l, ●—●; 4 g Tween 80/l, □—□. *M. parvicella* had been grown for 10 days in medium D at 25°C.

Table 2.

Inhibitory concentration of oleic acid in relation to density of *Microthrix parvicella* cells in suspension.

Cell dry weight of washed cells (g/l)[a]	Final oleic acid concentration (mg/l)					
	5	10	20	40	80	160
0.25	-[b]	+[c]	+	+	+	+
0.50	-	-	+	+	+	+
1.0	-	-	-	+	+	+
2.0	-	-	-	-	+	+
4.0	-	-	-	-	-	+

a) Washed cells contained 0.31 g protein/g cell dry weight.

b) No inhibition: initial oxygen uptake rate higher than (equal to) initial respiration rate at lower oleic acid concentration.

c) Inhibition: initial oxygen uptake lower than initial respiration rate at lower oleic acid concentration.

The response of the respiration of *M. parvicella* to various organic compounds was as follows. The oxygen consumption increased after the addition of fatty acids and soluble esters of long chain fatty acids (Tweens). Simple organic acids like succinic and citric acids, carbohydrates and a mixture of amino acids did not increase the oxygen uptake rate. The oxygen consumption rates were determined at pH 8 (Table 3) as well as at pH 6. At the latter value, respiration rates of the fatty acids and Tweens were considerably lower than at pH 8. Also at pH 6 the oxygen uptake rate did not respond to the addition of the other compounds.

Tweens and fatty acids are surface-active compounds which lower the surface tension of the suspension and accumulate at the cell surfaces. The effect of a number of surfactants on the growth of *M. parvicella* in medium D and the respiration of washed cells was studied (Tables 4 and 5). The anionic surfactant, sodium dodecylsulphate (SDS) added at a concentration of 1.0 g/l to medium D, completely inhibited the growth of *M. parvicella*. However, at a concentration of 0.05 g/l, SDS slightly increased the cell yield in medium D. Cell yield was considerably decreased by non-ionic surfactants containing a lipophilic and a hydrophilic moiety (Triton X-100, Brij 96, Brij 99) supplemented to the medium at the concentration of 4 g/l. At 0.2 g/l, two of these compounds were inactive, as was the case with the non-ionic surfactant polyoxyethylene at both concentrations (Table 4). The oxygen uptake of washed cells was considerably stimulated by these surfactants, polyoxyethylene excepted (Table 5). The increase of the respiration rate of washed cells in the presence of one of the surfactants (2.5 g/l) by subsequent addition of Tween 80 is given in Table 6.

Tween 80 contains approximately 24% oleic acid and complete oxidation of 1 µmol oleic acid stoichiometrically requires 25.5 µmol O_2. The oxygen demand for the oxidation of the oleic acid moiety of 1 mg Tween 80 is 22 µmol O_2. However, an experimental determination resulted in an oxygen consumption of only 10-30% of this value (Fig. 4). After the addition of Tween 80 at a low concentration the respiration rate of washed cells returned almost entirely to the endogenous value. Similar experiments with oleic acid and SDS showed identical results but the stimulation by Triton X-100, Brij 96 and Brij 99 at low concentrations only slowly decreased with time (Table 7).

Table 3.

Respiration of various organic compounds by washed-cell suspensions of *Microthrix parvicella* grown for 10 days in medium D.

Substrate	Oxygen uptake rate[a] (μmol O_2/mg protein.h)			
	Substrate concentration in vial (g/l)			
	0.01	0.10	1.0	2.5
Tween 80 (polyoxyethylene-sorbitan mono-oleate)	0.8	1.5	1.6	1.6
Tween 60 (polyoxyethylene-sorbitan mono-stearate)	0.7	0.9	1.1	1.1
Tween 40 (polyoxyethylene-sorbitan mono-palmitate)	0.7	1.1	1.2	1.3
Tween 20 (polyoxyethylene-sorbitan mono-laurate)	0.6	1.0	1.0	1.0
Oleic acid	1.0	-[b]	-	-
Stearic acid	1.1	1.8	nd[c]	nd
Palmitic acid	1.2	1.7	nd	nd
Lauric acid	0.9	1.1	nd	nd
Caprinic acid	0.6	0.8	nd	nd
Caprylic acid	0.7	0.8	0.8	nd
Caproic acid	0.5	0.6	0.7	nd
Butyric acid	0.3	0.3	0.3	0.3
Acetic acid	0.1	0.2	0.3	0.3

The following compounds were found to have no effect at these concentrations on the oxygen consumption: fructose, glucose, sorbitol, citric acid, succinic acid, lactic acid and Casamino acids.

a) Corrected for endogenous respiration rate.

b) Inhibition of the oxygen consumption.

c) Not determined.

Table 4.

Effect of various surfactants on the growth yield of *Microthrix parvicella* in medium D.

Surfactant	Concentration (g/l)	Cell yield[a] (g cell dry weight/l)
-	-	1.30
Oleic acid	0.05	1.37
Sodium dodecylsulphate	0.05	1.35
	1.0	0.15
Triton X-100	0.05	1.27
	0.20	0.92
	4.0	0.40
Polyoxyethylene (10) oleyl ether (Brij 96)	0.2	1.30
	4.0	0.60
Polyoxyethylene (20) oleyl ether (Brij 99)	0.2	1.30
	4.0	0.77
Polyoxyethylene 400	0.2	1.30
	4.0	1.30

a) Determined after incubation for 10 days at 25°C.

Table 5.

Effect of various surfactants on the oxygen uptake rate of washed cells of *Microthrix parvicella*.

Surfactant	Respiration rate[a] (μmol O_2/mg protein.h)			
	Concentration in vial (g/l)			
	0.01	0.10	1.0	2.5
Tween 80	nd[b]	nd	nd	1.45
Oleic acid	0.95	--[c]	--	--
Sodium dodecylsulphate	1.04	1.04	--	--
Triton X-100	0.02	0.13	0.45	0.56
Polyoxyethylene (10) oleyl ether (Brij 96)	0.58	1.28	1.60	1.60
Polyoxyethylene (20) oleyl ether (Brij 99)	0.13	0.71	1.15	1.36
Polyoxyethylene	0.0	0.0	0.0	0.0

a) Values of initial rates after surfactants addition corrected for endogenous respiration.

b) Not determined.

c) Inhibition of the oxygen consumption.

Table 6.

Increase of the oxygen consumption of washed cells of *Microthrix parvicella* by Tween 80 after addition of various non-utilizable surfactants.

Surfactant	Concentration in vial (g/l)	Oxygen uptake rate after addition of 2.5 g Tween 80/l [a] (µmol/mg protein.h)
-	-	1.45
Triton X-100	2.5	1.36
Polyoxyethylene(10) oleyl ether (Brij 96)	2.5	1.86
Polyoxyethylene(20) oleyl ether (Brij 99)	2.5	1.73
Polyoxyethylene 400	2.5	1.21

[a] Tween 80 added 4 min after the addition of the surfactant; data corrected for endogenous respiration.

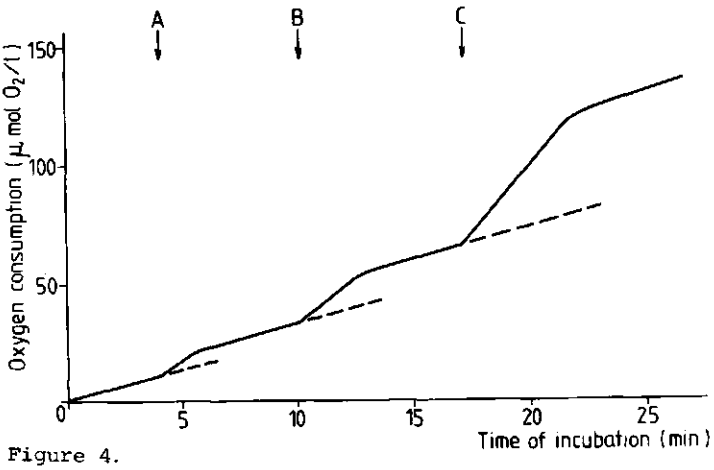

Figure 4.

Response of the oxygen consumption of a washed cell suspension of *Microthrix parvicella* to added substrate. Additions of Tween 80: 1 mg/l (A); 5 mg/l (B) and 25 mg/l (C). Suspension contained 0.66 g protein/l and *M. parvicella* had been grown for 10 days in medium D at 25°C.

Table 7.

Effect of various surface-active compounds at low concentrations on the oxygen uptake rates of washed cells of *Microthrix parvicella*.

Surface-active compound	Concentration in vial (mg/l)	Time after surfactant addition (min)	Oxygen uptake[a] (µmol O_2/ mg protein.h)
Oleic acid	0.4	0	0.30
		5	0.02
	2.0	0	0.50
		7	0.07
	10.0	0	1.30
		10	0.14
Sodium dodecyl-sulphate	0.4	0	0.29
		5	0.00
	2.0	0	0.91
		7	0.01
	10.0	0	1.12
		10	0.16
Tween 80	25	0	1.20
		10	0.15
Triton X-100	25	0	0.05
		10	0.05
Polyoxyethylene (10) oleyl ether (Brij 96)	25	0	0.87
		10	0.87
		25	0.63
Polyoxyethylene (20) oleyl ether (Brij 99)	25	0	0.43
		10	0.43
		25	0.36

a) Corrected for endogenous respiration.

The inhibitory action of oleic acid (200 mg/l) on the respiration of washed *M. parvicella* cells (2 g cell dry weight/l) was prevented by the addition of various surfactants and of Ca and Mg ions. However, once cell respiration had been inhibited by oleic acid, it was not restored within 10 min by a subsequent addition of the above-mentioned surfactants or of Ca and Mg ions (Table 8).

Table 8.
Inhibitory action of oleic acid on the oxygen uptake of *Microthrix parvicella* as affected by surfactants and cations.

Additions				Oxygen uptake rate[a] (μmol O_2/mg protein.h)	
A	Concentration in vial (g/l)	B[b]	Concentration in vial (g/l)	A	B[b]
Tween 80	2.5	Oleic acid	0.2	1.00	1.35
Oleic acid	0.2	Tween 80	2.5	-[c]	-
SDS[d]	0.1	Oleic acid	0.2	0.95	-
Oleic acid	0.2	SDS	0.1	-	-
Triton X-100	2.5	Oleic acid	0.2	0.73	1.22
Oleic acid	0.2	Triton X-100	2.5	-	0.06
Brij 96[d]	2.5	Oleic acid	0.2	1.32	1.54
Oleic acid	0.2	Brij 96	2.5	-	0.22
Polyoxyethylene 400	2.5	Oleic acid	0.2	0.00	-
Oleic acid	0.2	Polyoxyethylene 400	2.5	-	-
$MgCl_2$[e]	0.25	Oleic acid	0.2	0.00	1.39
Oleic acid[e]	0.2	$MgCl_2$	0.25	-	-
$CaCl_2$[e]	0.25	Oleic acid	0.2	0.00	1.22
Oleic acid[e]	0.2	$CaCl_2$	0.25	-	-

a) Corrected for endogenous respiration.
b) Added 4 min after first addition (A).
c) Inhibition of the oxygen consumption, i.e. below initial value for endogenous respiration.
d) SDS = Sodium dodecylsulphate; Brij 96 = Polyoxyethylene (10) oleyl ether.
e) Determined in 25 mM Tris-HCl buffer pH 7.8.

M. parvicella is a strictly aerobic bacterium but even at a very low oxygen concentration, the organism was found to consume oxygen at a maximum rate. Under the conditions of the experiment, the consumption rate was only reduced to half the maximum value at an oxygen concentration in the liquor of less than 0.5 µmol/l. The determination of the apparent K_S value for oxygen of an *Acinetobacter* sp., another typical activated sludge bacterium, resulted in a value of 2.6 µmol O_2/l. The results of these experiments (Fig. 5) were calculated according to equation (2). In this figure the data on carbon-substrate respiration are plotted but the K_S values calculated from the data on the endogenous respiration rate at various oxygen concentrations resulted in the same values of the K_S for oxygen.

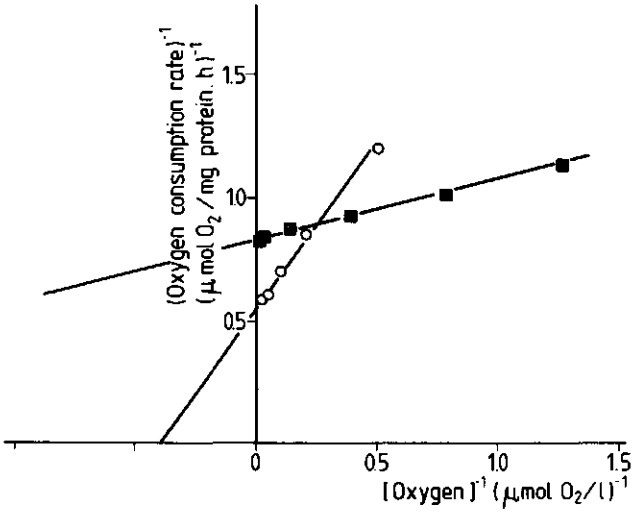

Figure 5.
Oxygen uptake rates of washed-cell suspensions of *Microthrix parvicella* and of an *Acinetobacter* sp. at various oxygen concentrations. Oxygen uptake by *M. parvicella* in the presence of 4 g Tween 80/l, ■——■ and of an *Acinetobacter* sp. in the presence of 2 g Na butyrate/l, O——O. *M. parvicella* had been grown for 10 days in medium D at 25°C and the *Acinetobacter* sp. had been grown for 4 days in butyrate medium at 25°C.

DISCUSSION

The oxygen uptake of *Microthrix parvicella* during the early growth phase only slightly increased upon the addition of Tween 80. This result might have been due to extracellular Tween 80 because the medium is not completely exhausted after incubation for 5 days (Slijkhuis et al., 1983). However, a washed-cell suspension of a young culture also showed hardly any substrate respiration. The endogenous respiration of washed cells of a young culture was high (Table 1) and it may be assumed that the fatty acids accumulating in the cells during the early growth phase (Slijkhuis et al., 1983) acted as an internal substrate for *M. parvicella*. Cell activity determined as oxygen uptake per mg protein had a maximum value during the early growth phase but these cells could not be applied for respiration experiments due to the high endogenous respiration.

Tween 80 and oleic acid added at low concentrations to washed cells grown for several days in medium D were readily utilized. The apparent K_S values of these substrates were very low, probably resulting from their surface-active properties, so that their concentration at the cell surface is considerably higher than in the ambient fluid. Because of the surface activity of Tween 80 and oleic acid, it is not feasible to determine K_S values. These values will not be constant, but depend on cell density. The K_S values for these substrates will increase with increasing cell density and it is more feasible to calculate the substrate to biomass ratio at which the oxygen uptake rate is half the maximum value. This substrate to biomass ratio of *M. parvicella* for oleic acid and Tween 80 was found to be 4 and 8 mg substrate/g of cell dry weight, respectively. Evidently, the apparent K_S values of the substrates may not be compared with values of other substrates for bacteria.

Oleic acid added to a washed-cell suspension (2 g of cell dry weight/l) at a concentration exceeding 50 mg/l inhibited the respiration of this suspension as it also inhibited the growth of *M. parvicella* in a medium containing 0.25 g of oleic acid - C/l (Slijkhuis, 1983). Both experiments cannot be compared as cell density in the experiments reported in this paper is much higher than in an inoculated medium and the protective action of Ca and Mg ions present in medium D is lacking in washed-cell suspensions.

Inhibition of growth and respiration of microorganisms by surfactants has often been reported. Cationic surfactants are bactericidal to a large

variety of species whereas anionic surfactants are effective upon Gram-positive bacteria (Hotchkiss, 1946). Many reports deal with the inhibitory action of long chain fatty acids on tubercle bacteria and other Gram-positive bacteria (Bergström et al., 1946; Boissevain, 1926; Minami, 1957; Karlson, 1954; Galbraith and Miller, 1973a,b,c; Willet and Morse, 1966; Laser, 1952; Wynne and Foster, 1950; Camien and Dunn, 1957; Kodicek, 1949).

The respiration rate of oleic acid by *M. parvicella* decreased more rapidly with pH at 50 than that at 5 mg/l indicating that cell respiration was inhibited more effectively by undissociated oleic acid than by oleate (Fig. 3). In agreement with this observation the insoluble salts (Ca oleate, Mg oleate) were not inhibitory at all (Table 8). The ratio oleic acid to oleate in the suspension decreases with increasing pH, but the value of this ratio at various pH values cannot be calculated because oleic acid is insoluble in water. Similar results concerning the inhibitory effects of fatty acids and fatty acid salts on Gram-positive bacteria were obtained by Eggerth (1926) and prevention of bactericidal action of various long chain fatty acids by Ca and Mg ions was reported by Galbraith and co-workers (1971, 1973a).

Bergström et al. (1946), Laser (1952) and Karlsson (1954) reported that the bactericidal activity of fatty acids depended on the ratio of the quantity of these acids to the amount of biomass present, rather than on the calculated concentration in the medium, because of the adsorption of the fatty acid onto the cells. This phenomenon also occurs with oleic acid and *M. parvicella* (Table 2).

The oxygen uptake by washed cells of *M. parvicella* due to added Tween 80 at a low concentration was very low; the amount of oxygen taken up relative to the quantity of Tween 80 supplied decreased with increasing substrate supply (Fig. 4). The major part of the consumed substrate is presumably stored within the cells and therefore protected against ready oxidation. As has been observed in young growing cultures of the organism, the oleic acid moiety of Tween 80, was readily taken up and stored in the cells as lipids. In the growing cells the content of internal lipids in the biomass subsequently decreased whereas the content of cell protein increased (Slijkhuis et al., 1983).

The non-ionic surfactants with a hydrophilic as well as a lipophilic moiety (Triton X-100, Brij 96, Brij 99) which are not utilized by *M. parvicella* are not bactericidal to this bacterium, although at high concen-

trations they decreased cell yield on Tween 80 (Table 4). On the other hand, the oxygen uptake by washed cells was activated by these surfactants (Table 5). As the stimulation was not due to the oxidation of these compounds, the increased oxygen uptake must have been due to the activated oxidation of endogenous substrates. This effect might have depended on the uncoupling of the oxydative phosphorylation as has been reported for surface-active compounds (long chain fatty acids) on mitochondria (Borst et al., 1962). The chemical structure of Tween 80 resembles the structure of Brij 99 and the activation of the oxygen uptake rate by Tween 80 is presumably not only due to substrate respiration, but also to a non-specific surfactant activation. The latter activation cannot be quantified as it depends on the concentration as well as on the type of the surfactant. The increased oxygen uptake by Tween 80 after a maximum stimulation by various non-ionic surfactants depends also on the type of surfactant (Table 6). The anionic surfactants like long chain fatty acids and SDS may have similar effects on *M. parvicella*, although it could not be concluded from the data on oleic acid and SDS respiration. SDS is probably metabolized by *M. parvicella* as it acted like oleic acid, increasing cell yield on medium D (Table 4) and activating the oxygen uptake rate only during a short period of time after supplying it (Table 7).

In view of the stimulating effects of non-respirable surfactants on oxygen uptake, the response to various fatty acids is thought to be not only based on substrate respiration. The decrease of oxygen uptake rate with chain length of fatty acids may be caused by a decreased substrate affinity of various enzymes involved in fatty acid oxidation, in particular the fatty acid activating enzyme (Table 3). However, the involvement of decreasing surface activity with decreasing chain length should not be excluded. The absence of oxygen uptake following the supply of Casamino acids indicates that the amino acids in medium D are hardly utilized by *M. parvicella*. This conclusion agrees with the result of growth studies (Slijkhuis et al., 1983). *M. parvicella* fails to respire carbohydrates and simple organic acids like citric and succinic acids; these results are in agreement with the observed absence of yield response to these compounds (Slijkhuis, 1983).

The low apparent K_S for oxygen of *M. parvicella* may be an important characteristic as in activated-sludge plants, the habitat of this bacterium, oxygen is often present in a limited amount. Comparison with the K_S

values for oxygen of other typical activated-sludge organisms is impossible as such data are not available. The apparent K_S of an *Acinetobacter* sp., isolated from activated sludge, was at least 5 times the concentration as estimated for *M. parvicella*.

A remarkable result of the present investigation, the promotion of the oxygen uptake by *M. parvicella* by non-ionic, non-utilizable surfactants, may contribute to the better understanding of the unusual lipid accumulation in the cells during the early growth phase. Assuming that Tween 80 would act like Brij 96 and Brij 99, viz. stimulating endogenous respiration for a prolonged period (Table 7), the inoculated cells would be exhausted of endogenous substrates when Tween 80 uptake would proceed slowly (e.g. proportional to protein production). However, when Tween 80 is readily taken up and hydrolyzed and the liberated oleic acid temporarily stored in the cells in the form of neutral lipids, the non-specific surfactant effect of Tween 80 would be rapidly reduced (Table 7). The oleic acid moiety of Tween 80, the nutritional part of Tween 80, is preserved for growth and the remaining part, mainly polyoxyethylene does not affect the growth and respiration of *M. parvicella* (Tables 4,5). The requirement for a large inoculum of *M. parvicella* in media containing 4 g of Tween 80/l may also be based on this phenomenon, as it reduces the ratio of Tween 80 to inoculated biomass and consequently the unfavourable effect of this compound.

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Effect of environmental conditions on the occurrence of the filamentous bacterium *Microthrix parvicella* in activated sludge.

INTRODUCTION

The occurrence of bulking sludge is a severe problem in the operation of activated-sludge plants. The activated sludge of more than 50% of the plants included in the survey of 65 plants in Great Britain had poor settling properties (Tomlinson, 1976). Bulking of the sludge usually depends on abundant growth of filamentous organisms. The type of filamentous organism occurring in activated sludge may vary widely. More than 30 types have been detected by Eikelboom (1975) in this environment, but only few of them are frequently encountered in bulking sludge as the predominant organism.

Microthrix parvicella is the filamentous organism that predominates in bulking sludge of low-loaded activated-sludge plants (e.g. oxidation ditches) served with domestic sewage. When dairy waste water is supplied in such plants under similar conditions, bulking is often caused by *Haliscomenobacter hydrossis* (Eikelboom, 1982). *Sphaerotilus natans* and a filamentous organism described by Eikelboom as type 021N are usually the bacteria responsible for bulking sludge of conventional purification plants with a moderate or high sludge loading (Eikelboom, pers comm).

From a superficial point of view the growth of filamentous bacteria in activated sludge may be explained by the general supposition that these organisms have advantage over floc-forming types because they protrude from the flocs into the mixed liquor. Therefore, substrates and oxygen have access to the filaments more rapidly than to organisms growing in the centre of a floc. However, the competition between organisms for substrates and oxygen depends not only on the morphological structure of the organism but also on the composition of the waste water (types of substrate), on plant parameters (oxygen supply, sludge loading rate) and on the physiological characteristics of the activated-sludge organisms. The relation between physiological characteristics and growth in activated sludge of filamentous organisms has only rarely been demonstrated as only

few of such organisms have been studied in pure culture.

Sphaerotilus natans has been investigated extensively in pure culture. The organism prefers organic nitrogen compounds for growth and, although being an aerobic bacterium, is able to grow at a very low oxygen concentration as it was observed by cultivating the organism in non-aerated nutrient medium. This was in contrast to *Arthrobacter globiformis* that grew very poorly under such conditions (Mulder, 1964). Houtmeyers (1978) demonstrated that *S. natans* predominated over an *Arthrobacter* sp. when grown in a continuous culture with glucose as the limiting substrate. These results may partly explain the dominating occurrence of *S. natans* in a number of activated sludge plants. Excessive growth of this organism was reported in activated sludge supplied with a limited amount of oxygen (Ruchhoft and Kachmar, 1941), in systems fed with waste water containing a high concentration of carbohydrates (Lackey and Wattie, 1958), with a high carbon to nitrogen ratio (Hattingh, 1963), in the presence of organic nitrogen compounds (Harisson and Heukelekian, 1958) or fed with waste water at a high sludge loading rate (Rensink, 1974). Reduced oxygen supply and high C/N ratio of dairy waste were responsible for bulking activated sludge caused by *S. natans* in laboratory experiments carried out by Adamse (1968).

Haliscomenobacter hydrossis was studied in pure culture and in a mixed culture with *Zoogloea ramigera* (Krul, 1977; van Veen et al., 1982) but the abundant growth of this bacterium in activated sludge plants served with dairy waste water cannot be explained by their results. Dairy waste water contains mainly milk components (lactose, protein and fats) and the effect of these substrates and of oxygen on the growth of *H. hydrossis* has not been studied.

Beggiatoa and *Thiothrix* spp. often show sulphur globules in the cells and their occurrence in activated sludge can be explained by the presence of sulphides in the mixed liquor derived from the waste water or from reduction of sulphates in the sludge flocs during poor oxygen supply (unpublished results).

The physiology of *Microthrix parvicella* grown in axenic culture was extensively studied in this laboratory (Slijkhuis, 1983a,b,c; Slijkhuis and ten Brummeler, 1983; Slijkhuis et al., 1983). This organism was found to utilize only long chain fatty acids, preferably in esterified form, as carbon and energy source. In batch culture the carbon substrate (oleic

acid) was stored in esterified form as lipid globules during the early growth phase. Simple organic compounds, like glucose, fructose, succinic and citric acids, amino acids, which are excellent substrates for micro-organisms were not utilized and short chain fatty acids like acetic and butyric acids and glycerol were utilized only if supplied along with a long chain fatty acid. *M. parvicella* requires ammonium nitrogen for growth, nitrates are not utilized. Sulphur is assimilated only if it is supplied as a reduced S compound like L-methionine, L-cysteine, sodium thiosulphate or sodium sulphide. *M. parvicella* is a strictly aerobic bacterium; it was found to have a high affinity for oxygen as well as for carbon substrates. The cell yield on oleic acid was high (1.4 g cell dry weight/g of oleic acid consumed) and its maximum specific growth rate was approximately 0.06 h^{-1}.

M. parvicella often causes bulking problems in oxidation ditches served with domestic sewage (Eikelboom, 1982). Municipal waste water contains a considerable amount of fatty matter, originating from garbage, human excrements and kitchen waste. The concentration of grease in this type of sewage varies considerably, values from 16 to 200 mg/l (Mahlie, 1940) and from 23 to 52% of the organic matter in sewage (Hunter and Heukelekian, 1965) being reported. From this survey it may be concluded that the requirement of *M. parvicella* for fatty acids is met with in an activated sludge process served with domestic sewage. However, various other parameters will affect the growth of *M. parvicella* in sludge as this organism is not the predominant organism in all plants served with domestic waste water.

This report deals with the effect of nutrient and oxygen supply on the growth of *M. parvicella* in the activated sludge of an experimental plant.

7.2 MATERIALS AND METHODS

Organism and growth conditions. M. parvicella, strain B, was isolated from activated sludge of the oxidation ditch of Bennekom, The Netherlands. Stock cultures were kept on slants of medium A agar. The organism was cultivated in shake cultures containing medium A at 25°C for 8-10 days. The media were inoculated with 10% of a full-grown culture (Slijkhuis, 1983a).

Media. Media of varying composition were used to study the growth of *M. parvicella* on agar plates. G agar contained per liter of distilled water: Tween 80 (polyoxyethylenesorbitan mono-oleate, Sigma), 0.25 g; $(NH_4)_2SO_4$, 0.8 g; $Na_2S_2O_3.5H_2O$, 0.12 g; $MgSO_4.7H_2O$, 0.075g; $CaCl_2$, 0.05 g; $FeCl_3.6H_2O$, 5 mg; $MnSO_4.H_2O$, 3 mg; $ZnSO_4.7H_2O$, 0.1 mg; $CuSO_4.5H_2O$, 0.1 mg; H_3BO_3, 0.1 mg; $Na_2MoO_4.2H_2O$, 0.05 mg; $CoCl_2.6H_2O$, 0.05 mg; phosphate solution, 100 ml; vitamin stock solution, 10 ml; agar, 7.5 g; pH = 8. The phosphate solution contained per 1: K_2HPO_4, 82 g and KH_2PO_4, 3.5 g; the vitamin stock solution (adjusted to pH 3 with HCl) contained per liter: thiamin, 10 mg and cyanocobalamin, 0.5 mg. The medium was sterilized by autoclaving (15 min, 121°C); phosphates (sterilized separately) and vitamins (sterilized by Seitz filtration) were added to the agar medium after cooling to 60°C. H agar: the concentration of Tween 80 was decreased (0.10 g). I and J agar: Tween 80 was replaced by Na oleate, 55 mg and 22 mg, respectively. Agar media G', H', I' and J' contained bovine albumin serum (5 mg/l), sterilized according to Dubos and Middlebrook (1947).

Washed-cell suspensions. Cultures grown in medium A for 8-10 days were centrifuged (20 min at 4000 x g) and the supernatant partly removed by decanting. The loosely packed pellet was filtered by membrane filtration and washed twice with NaCl solution (2.5 g/l). The biomass of 1 l culture (about 1.3 g dry weight) was taken up in 100 ml of this solution.

Activated sludge. Activated sludge was obtained from the oxidation ditch of Bennekom. The sludge was sieved (mesh width, 4 mm) to prevent blocking of the sludge recycling tubes. The oxidation ditch is supplied with only domestic sewage from the community of Bennekom (population 16,500) at a sludge loading rate of 0.06 g COD/g dry solids.d.

Pilot plants. Two plants (Fig. 1) were installed near the oxidation ditch. The influent of pilot plant A was untreated (raw) sewage. The influent of plant B was settled sewage passed through an inclined parallel plates grease separator to decrease the amount of fatty matter of the waste water (Fig. 2). The maximum hydraulic loading rate of the grease separator was 2 $m^3/m^2.h$. The plants were operated for 1 year at two sludge loading rates. The activated sludge was completely mixed with the waste water by means of a diffused-air system. The oxygen concentration of the mixed liquor did not drop below 5 mg/l during the experiments and the pH was

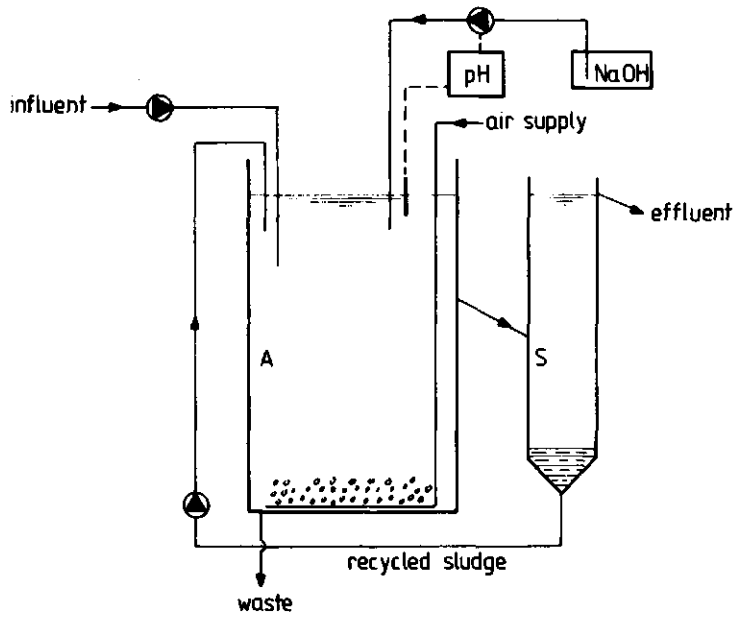

Figure 1.
Outline of a pilot scale activated-sludge plant. A = aeration tank (280 l); S = settling tank (24 l).

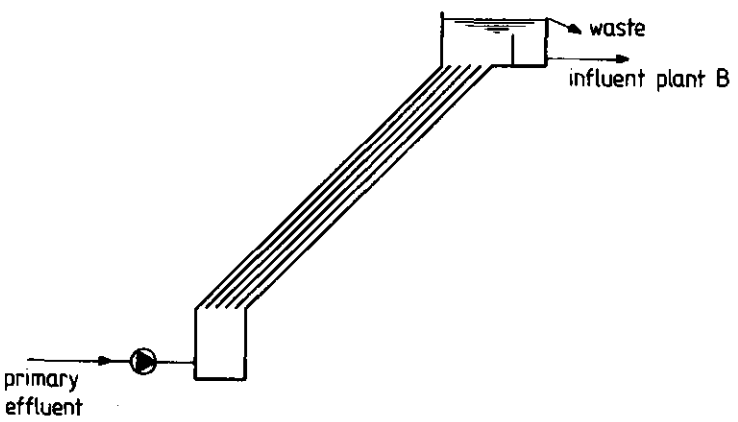

Figure 2.
Outline of a parallel plates grease separator. Dimensions of parallel plates compartment: 1 m (length) x 0.35 x 0.35 m.

adjusted automatically at 7.2 by adding 4 M NaOH. The temperature of the mixed liquor varied between 10 - 17°C; it was usually 3°C higher than in the oxidation ditch. Excess sludge was wasted discontinuously to maintain a concentration of suspended solids in the mixed liquor of 3 g/l. The sludge recirculation factor was 1, resulting in a low amount of solids in the settling tank.

Bench scale plants. Two identical bench-scale activated-sludge plants were fed with two types of synthetic waste water. The capacity of the aeration tank was 5 l and that of the settling tank 2 l (both tanks constructed of perspex). The activated sludge was completely mixed with the waste water using a diffused-air system. The oxygen concentration was at least 5 mg/l, the pH was kept at 7.2 by adding 4 M NaOH and the temperature was 15-20°C. The sludge was wasted discontinuously to maintain a concentration of 3 g/l suspended solids in the mixed liquor. The synthetic waste water contained as the main source of carbon and energy Na oleate and glucose, respectively. The oleate influent contained per l: Na oleate, 140 mg; $(NH_4)_2SO_4$, 165 mg; Bacteriological peptone (Oxoid), 60 mg; K_2HPO_4, 40 mg; $MgSO_4.7H_2O$, 30 mg; $CaCl_2$, 25 mg; vitamin stock solution, 0.5 ml; tap water, 800 ml. The vitamin stock solution (Slijkhuis, 1983a) contained among other vitamins, thiamin and cyanocobalamin, essential vitamins for *M. parvicella*. Na oleate was dissolved in 200 ml deionized water and added to the rest of the influent. The glucose influent was similar to the oleate influent with Na oleate replaced by glucose, 330 mg/l. Both media were stored at 4°C while oleate influent was continuously stirred to prevent precipitation of Ca and Mg oleates. The chemical oxygen demand (COD) and total organic nitrogen (Kj-N) of the media were 400 mg O_2/l and 42 mg N/l, respectively.

Activated sludge enriched with M. parvicella. After one year of operation of the bench scale plants, the following alterations were carried out. Activated sludge (5 l) of the oxidation ditch was seeded with a washed-cell suspension of *M. parvicella* containing 1.3 g of cell dry weight and the sludge mixed with the waste water by a propeller stirrer. Oxygen was brought into the mixed liquor by the diffused-air system (usually an intermittent supply). The composition of the synthetic waste water was mainly as described above but the final concentration of the components (the vit-

amin stock solution excepted) was increased six-fold. The concentrated carbon source was fed to the activated sludge independently of the rest of the influent.

Analyses. Suspended and volatile suspended solids, grease, chemical oxygen demand (COD), sludge volume index (SVI), ammonium nitrogen (NH_4^+-N), total organic nitrogen (Kj-N), nitrite nitrogen (NO_2^--N) and nitrate nitrogen (NO_3^--N) were determined according to the procedures outlined in NEN (1982).

The composition of fatty acids in the waste water was determined gas-chromatographically. Chloroform extracts of waste water were saponified and methylated (Brian and Gardner, 1967). Methyl esters of fatty acids were separated in a gas chromatograph equipped with a flame ionization detector at a gas flow rate of 20 ml nitrogen per min. Column: 2000 x 4 mm stainless steel containing 10% (w/w) of diethyleneglycolsuccinate on Chromosorb G-HP (80-100 mesh) and operating at 180°C. Methyl esters of various pure fatty acids were used as standards.

To determine the amount of oleic acid in the mixed liquor, a sample of 5 ml was digested in 1 M HCl at 100°C for 2 h and extracted with chloroform. The oleic acid concentration was determined spectrophotometrically after reaction with concentrated H_2SO_4 (Zevenhuizen, 1974; Zevenhuizen and Ebbink, 1974).

7.3 RESULTS AND DISCUSSION

Plate count of Microthrix parvicella

Undiluted and $1:10^2$ diluted samples of a *M. parvicella* culture, grown for 10 days in medium A and containing 1.3 g of biomass (dry wt) per l, gave abundant growth on G, H, I and J agar. At dilutions 10^{-4} and 10^{-6}, however, only very poor colony growth was observed on G and H agar and no growth at all on I and J plates after incubation for 30 days at 25°C. These poor results are explained by the inhibiting action of oleic acid. The adverse effect of this compound on growth and respiration of *M. parvicella* was in an earlier paper shown to exist (Slijkhuis, 1983a,c). Oleic acid is the substrate of media I and J and it may also have been present in media G and H as contaminant in the commercial Tween 80, the substrate of these media, or it may have been liberated from Tween 80 by

enzyme activity. Inhibition of Gram-positive bacteria by oleic acid has often been reported (Boissevain, 1926; Kodicek, 1949; Wynne and Foster, 1950); this effect was prevented by the addition of bovine albumin serum (Dubos, 1947; Davis and Dubos, 1947). In agreement with this observation, *M. parvicella* on agar media containing albumin (G', H', I' and J') was found to give colony counts in agreement with the degree of dilution (Table 1).

Table 1.
Growth of a pure culture of *M. parvicella* on agar plates.

Agar medium	Number of *M. parvicella* colonies from 0.1 ml of a diluted sample[1)]		
	10^{-4}	10^{-5}	10^{-6}
G'	nd[2)]	nd	4
H'	nd	nd	4
I'	>200	50-140	6-17
J'	nd	nd	2-7

1) Determined after incubation for 20 days at 25°C. The culture contained about 1.3 g cell material dry weight/l.

2) Not determined.

Undiluted and diluted samples (10^{-2} and 10^{-4}) of homogenized activated sludge containing *M. parvicella* filaments were plated on medium I'. After incubation for 20 days at 25°C some *M. parvicella* colonies were detected at the highest dilution between more than one hundred colonies of other activated-sludge bacteria. These results clearly show that the presence of *M. parvicella* in activated sludge cannot be estimated quantitatively by the plate count procedure; however, the method may be employed for the isolation of the bacterium from activated sludge.

In this investigation the occurrence of *M. parvicella* in activated sludge was determined by microscopic observation and by the staining methods as described by Eikelboom and Buijsen (1981).

Effect of environmental conditions on the occurrence of M. parvicella in activated sludge.

a. Effect of lipids and fatty acids contained in the waste water.

The effect of fatty matter in the waste water on the growth of *M. parvicella* in activated sludge was studied simultaneously in the pilot plants supplied with raw and "treated" sewage, respectively, and the bench scale plants supplied with glucose and oleic acid influent, respectively. The experiments were carried out at various sludge loading rates.

Table 2.
Grease and COD content of grab samples of raw domestic sewage taken at different times.

Grease mg/l	COD mg O_2/l	Grease/COD mg/mg O_2
18	132	0.136
21	165	0.127
39	284	0.137
44	311	0.141
48	350	0.137
77	650	0.118
118	920	0.128

Pilot scale plants. The concentration of organic substrates in the waste water varied considerably during the period of plant operation. The amount of chloroform-extractable compounds (grease) was approximately proportional to the COD of the waste water (Table 2). The grease content of this domestic sewage was within the wide range of values reported (viz. 20-150 mg/l) for domestic sewage (Mahlie, 1940; Painter and Viney, 1959; Viswanathan et al., 1962; Hunter and Heukelekian, 1965; Loehr and Kukar, 1965; Loehr and Navarra, 1969; Young, 1979). The same was true of the grease to COD ratio for this type of waste water (Table 3). The fatty acid composition of the grease present in the domestic sewage and in that from settled sewage

after passage through the parallel plates grease separator was analyzed gas chromatographically (Table 4). Allthough the total amount of grease varied widely, its composition was constant. The distribution of the various types of fatty acids present in the waste water was similar to the values reported by various other investigators (Viswanathan et al., 1962; Pierson et al., 1980; Hrudey, 1981).

Table 3.

Grease content and grease to COD ratios of domestic waste water.

Grease mg/l	COD mg O_2/l	Grease/COD mg/mg O_2	Reference
26.4	225	0.117	Hunter and Heukelekian (1965)
147[1]	640	0.230	Loehr and Navarra (1969)
3.1	128	0.025	Pierson et al. (1980)
37.6	504	0.075	Pierson et al. (1980)
26.2	537	0.049	Pierson et al. (1980)
35.3	761	0.046	Pierson et al. (1980)
146	760[2]	0.192	Painter and Viney (1959)
129	770[2]	0.168	Painter and Viney (1959)

[1] Sewage contained waste water of meat-processing plants.

[2] COD values were calculated from organic-carbon values by multiplying these values with 3.3.

Table 4.

Composition of fatty acids present in grease extracted from raw domestic sewage and in that from settled sewage after passage through parallel plates grease separator.

Source of grease	% of total fatty acids[1]					
	C12:0	C14:0	C16:0	C18:0	C18:1	C18:2
Raw sewage	3	4	31	25	30	6
"Treated" sewage	2	4	33	25	32	2

[1] Averages of 5 determinations; saturated and unsaturated fatty acids with a chain length from 12 to 18 carbon atoms were recorded.

Table 5.
Characteristics of the waste water.[1)]

	COD mg O_2/l	Kj-N mg N/l	Grease mg/l	Kj-N/COD mg N/mg O_2	Grease/COD mg/mg O_2
Raw sewage	490	53.3	52	0.11	0.106
Treated sewage[2)]	390	49.6	32	0.13	0.082

1) Averages of 20 grab samples.
2) After primary settling and passage through a parallel plates grease separator.

The effect of the primary treatment of the sewage affected the grease to COD ratio to a limited extent (Table 5). The ratio settleable solids to non-settleable waste water compounds was considerably reduced by the primary treatment but the chemical composition of the waste water determined as Kj-N to COD ratio and grease to COD ratio only moderately changed. The effect of the parallel plates hardly decreased the relative amount of grease in this type of waste water. The efficiency of grease removal was in the same range as it is in normal primary treatment (primary settling) (Table 6). This grease separator proved to be an excellent apparatus in the removal of grease from certain industrial waste water with a high lipid content (Chin and Wong, 1981). It is likely, that the lipids in domestic waste water are adsorbed to or incorporated in other sewage compounds and that these conglomerates were not removed from the sewage to a great extent by the primary treatment including settling and passage through parallel plates. Consequently, the initial aim to study the performance of activated-sludge plants served with raw domestic sewage and domestic sewage stripped of grease, respectively, was not realized, so that both pilot plants have been operated under similar conditions with respect to chemical composition of the waste water.

The performance of the pilot plants was identical at both sludge loading rates and both plants efficiently removed the organic compounds from the waste water (Table 7). The characteristics of the activated sludge itself hardly varied during the experimental period. The flocs were compact

Table 6.

Efficiency of grease removal from domestic sewage by primary treatment.

Raw sewage		Primary effluent		Efficiency of grease removal[1]	Reference
grease (mg/l)	organic matter (mg/l)	grease (mg/l)	organic matter (mg/l)	%	
147	640 COD	82	430 COD	17	Loehr and Navarra (1969)
33	229 BOD	15	137 BOD	26	Young (1979)
26	225 COD	15	142 COD	10	Hunter and Heukelekian (1965)
52	491 COD	32	391 COD	23	this study

[1] Calculated by using the equation:

$$\frac{(\text{grease/organic matter})_I - (\text{grease/organic matter})_P}{(\text{grease/organic matter})_I} \times 100\%$$

I = influent

P = primary effluent

Table 7.

Characteristics of pilot scale activated-sludge plants.

	Pilot plant A		Pilot plant B		Oxidation ditch Bennekom
Sludge loading rate (g COD/g dry weight.d)	0.043	0.083	0.040	0.089	0.060
Influent					
COD (mg O_2/l)	491	495	391	399	492
Kj-N (mg N/l)	53.6	54	49.6	51	54
Mixed liquor					
Suspended solids (g/l)	2.5	3.0	2.4	3.0	3.9
Volatile matter					
% of suspended solids	77.9	80.6	80.6	81.4	79.9
pH	7.2	7.2	7.2	7.2	6.9
SVI (ml/g)	133	117	110	80	224
Oxygen concentration (mg/l)	>6	>6	>6	>6	0-2
Effluent					
COD (mg O_2/l)	66	48	63	55	42
Kj-N (mg N/l)	-[1]	1.4	-	1.6	5.7
NO_3^--N (mg N/l)	-	52	-	49	11
COD reduction %	87	90	84	86	91
Kj-N reduction %	-	97	-	97	89
N-reduction %	-	1	-	0	69

1) Not determined.

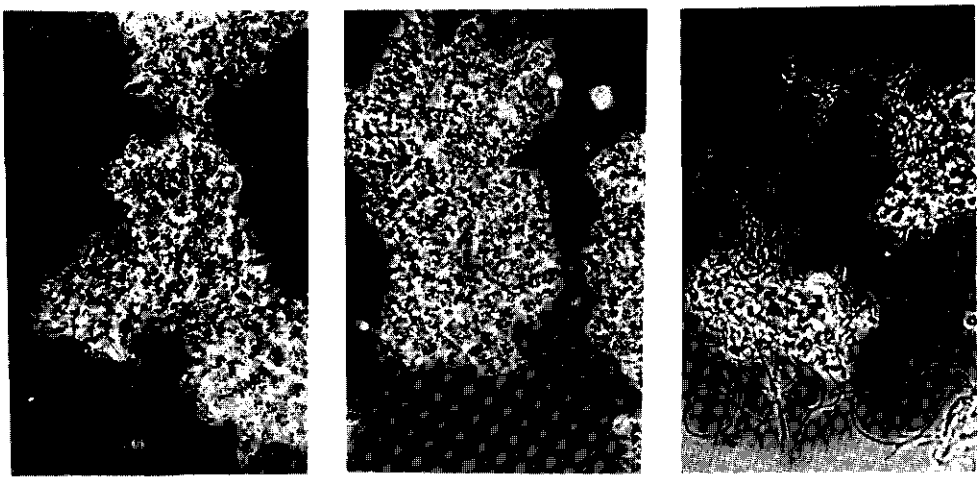

Figure 3.
Activated-sludge flocs (625x) in pilot plant fed with raw domestic sewage (a); pilot plant fed with settled domestic sewage after passage through grease separator (b) and oxidation ditch of Bennekom fed with raw domestic sewage (c).

(Fig. 3a,b) and settling of the sludge was good. The growth of filamentous bacteria in the activated sludge was poor. Only at the end of the experimental period, the sludge in pilot plant B (fed with "treated" sewage) contained a considerable amount of a filamentous bacterium, but the characteristics of this organism showed it to be no *M. parvicella*. It is important to note that the occurrence of *M. parvicella* in the sludge of the pilot plant was poor, whereas the organism was predominant in the sludge of the oxidation ditch of Bennekom (Fig. 3c). The presence of the organism in the latter plant resulted in a poor settling of the sludge, showing a SVI value of 224 ml/g (Table 7). The waste water treated in pilot plant A was identical with that treated in the full scale plant and sludge loading rates and other process parameters like pH and temperature were in the same range. The mixing of sludge and waste water and the oxygen concentration in the mixed liquor were the only parameters which clearly differed in the pilot plants and the full scale plant. Mixing of sludge and waste water in the pilot plants was by means of a diffused-air system resulting in complete mixing and high oxygen concentration in the mixed liquor at the low sludge loading rates applied in this study. Nitrification in the sludge system was high due to the high oxygen concentration and the high sludge age (>15 days)

and denitrification was poor as a result of the high oxygen supply. The oxygen concentration in the mixed liquor of the full scale plant varied between 0-2 mg O_2/l and the mixed liquor was anoxic for a considerable period during the circulation in the plant before it was reaerated by the brushes. Consequently, nitrification was less complete and denitrification in the anoxic zone was considerable. The effect of a low oxygen supply on the nitrogen removal from the waste water was clearly demonstrated (Table 7) and must be explained by nitrification followed by denitrification. From the comparison of both types of purification systems it is concluded that the differences in sludge characteristics between the pilot plants and the full scale plant, such as microscopic picture and sludge volume index (SVI) were probably due to differences in oxygen supply.

Bench scale plants. These plants, fed with synthetic waste water containing glucose and oleic acid, respectively, as the main source of carbon and energy, were operated for a prolonged period at two sludge loading rates. These rates were higher than those of the pilot plant study because the temperature and consequently the maintenance of the activated-sludge organisms was higher. Experiments at a lower sludge loading rate (0.05 g COD/g dry weight.d) resulted in a decrease of the organic matter content of the sludge at a prolonged period.

The influent contained about 400 mg COD and 42 mg Kj-N/l. The characteristics of the activated sludge, those of the final effluent and those of the treatment performance, are presented in Table 8. The plant performance was good, as COD and Kj-N removal were high. Nitrification of the biomass was high and denitrification was very low due to the high oxygen concentration, consequently total nitrogen removal was low. Presumably there was no denitrification at all because wasted sludge also contained nitrogen and this value added to total nitrogen in the effluent probably balanced the nitrogen flow.

The activated sludge of both plants compacted poorly as represented by the high SVI values, especially at the low sludge loading rate. The bulking of the sludge fed with glucose influent was entirely explained by abundant growth of a filamentous microorganism. The growth of this organism started in the second week of the experimental period and did not disappear with increased sludge loading. After a few weeks of operation the sludge consisted mainly of this bacterium whilst the sludge had a red-brownish colour. The causative organism was a thin, Gram-negative, sheath-

Table 8.

Characteristics of bench-scale activated-sludge plants.

	Waste water			
	Glucose influent		Oleic acid influent	
Sludge loading rate (g COD/g dry weight.d)	0.065	0.097	0.062	0.103
Mixed liquor				
Suspended solids (g/l)	2.7	2.8	2.8	2.6
Volatile matter				
% of suspended solids	61	65	61	70
pH	7.2	7.2	7.2	7.2
SVI (ml/g)	>400	335	223	131
Oxygen concentration (mg/l)	>6	>6	>6	>6
Effluent				
COD (mg O_2/l)	30	21	28	28
Kj-N (mg N/l)	-[1)]	0.6	-	1.1
NO_2^--N (mg N/l)	-	0.1	-	0.1
NO_3^--N (mg N/l)	-	33	-	31.5
COD reduction (%)	93	95	93	93
Kj-N reduction (%)	-	98	-	97
N-reduction (%)	-	20	-	22

1) Not determined.

forming filamentous bacterium. Characteristic shape of the organism, sheath formation and result of the Gram-staining clearly demonstrated that this bacterium was no *M. parvicella* (Fig. 4a). The poor compaction of the sludge fed with oleic acid was not due to abundant growth of filamentous organisms (Fig. 4b). The *M. parvicella* filaments present in the initial sludge had almost disappeared after two weeks of operation. Within a few days after starting up the plant, the condition of the *M. parvicella* filaments deteriorated. Irregular shape of the filaments due to globules, probably lipid inclusions, were clearly visible by microscopic examination.

In an earlier paper (Slijkhuis, 1983a) it was reported that *M. parvicella* could be cultivated in shaken pure culture in a medium containing peptone and oleic acid. In the present study it was shown that the organism is unable to compete successfully with other organisms for these substrates in the activated-sludge process as applied in these experiments. The low sludge loading used does not explain the absence of *M. parvicella* as in full scale activated-sludge plants bulking caused by this bacterium usually occurs at low or moderate sludge loadings.

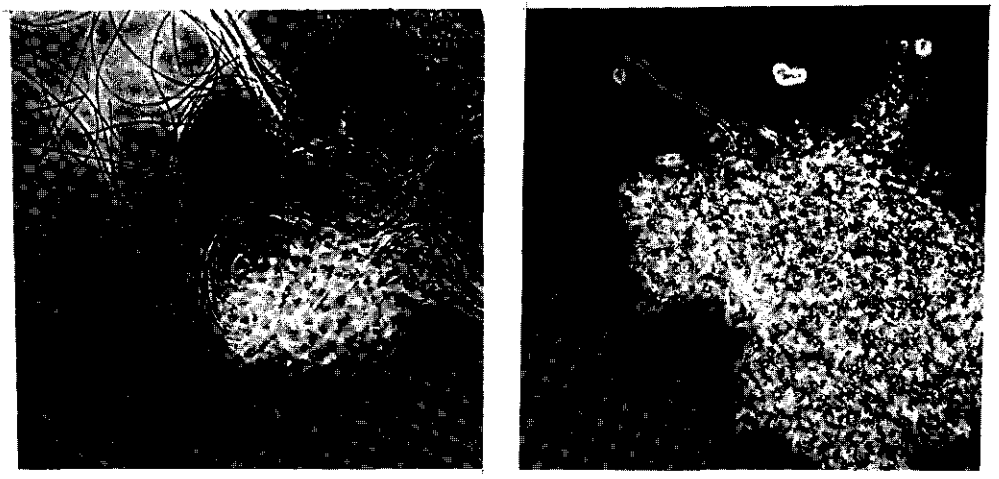

Figure 4.
Activated-sludge flocs (625x) in bench-scale plants fed with glucose influent (a) and oleate influent (b).

Abundant growth of *M. parvicella* was not achieved in activated-sludge plants (pilot plants and bench-scale plants) supplied with waste water containing considerable amounts of fatty matter. *M. parvicella* requires long chain fatty acids for growth (Slijkhuis, 1983a,b) but the results of the present study show that the occurrence of the bacterium in activated sludge does not depend only on the presence of these substrates in the waste water. The experimental plants were operated similarly to full scale oxidation ditches concerning sludge loading rate, pH and as far as possible, temperature of the mixed liquor. However, the supply of oxygen to the mixed liquor and the mixing characteristics of the experimental plants were markedly different from those of the full scale plant. As a result of the excessive oxygen supply of the former compared to the latter system, nitrifying activity of the biomass of the experimental plant high resulting in a high sodium hydroxide demand of the activated sludge for buffering. Denitrifying activity of the biomass in the experimental plants was low compared to that of the full scale plant (Tables 7 and 8). As a result of nitrification the concentration of reduced nitrogenous compounds in the mixed liquor of the experimental plants was low (determined as Kj-N in the effluents; Tables 7 and 8). It is likely that owing to the excessive oxygen supply also the concentration of reduced-sulphur compounds in the experimental plants has been very low. As *M. parvicella* requires both reduced-nitrogen compounds and reduced-sulphur compounds for growth (Slijkhuis, 1983a), the absence of the organisms from the activated sludge of the experimental plants may have been due to the lack of these compounds. Experiments were subsequently set up to test this hypothesis.

b. Effect of oxygen supply and of reduced-sulphur compounds of the waste water.

These experiments were carried out in the bench scale plants with activated sludge from the oxidation ditch of Bennekom. The amount of *M. parvicella* filaments in the sludge was low at the time of the experiments. Therefore the sludge was enriched with a pure culture of *M. parvicella* (Fig. 5); the applied sludge loading rate was 230 mg COD/g dry weight.d to obtain a clear response during the limited period of operation (15-20 days). The activated sludge (3 g dry weight/l) was completely mixed with

the synthetic waste water containing 2400 mg COD/l and 250 mg Kj-N/l.

Figure 5.
Microthrix parvicella enriched activated sludge (625x).

The variations in conditions and the results of the experiments are summarized in Table 9. In experiment 1 the activated sludge was fed with glucose influent and oleic acid influent, respectively; oxygen was intermittently supplied. During this experiment the excess sludge was not wasted, consequently the COD sludge loading rate decreased with time. *M. parvicella* filaments in the sludge fed with glucose influent did not increase, whereas the growth of the bacterium in the sludge fed with oleic acid influent was abundant (Fig. 6). Growth of a filamentous bacterium in the sludge fed with glucose influent occurred after 10-15 days, but this was no *M. parvicella*. Identical results were obtained in an experiment carried out at a constant sludge loading rate (exp. 2).

The oxygen concentration considerably affected the growth of *M. parvicella* in activated sludge fed with oleic acid influent. In the system with continuous oxygen supply and consequently a high oxygen concentration (exp. 3A), *M. parvicella* did not grow, whereas in the system with intermittent oxygen supply the growth was similar to the growth observed in

Table 9.

Survey of the experiments in bench scale plants with activated sludge enriched with *M. parvicella*.

Exp. no.	Type of influent	Sludge loading rate g COD/g dry wt.d	Oxygen supply[1]	Mixed liquor characteristics		Effluent characteristics		
				Oxygen conc.	Growth of *M.parvicella*[2]	COD (mg O_2/l)	NH_4^+-N (mg N/l)	NO_3^--N (mg N/l)
1A	Gluc[3]	0.23→0.15	I (0.5/2.5)	±[4]	-	nd	nd	nd
1B	Olea[3]	0.23→0.15	I (0.5/2.5	±	+	nd	nd	nd
2A	Gluc	0.23	I (0.5/2.5)	±	-	8-20	20-50	0
2B	Olea	0.23	I (0.5/2.5)	±	+	10-40	10-40	0
3A	Olea	0.23	C	>6	-	65-75	3-10	160
3B	Olea	0.23	I (0.5/2.5)	±	+	80-160	100-110	1-3
4A	Olea	0.23	I (0.5/4.5)	±	+	100	120	<1
4B	olea	0.23	I (0.5/4.5)	±	+	100	150	0
5A	Olea	0.23	I (3/15)	±	+	140	115	0
5B	Olea	0.23	I (3/15)	±	+	62	70	1
6A	Olea	0.23	I (0.5/2.5)	±	+	40-55	3-12	3-25
6B	Olea+[5]	0.23	I (0.5/2.5)	±	+	45-75	7-12	4-60
7A	Olea	0.23	I (0.5/2.5)	±	+	nd	70	nd
7B	Olea+[5]	0.23	I (0.5/2.5)	±	+	nd	72	nd

1) Oxygen was supplied intermittently (I) or continuously (C), the pulse/cycle ratio in minutes is given in parentheses.

2) By microscopic observation.

3) Gluc = glucose influent; Olea = oleic acid influent.

4) Most of the period the system was anoxic, only during oxygen supply the concentration was 0.5-2 mg O_2/l.

5) Olea influent supplemented with $Na_2S_2O_3.5H_2O$ (0.2 g/l).

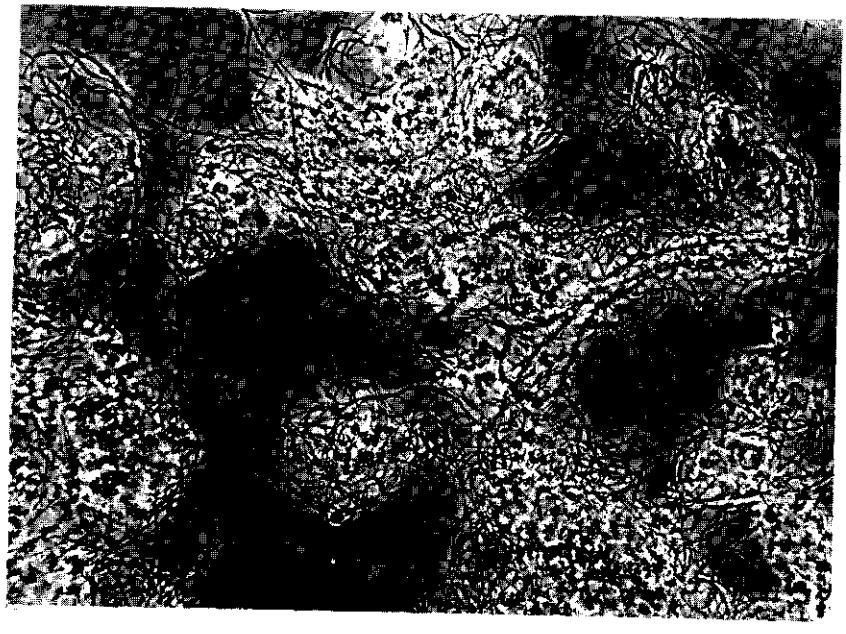

Figure 6.
Activated sludge (625x) in bench-scale plant supplied with oleate influent and a limiting amount of oxygen.

experiments 1B and 2B (exp. 3B). The effect of the period of oxygen supply on the growth of *M. parvicella* was studied in the experiments 4 and 5. Reducing the amount of oxygen supplied to the activated sludge affected the growth of *M. parvicella* to some extent. The outgrow of the filaments was excellent during the first week of operation but after a prolonged period the condition of the cells deteriorated. The protrusion of the filaments from the flocs decreased and lipid accumulation in the cells occurred as determined by microscopic examination. Accumulation of lipids was observed in *M. parvicella* cells of a batch culture during the early growth phase. These lipids may account for 35% of the cell dry weight (Slijkhuis et al., 1983). In the present investigation the lipid content of the sludge increased significantly during the experimental period, whereas the Kj-N content of the dry weight hardly varied (Table 10). The effect of the oxygen supply was reflected in the composition of the nitrogen compounds in the effluent (Table 9). Nitrification without an extreme denitrification of the biomass was observed at a high oxygen concentration (exp. 3A). A limited oxygen supply resulted in a decreased nitrifying activity whereas denitrifying activity was high (exps. 3B; 4A,B; 5A,B).

Table 10.

Total organic nitrogen and oleic acid in activated sludge after various periods of oleic acid feeding.

Time of oleic acid feeding (d)	Total organic nitrogen in sludge g N/g dry weight			Oleic acid in sludge g oleic acid/g dry weight		
	0	7	16	0	7	16
Exp.nr.[1)]						
2A	0.078	0.072	0.080			
2B	0.071	0.068	0.083			
3A		0.070	0.073	0.074		0.099
3B		0.068	0.088	0.074		0.139
4A	0.078	0.071		0.074	0.241	
4B	0.071	0.069		0.074	0.235	
5A	0.072	0.072				
5B	0.075	0.070				
6A		0.082	0.076			
6B		0.070	0.080			

1) Experiment numbers refer to Table 9.

The addition of a reduced-sulphur compound to the influent was not required for the growth of *M. parvicella* filaments in activated sludge of systems operating under a limited oxygen supply. A pronounced development of the organism was observed in sludge fed with oleic acid both with and without added sodium thiosulphate (exps. 6 and 7). These results show that the concentration of reduced-sulphur compounds in the liquor was sufficient and that sulphur was not the rate-limiting nutrient of *M. parvicella* growing in activated sludge supplied with a limited amount of oxygen.

Experiments carried out under identical conditions (exps. 2B; 3B; 6A; 7A) resulted in a similar response to oleic acid as concerns the occurrence of *M. parvicella* in activated sludge. On the other hand, the composition of the effluent (COD; NH_4^+-N; NO_3^--N) varied considerably in these experiments (Table 9), probably due to variations in the oxygen supply or in the microbial composition of the initial sludge.

The condition of *M. parvicella* filaments sometimes deteriorated after a prolonged period of operation. The supply of oxygen was usually not sufficient for the complete oxidation of the fatty acids, resulting in the accumulation of these acids in the sludge. Oleic acid is inhibitory to a variety of microorganisms, in particular Gram-positive bacteria (Boissevain, 1926; Kodicek, 1949; Wynne and Foster, 1950). The deterioration of *M. parvicella* like the decreasing protrusion of the filaments from the sludge flocs into the liquor and the accumulation of lipids in the cells may have been due to this phenomenon. Earlier results have shown that oleic acid considerably inhibited the growth and respiration of the organism when added at a relatively high oleic acid to biomass ratio (Slijkhuis, 1983c). The oleic acid content of the sludge increased during the experimental period (Table 10) and the ratio of oleic acid to biomass may have reached the inhibitory level for *M. parvicella*. The results of Table 10 cannot be compared with those of the respiration experiments (Slijkhuis, 1983c) as Ca and Mg oleates, the salts in which oleic acid probably occurs in the sludge, are insoluble and consequently not inhibitory to *M. parvicella*. The effect of "oleic-acid overloading" of the sludge did not interfere with the effects of oxygen supply or waste water composition on activated sludge as the initial increase of *M. parvicella* filaments was clearly demonstrated in oxygen-limited, oleic acid-fed activated sludge.

The growth of *M. parvicella* in activated sludge depends on the presence of long chain fatty acids in the waste water and on a limited oxygen supply of the system. The latter may depend on a direct or an indirect effect. *M. parvicella* has a high affinity for oxygen (Slijkhuis, 1983c) and it may therefore successfully compete with other bacteria for this compound at a low concentration, whereas at a high oxygen concentration other types may prevail. On the other hand, *M. parvicella* requires reduced-nitrogen and reduced-sulphur compounds for growth and the concentration of these compounds in the mixed liquor at a high oxygen concentration may be too low to enable the growth of the organism.

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8. Summary.

The activated-sludge process is a treatment system in which the waste water (influent) is mixed for some time with activated sludge. Subsequently, the sludge is separated from the treated waste water by settling. Activated-sludge flocs contain active biomass, mainly bacteria, dead organic compounds and inorganic material. The components are removed from the waste water by adsorption onto the flocs or by direct uptake by microorganisms. The mixed liquor (mixture of sludge and waste water) is supplied with oxygen. During the mixing the biodegradable matter is partly oxidized, whereas another part is utilized for growth of the biomass. After the period of mixing (some hours to some days) the treated waste water (effluent) is separated from the sludge by settling. The effluent can usually be disposed off to surface water without disturbing the ecology of the environment to a great extent. The settled sludge is returned to the aeration tank in which it is mixed again with waste water. Excess sludge is continuously or intermittently withdrawn from the system.

The activated-sludge process is widely used in waste-water treatment because of its high efficiency and rapid adaptation to variations in waste-water composition. However, the process often meets operational problems due to the poor settling of the sludge flocs from the mixed liquor. Poor settleability is usually caused by abundant growth of filamentous organisms in the sludge ("bulking" sludge).

The microbial composition of activated sludge is determined by the composition of the waste water, by plant operation (oxygen supply, mixing characteristics, sludge loading) and by the physiological characteristics of the organisms. Bulking of sludge can only be controlled by alteration of the plant operation as the composition of the waste water cannot be changed. The methods used to control abundant growth of filamentous organisms in sludge are generally not selective because little is known about the causative organisms.

This thesis describes a number of physiological characteristics of *Microthrix parvicella*, a filamentous bacterium that often causes bulking

sludge in low-loaded activated sludge plants (e.g. oxidation ditches), particularly when supplied with domestic sewage. Low-loaded activated-sludge plants account for more than 50% of the treatment plants in the Netherlands.

M. parvicella was shown to have remarkable nutritional requirements. The organism utilizes as carbon and energy source only long chain fatty acids, preferably in esterified form. Simple carbon sources like carbohydrates and various organic acids are not utilized. Short chain fatty acids and glycerol are only consumed if supplied together with a long chain fatty acid. *M. parvicella* requires an ammonium salt as nitrogen source. Sulphates cannot be utilized as sulphur source; the organism requires a reduced-sulphur compound for growth (L-methionine, L-cysteine, sulphides, elemental sulphur, thiosulphates). The vitamins thiamin and cyanocobalamin are essential for growth. The requirement for trace elements has not been studied.

The organism grows readily in batch culture at 25°C and above pH 7 on Tween 80 (polyoxyethylenesorbitan mono-oleate, 4 g/l) as source of carbon and energy. To obtain growth basal medium containing Tween 80 and the essential vitamins should be inoculated with 10% (v/v) of a full-grown culture. During the early growth phase the available oleic acid is readily taken up by the cells and stored in esterified form in globules, resulting in a high lipid content of the cells (up to 35% of the cell dry weight). In a subsequent period, the lipids are applied for energy supply and synthesis of cell constituents such as protein.

Growth kinetics of *M. parvicella* have been determined by cultivating the organism in a chemostat with Tween 80 as the limiting substrate. The maximum specific growth rate is 0.06 h^{-1} (mean doubling time about 12 h). The substrate constant (K_S) for Tween 80 is very low but an exact value could not properly be determined. The maximum yield coefficient is 1.4 g of cell dry weight/g of oleic acid consumed and the maintenance coefficient was found to be 1.5 mg oleic acid/g cell dry weight.h.

Short chain fatty acids and glycerol cannot be utilized as the only carbon and energy source, presumably because *M. parvicella* lacks the system to synthesize long chain fatty acids.

Oleic acid at very low concentrations and the oleic acid moiety of Tween 80 are excellent substrates for *M. parvicella*. However, the growth of the organism is inhibited by the free acid already at a low concentration.

The respiration of washed cells is also inhibited by oleic acid. The inhibition increases with decreasing pH. The concentration of oleic acid causing inhibition is proportional to the concentration of biomass. Ca and Mg oleates which are insoluble salts of oleic acid are not inhibitory to the organism. Substrates (oleic acid, Tween 80) are probably not directly oxidized by washed cells; the major part is stored in the cells. Tween 80 has surface-active properties. Compounds having similar characteristics but which cannot be utilized by the organism for growth enhance the oxygen uptake of washed cells.

The increased oxygen uptake due to added Tween 80 probably consists of a substrate-specific respiration and a non-substrate-specific oxygen uptake. The apparent substrate constant (K_S) for oxygen determined with a washed-cell suspension (2 g of cell dry weight/l) is very low (< 0.016 mg O_2/l).

The above-described characteristics determine to a great extent the occurrence of *M. parvicella* in activated sludge, as was demonstrated in experiments with bench-scale plants. Abundant growth of the organism was observed in sludge fed with waste water containing oleic acid and supplied with a limited amount of oxygen.

The growth of *M. parvicella* in full-scale activated-sludge plants fed with domestic sewage is explained by the reported results. Domestic sewage contains a large amount of fatty matter (up to 50% of the organic carbon present in the waste water). Oxygen is usually supplied in a limited amount; consequently, reduced-nitrogen compounds and reduced-sulphur compounds are present in considerable amounts in the mixed liquor.

Excessive growth of the organism may be prevented by decreasing the amount of fatty matter in the waste water and by reducing the concentration of reduced nitrogen and reduced sulphur in the mixed liquor. Fatty matter can be removed from the waste water as its density is lower than that of water. However, the fatty matter in domestic sewage is adsorbed onto or incorporated within other sewage components and therefore not easily to remove from the sewage. The concentration of reduced N and S compounds depends to a great extent on the oxygen concentration in the mixed liquor. Therefore, growth of *M. parvicella* can be prevented by an enhanced supply of oxygen. The minimum oxygen concentration in the mixed liquor at which

growth of *M. parvicella* is limited has to be studied. A high oxygen concentration is not advisable because of high energy costs and of a decreased denitrifying activity of the sludge. Denitrification within the floc is required for the elimination of nitrate present in the mixed liquor and for the stabilization of the pH of the mixed liquor.

9. Samenvatting

Het actief-slibproces is een zuiveringssysteem waarbij het afvalwater ("influent") gedurende enige tijd wordt gemengd met actief slib, waarna dit slib door middel van bezinking wordt afgescheiden van het behandelde afvalwater. Actief slib bestaat uit vlokken met actieve organismen, met name bacteriën, dood organisch materiaal en anorganische verbindingen. Veel bestanddelen van afvalwater worden in het actief-slib proces door de vlokken weggenomen, hetzij door direkte opname door de organismen, hetzij door adsorptie aan de vlokken. Biologisch afbreekbaar materiaal wordt tijdens het contact van slib en afvalwater voor een gedeelte door ingebrachte zuurstof geoxydeerd en voor een gedeelte omgezet in bacteriecellen, dat wil zeggen nieuw biologisch actief materiaal. Na enige tijd (enkele uren tot enkele dagen) wordt het slib door bezinking gescheiden van het gezuiverde afvalwater (het "effluent"), dat meestal zonder milieubezwaren op het oppervlaktewater kan worden geloosd. Het bezonken slib wordt teruggevoerd naar de installatie waar het opnieuw wordt gemengd met afvalwater. De overmaat aan actief slib wordt continue of discontinue afgevoerd en behandeld. Het actief-slibproces is het meest toegepaste zuiveringssysteem vanwege de hoge efficiëntie en de snelle aanpassing aan variaties van het aangevoerde afvalwater. Een goede procesvoering wordt echter vaak verstoord doordat de afscheiding van het slib uit het slib-watermengsel niet voldoende snel verloopt. Problemen met slechte bezinking worden meestal veroorzaakt door sterke groei van draadvormige micro-organismen in het slib ("licht slib"; "bulking sludge"). De microbiologische samenstelling van het actieve slib wordt bepaald door de samenstelling van het afvalwater, de wijze waarop het proces wordt uitgevoerd (zuurstofvoorziening, menging, slibbelasting) en de fysiologische eigenschappen van de organismen. In het geval van licht slib zijn de mogelijkheden om de moeilijkheden op te lossen beperkt. De samenstelling van het afvalwater is een vast gegeven, maar procesvariabelen

zijn in principe te variëren. De methoden om licht slib te bestrijden zijn in het algemeen weinig gericht, omdat van de veroorzakende organismen weinig bekend is.

Dit proefschrift beschrijft een aantal facetten van de fysiologie van *Microthrix parvicella*. Dit is een draadvormige bacterie die vaak licht slib veroorzaakt in laagbelaste actief-slibinstallaties, zoals oxydatiesloten, in het bijzonder bij voeding met huishoudelijk afvalwater. In Nederland is dit type installatie qua aantal het grootst.

M. parvicella heeft een merkwaardige substraatbehoefte. Het organisme gebruikt hogere vetzuren (bij voorkeur in veresterde vorm) als koolstof- en energiebron. Eenvoudige koolstofbronnen zoals suikers en verscheidene organische zuren kunnen in het geheel niet als substraat dienen. Lagere vetzuren en glycerol kunnen slechts samen met een hoger vetzuur als substraat worden benut. Gebleken is dat *M. parvicella* een gereduceerde anorganische stikstofverbinding (een ammoniumzout) nodig heeft. Sulfaten kunnen voor dit organisme niet als zwavelbron dienen; zwavelhoudende aminozuren, zoals L-methionine en L-cysteine, of anorganische verbindingen met gereduceerde zwavel (sulfiden, elementaire zwavel, thiosulfaten) kunnen wel worden benut. Van de vitaminen zijn thiamine en cyanocobalamine (vit. B12) nodig voor groei. De behoefte aan micro-elementen is niet onderzocht.

Het organisme groeit in batch-cultuur met Tween 80 (polyoxyethyleensorbitan mono-oleaat, 4 g/l) als koolstof- en energiebron, het snelst bij 25°C, in een medium met een pH > 7. Minimaal medium moet met 10% (volume %) van een volgroeide cultuur geënt worden om groei te verkrijgen. Gedurende de eerste fase van de groei wordt alle beschikbare oliezuur door de cellen opgenomen en in veresterde vorm opgeslagen, resulterend in een hoog vetgehalte van de cellen. In een volgende fase wordt het opgeslagen vet gebruikt voor celsynthese en energielevering.

Kinetische parameters van *M. parvicella* zijn in dit onderzoek bepaald in continucultuur met Tween 80 als limiterend substraat. De maximale specifieke groeisnelheid is 0.06 uur^{-1} (verdubbelingstijd ± 12 uur). De substraatconstante (K_S) voor Tween 80 is zeer laag, doch een waarde kon niet bepaald worden. De maximale opbrengstcoëfficient is 1,4 drooggewicht/g geconsumeerd oliezuur en voor de onderhoudscoëfficient werd een waarde van 1,5 mg oliezuur/g drooggewicht.uur gevonden.

Lagere vetzuren en glycerol kunnen niet als enige koolstof- en energiebron worden benut, waarschijnlijk omdat *M. parvicella* geen hogere vetzuren kan synthetiseren.

Ondanks het feit dat oliezuur een goed substraat is voor *M. parvicella* wordt de groei van het organisme reeds door een lage concentratie van dit zuur in het medium geremd. Ook de ademhaling van een gewassen celsuspensie wordt geremd door oliezuur. Deze remming is sterker bij lagere pH en bij een lagere concentratie van biomassa. De onoplosbare calcium- en magnesiumoleaten remmen de ademhaling niet. Het aangeboden substraat (oliezuur, Tween 80) wordt door gewassen cellen niet direct verademd, maar wordt waarschijnlijk grotendeels in de cellen opgeslagen. Tween 80 is een oppervlakte-actieve stof. Verbindingen, die qua fysisch-chemische eigenschappen lijken op Tween 80, maar niet als substraat voor het organisme kunnen dienen, verhogen de zuurstofopname van gewassen cellen. De verhoging van de zuurstofopname ten gevolge van de toevoeging van Tween 80 is waarschijnlijk een combinatie van substraatademhaling en een niet substraat-specifieke zuurstofopname. De substraatconstante (K_S) voor zuurstof gemeten met een gewassen celsuspensie (2 g drooggewicht/l) is zeer laag (< 0.0.16 mg O_2/l).

Het belang van de genoemde eigenschappen van *M. parvicella* bij het voorkomen van dit organisme in actief slib werd gedemonstreerd in proeven op laboratorium- en semi-praktijkschaal. Massale groei van het organisme werd verkregen in slib gevoed met oliezuurhoudend afvalwater bij een gelimiteerde zuurstoftoevoer.

De verkregen resultaten verklaren het optreden van *M. parvicella* in actief slib gevoed met huishoudelijk afvalwater. Dit afvalwater bevat een grote hoeveelheid vetachtig materiaal (tot 50% van de aanwezige organische koolstof) en de zuurstoftoevoer is in het algemeen beperkt. Stikstof en zwavelverbindingen zijn hierdoor in gereduceerde vorm aanwezig in het slib-watermengsel.

Massale groei van het organisme kan worden voorkomen door de toevoer van vetachtige verbindingen en de aanwezigheid van stikstof en zwavel in gereduceerde vorm in het slib-watermengsel te voorkomen. Vetachtig materiaal kan worden afgescheiden uit een waterig milieu omdat het een lagere dichtheid heeft dan water. In huishoudelijk afvalwater zijn deze verbindingen echter gebonden aan andere componenten en zijn dan slecht

af te scheiden. De concentratie van verbindingen met gereduceerde stikstof en zwavel in het slib-watermengsel is in hoge mate afhankelijk van de zuurstofconcentratie, zodat verhoging van de zuurstoftoevoer de groei van *M. parvicella* in actief slib kan verhinderen. De minimale zuurstofconcentratie waarbij het optreden van dit organisme wordt beperkt, moet worden onderzocht. Een hoge zuurstofconcentratie is niet gewenst vanwege de hoge energiekosten en vanwege de verminderde denitrificatie. Denitrificatie in de vlok is van groot belang voor de verwijdering van nitraatstikstof en voor stabilisatie van de pH van het slib-watermengsel.

Curriculum Vitae.

De auteur, geboren op 19 september 1953 in Leiden, behaalde in 1970 het diploma H.B.S.-B aan het Christelijk Lyceum te Voorburg. In dat jaar begon hij met de studie aan de Landbouwhogeschool in Wageningen. Hij behaalde met lof het ingenieursdiploma Milieuhygiëne in 1978 en Moleculaire wetenschappen in 1979. Het vakkenpakket in de ingenieursfase bestond uit microbiologie, waterzuivering en biochemie. Van 1979 tot en met 1982 was hij als promotieassistent verbonden aan de vakgroep Microbiologie van de Landbouwhogeschool. Sinds februari 1983 is de auteur werkzaam bij de Research en Development Organisatie van Gist-Brocades N.V. in Delft.