The growth of bacteria on organic compounds in drinking water

CENTRALE LANDBOUWCATALOGUS

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The growth of bacteria on organic compounds in drinking water

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Stellingen

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1.

Nagroei van bacteriën in drinkwater tijdens distributie en de daarmee samenhangende kwaliteitsverslechtering van dit water kunnen worden beperkt door toepassing van een biologische filtratie als laatste behandelingsfase.

Dit proefschrift

2.

Ten onrechte wordt door voorstanders van de continue cultuur gesteld dat selectie van micro-organismen door ophoping in batch-cultures altijd berust op verschillen in de maximale groeisnelheid van de aanwezige organismen.

H. Veldkamp, Adv. Microbial Ecol. 1 (1977): 59-94
W. Harder en L. Dijkhuizen. Phil.Trans R. Soc. Lond. B 297 (1982): 459-480
Dit proefschrift

3.

De kinetiek van de groei van een micro-organisme op een groeibeperkend substraat kan met behulp van batch-cultures op een eenvoudige wijze worden vastgesteld, mits de groei van het organisme kan worden gemeten met behulp van koloniegetalbepalingen.

Dit proefschrift

4.

Het is onwaarschijnlijk dat de substraatverzadigingsconstanten (K_s) van bacteriën die worden aangetroffen in drinkwater in het algemeen lager zijn dan die van bacteriën in actief slib.

5.

Glijmiddelen die worden toegepast bij het koppelen van waterleidingbuizen dienen als zodanig bacteriedodend te zijn, terwijl lage concentraties van deze middelen de vermeerdering van bacteriën in drinkwater niet mogen bevorderen.

6.

Berichtgevingen in de nieuwsmedia over onderwerpen waarvan de lezer gedetailleerde kennis heeft, doen vragen rijzen over de mate van betrouwbaarheid van het overige hem, of haar, aangeboden nieuws.

7.

Bij de bestrijding van *Legionella pneumophila*, de veroorzaker van de zogenaamde "veteranenziekte", in warmtapwatersystemen is het verhogen van de watertemperatuur niet de enige remedie.

J.S. Colbourne, D.J. Pratt,
M.G. Smith, S.P. Fischer-Hock,
D. Harper. Lancet, (1984): 210-213

8.

Nieuwsmedia vormen een betere "voedingsbodem" voor de veroorzaker van de veteranenziekte dan de bij het onderzoek van drinkwater gebruikelijke voedingsmedia.

9.

De geconstateerde aanwezigheid van genotoxische biotische stoffen in een aantal natuurlijke voedingsmiddelen, noopt tot verschuiving in waardering van het begrip "puur natuur".

R. Sijmons. Vrij Nederland 44
(1983): 7, 10

10.

Indien bij filtratie van water door actieve kool een CO_2-produktie optreedt die groter is dan overeenkomt met de verwijdering van organische koolstof uit dit water, dan kan dit niet zonder meer als bewijs dienen voor het optreden van een bioregeneratie van de adsorptie-capaciteit van de actieve kool.

M. Eberhardt, S. Madsen en
H. Sontheimer. GWF Wasser/Abwasser
116 (1975): 245-247
M. Jekel. Veröffentlichungen
Wasserchemie 11 (1979) Karlsruhe

11.

De beantwoording van de vraag welke toxicologische betekenis moet worden toegekend aan de aanwezigheid van individuele xenobiotische stoffen in drinkwater, wordt nagenoeg geheel bepaald door de genotoxische eigenschappen van deze verbindingen.

H.J. Kool, C.F. van Kreyl,
B.C.J. Zoeteman. CRC Crit. Rev.
Env. Control 12 (1983): 307-357

D. van der Kooij.
The growth of bacteria on organic compounds in drinking water.

Wageningen, 27 april 1984.

Aan Annette,
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Deze publikatie is een uitgave van:

Keuringsinstituut voor Waterleidingartikelen
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Abstract

Van der Kooij, D. (1984). The growth of bacteria on organic compounds in drinking water.

Growth ("regrowth") of bacteria in drinking water distribution systems results in a deterioration of the water quality. Regrowth of chemoheterotrophic bacteria depends on the presence of organic compounds that serve as a nutrient source for these bacteria.
A batch-culture technique was developed to study the growth of bacteria in drinking water. The maximum colony counts of selected pure cultures of bacteria grown in drinking water were used as a measure for the concentration of easily assimilable organic carbon compounds (AOC). Three strains of bacteria isolated from drinking water were selected for AOC determinations: (1) *Pseudomonas fluorescens* strain P17, an organism with a very great nutritional versatility, for general use; (2) a *Flavobacterium* sp. strain S12, to determine the concentration of maltose and starch-like compounds, and (3) a *Spirillum* sp. strain NOX to determine the concentration of carboxylic acids, including oxalic acid. The properties of these bacteria were extensively studied in batch-culture experiments with drinking water supplemented with very low amounts of selected organic compounds. The obtained results prove that growth and growth kinetics of bacteria at concentrations between 1 and 1000 µg of C per liter can be determined in simple experiments using simple equipment. The strains mentioned above were well adapted to growth at concentrations of a few micrograms of substrate C per liter; their substrate saturation constants (K_s) were below 1 µM for many compounds tested. K_s values below 0.1 µM were also obtained. Growth measurements with strain P17 in water sampled from various treatment stages revealed that the AOC concentrations in these water types usually varied between 10 and 100 µg of acetate C equivalents per liter. Ozonation caused a strong increase of the AOC concentration. Strain NOX was better suited for the determination of the AOC concentration in ozonated water because of its ability to utilize oxalate. Filtration processes caused significant reductions of the AOC concentrations, at low AOC concentrations (< 25 µg of acetate C equivalents per liter) this reduction was less than 50 %. The use of starch-based coagulant aids in water treatment gave increased AOC concentrations as determined with *Flavobacterium* strain S12. The concentration of these compounds was reduced to non-detectable levels (< 1 µg of starch C equivalents per liter) by biological

filtration processes. These findings demonstrate that biological filtration processes are effective in reducing AOC concentrations to levels where excessive regrowth is not possible. This conclusion is supported by the very poor growth of *Pseudomonas aeruginosa* in the filtrate of slow sand filters, and the inability of *Aeromonas hydrophila* to multiply in this water type.

1. General introduction

SIGNIFICANCE OF BACTERIA IN DRINKING WATER SUPPLY

The removal of bacteria of faecal origin from the raw water, and the prevention of their introduction into drinking water during distribution are of paramount importance for a safe drinking water supply. Therefore, freshly prepared drinking water and drinking water in distribution systems are frequently investigated by selective techniques which enable the detection of faecal indicator bacteria (thermotolerant coliforms, faecal streptococci) in volumes up to a few hundreds ml of water.

Nearly 100 years ago, Koch (36) observed that river water polluted with sewage did not cause disease when the number of bacteria, cultivated on a non-selective nutrient-rich medium, had been reduced to less than 100 colony-forming units per ml by slow sand filtration. Based on this experience, the colony count of bacteria on a nutrient-rich medium has initially been used as an important water quality parameter. Gradually bacteriological techniques were developed for the selective cultivation of bacteria of faecal origin. At present, colony counts on a nutrient-rich medium have only a limited significance as a water quality parameter (3).

Still, bacteria which contribute to these colony counts, and more generally those present in water, play a very important role in water supply. Such organisms are present in rivers, lakes and open storage reservoirs, but also in water during underground passage and various filtration processes, including rapid sand filtration, dry filtration, dual media filtration, granular activated-carbon filtration and slow sand filtration. A large variety of organic compounds are removed from the water by aerobic chemoheterotrophic aquatic bacteria utilizing these compounds as sources of carbon and energy for growth. In recent years, much attention has been paid to the microbial activities in granular activated-carbon filters, which are applied for the removal of recalcitrant organic compounds (15).

The pollution of the aquatic environment with anthropogenic organic compounds, including organochlorine compounds, has evoked a growing interest in the capabilities of bacteria in removing these compounds (79). Also anaerobic chemoheterotrophic bacteria are able to utilize such compounds (9). Recently, there is also much interest in using bacteria for the removal of excess nitrate from water (23). Of great importance for water treatment are also the autotrophic nitrifying bacteria which remove ammonia from water by oxidizing it to nitrate.

The occurrence in drinking water of large numbers of bacteria, grown on organic compounds in the raw water or during water treatment, may be prevented either by an appropriate sequence of treatment procedures or by the application of a disinfectant, of which chlorine is most widely used. The effect of chlorination on the taste of water, as well as the production of organochlorine compounds during chlorination of water (55), are limiting the use of chlorine in The Netherlands.

ENUMERATION OF BACTERIA IN WATER

Several techniques, including direct counting procedures, determination of most probable numbers by statistical interpretation of growth tests in various water volumes and the assessment of the number of colony forming units (CFU) per unit volume of water, are available for determining the numbers of bacteria in aquatic habitats. For enumeration of the viable chemoheterotrophic bacteria in drinking water, colony counts on nutrient-rich solid media are most generally used. The composition of the media and procedures used in routine, depend largely on legislation and standardization.

Colony count standards for drinking water are based on the application of nutrient-rich media and short incubation periods (2-3 days) at temperatures between 20-37 °C. With these techniques only those bacteria which can survive the great changes in environmental conditions (temperature, osmotic pressure, presence of nutrients) and multiply rapidly at a high substrate concentration, are enumerated. These organisms, the copiotrophic bacteria (50), are better adapted to nutrient-rich environments than the slow-growing bacteria, or those bacteria which cannot survive such environmental changes viz. the oligotrophic bacteria. The presence in drinking water of large numbers of the former group indicates that either the water treatment does not function properly with regard to the removal of bacteria, or that the distribution system is not sufficiently clean (54). Determination of faecal pollution indicator bacteria is necessary to evaluate the hygienic risks of such situations.

Longer incubation periods (47), a relatively low nutrient concentration (22, 65) and the use of the streak-plate method instead of the pour-plate method (14, 52) followed by incubation at 20-30 °C which allows multiplication of most bacteria, results in much higher colony counts than those obtained by the procedure

described above. Thus large propertions of the chemoheterotrophic bacteria are not included in the colony counts determined by techniques standardized for routine investigations of drinking water. Colony count procedures modified as indicated above may therefore be better suited to assess the efficiency of water treatment for removal of bacteria and give a more complete information about the numbers of bacteria present in the distribution system. However the long duration of the incubation period (1-3 weeks) of the improved procedures excludes taking rapid measures when unexpectedly high colony counts are found. Hence, their application seems mainly limited to research.

IDENTITY AND SIGNIFICANCE OF BACTERIA CONTRIBUTING TO COLONY COUNTS ON NON-SELECTIVE MEDIA

At the end of the 19th century, numerous investigations were conducted to identify the bacteria observed in drinking water by cultivation on the gelatine-broth medium developed by Koch (21, 28 34, 43, 73, 81). These investigations revealed that a great variety of bacteria were commonly present in drinking water. Moreover, the isolated bacteria appeared to be ordinary water bacteria, which were harmless for the consumer. These findings stimulated the development of methods for the selective isolation of bacteria of faecal origin.

Systematic investigations on the identity of bacteria contributing to the colony counts of drinking water on non-selective, nutrient-rich media have also more recently been conducted (7, 37, 45, 60, 71, 53). In addition, a great number of incidental observations concerning bacteria present in drinking water contributed significantly to the knowledge of the composition of the aerobic chemoheterotrophic bacterial flora of drinking water.

Genera and species of such bacteria which have frequently been isolated include: *Achromobacter, Acinetobacter, Aeromonas hydrophila, Alcaligenes, Arthrobacter, Bacillus, Caulobacter, Corynebacterium, Cytophaga, Flavobacterium, Moraxella, Pseudomonas alcaligenes, P.fluorescens, P.putida, Spirillum, Streptomyces* and *Staphylococcus*. Species of coliform bacteria, e.g. *Enterobacter aerogenes, E.cloacae, Citrobacter* spp. and *Klebsiella* spp. have also frequently been observed, particularly when selective media were used for cultivation (7,12). Representatives of chemoheterotrophic microorganisms, which require specific media for isolation, e.g. methylotrophic bacteria (46, 58, 69), *Sphaerotilus*

spp. (44, 74) and fungi (48) have also been observed in drinking water. These organisms do not contribute to the colony counts on routine media. The isolation of representatives of at least 31 biotypes of *P.fluorescens* and 14 biotypes of *P.putida* from various types of drinking water (72), in which these pseudomonads were only a small minority of the bacterial population (70), further demonstrates the great diversity of the bacterial flora of drinking water.

The results obtained by identification of bacteria isolated on nutrient-rich media do not give a correct impression of the composition of the population of chemoheterotrophic bacteria in drinking water because, for reasons mentioned above, only a small part of the population, in particular the copiotrophic bacteria, is isolated by these media. Furthermore, the characterization and identification of many bacteria are hampered for the following reasons: isolates do not always survive repeated inoculations required for cultivation (45); many "classical" characterization procedures are not suited for characterization of aquatic bacteria, and a large number of genera and species represented in drinking water, e.g. *Flavobacterium, Pseudomonas, Spirillum* are incompletely defined.

A number of species of the microorganisms mentioned above are causing hygienic, aesthetic or technical problems in drinking water supply. *Aeromonas hydrophila* may affect the coliform count when the membrane filtration technique is applied (38). Moreover, the organism may act as an opportunistic pathogen (77) or contribute to spoilage of food products (35). It has been suggested that *Arthrobacter* spp. are related to coloured-water problems (76). *Bacillus cereus* causes food spoilage and may subsequently act as a pathogen (20). *Flavobacterium* species also cause spoilage of food products (5). Hygienic problems related to the presence of specific *Flavobacterium* spp., e.g. *F.meningosepticum*, in water have been reviewed by Herman (30). Fluorescent pseudomonads may contribute to slime problems in industrial processes (51) and to spoilage of food products at low temperatures (35). *P.aeruginosa* is an opportunistic pathogen (31). *Streptomyces* species attack sealing rings of natural rubber (40, 41). Moreover, they produce compounds with earthy or musty smells such as geosmin and isoborneol (27) and may therefore contribute to taste and odour problems in stagnant water (11).The significance of specific groups of microorganisms has resulted in the development of selective or semi-

selective isolation techniques for most of the bacteria mentioned above.

Recently, a few types of aerobic chemoheterotrophic bacteria of pathogenic significance, that do not contribute to the colony counts on routine media, have been detected in drinking water, viz. *Mycobacterium kansasii* (10, 33) and *Legionella pneumophila* (and related species). *Mycobacterium kansasii* seems to multiply in drinking water pipes inside buildings (19). *Legionella pneumophila*, the causative agent of the legionnaires' disease has been observed in hot water systems, where water temperatures are below 60 °C (16, 63, 64, 66, 68, 78). The organism is able to multiply in water sampled from such systems (80).

GROWTH OF BACTERIA IN DRINKING WATER DURING STORAGE AND DISTRIBUTION

The colony count of drinking water during distribution is composed of bacteria which originate from (a) raw water, (b) water treatment, (c) growth ("regrowth") within the distribution system or (d) introduction into the distributed water during repair and construction activities. Introduction of large numbers of bacteria into the system by the freshly prepared drinking water, or by contamination during repair and construction activities indicate unsatisfactory situations from a hygienic point of view. They can be prevented by adequate water treatment and distribution procedures.

Regrowth is usually involved when large numbers of bacteria are observed in drinking water sampled from distribution systems which were supplied with water containing only a few bacteria. A large number of reports deal with regrowth in distribution systems (1, 2, 4, 6, 8, 25, 29, 32, 45, 49, 56, 75). The extent of this regrowth depends on (a) absence of a disinfectant residual, (b) the type and concentration of organic compounds which can be utilized as sources of carbon and energy for the growth of microorganisms, (c) the water temperature (d) the residence time of the water in the distribution system and (e) the presence of sediments in the system. Compounds serving as growth substrates may either originate from the raw water, may be introduced during treatment, e.g. by ozonation (17, 56, 62, 67) or are released from construction and plumbing materials (10, 18, 24, 57, 59).

Apart from the public health risks, aesthetic problems or technical problems evoked by the growth of specific groups of bacteria, the following additional negative aspects are connected

with the massive growth of typical aquatic bacteria in distribution systems.

(a) Oxygen consumption as a result of growth may cause anaerobic situations which give rise to increased corrosion of cast iron pipes followed by colour problems (29, 39, 49, 75).

(b) Development of animals which utilize bacteria as a food source. Such a development can lead to complaints of consumers when animals visible with the naked eye, viz. *Asellus aquaticus* are present (42, 61, 82).

(c) Permanently high colony counts of water in distribution systems reduce the value of routine colony count estimations as a water quality parameter.

(d) High colony counts seem to interfere with the detection of coliforms (26), but Clark (13) could not confirm this observation.

OBJECTIVES OF THE INVESTIGATIONS

Water quality deterioration related to regrowth of bacteria in the distribution system is a practical problem. Research was conducted to enable the resolution of this problem by the removal of organic compounds, which might serve as growth substrates, from the water entering the system. The objectives of the here described study were:

(a) The development of a bacteriological method for the estimation of the concentration of organic compounds that may be used as sources of carbon and energy for the growth of bacteria in drinking water.

(b) The assessment of the effects of water treatment procedures on the concentration of such compounds.

(c) The determination of the ability of typical aquatic oligotrophic bacteria and of bacteria with hygienic, aesthetic or technical consequences, to multiply at very low concentrations of substrate in water.

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2. Multiplication of fluorescent pseudomonads at low substrate concentrations in tap water

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Abstract

Two fluorescent pseudomonads, strains P17 and P500, belonging to different biotypes were tested for growth in tap water supplied with different concentrations of acetate and glutamate, low concentrations (10 and 20 µg of C per liter) of various other substrates and mixtures of related substrates, the latter being present each in amounts of 1 µg of C per liter. Amino acids appeared to be excellent substrates for both isolates, but also many other substrates were utilized at very low concentrations. Saturation constants (K_s) for P17 of acetate, arginine, aspartate, glutamate, lactate, succinate, malonate, *p*-hydroxybenzoate and glucose were all below 1 µM. The K_s values for P500 were about 5 times larger than those for P17. Since especially P17 is able to use a large number of different substrates at low concentrations, assessment of maximum colony counts of this organism by growth experiments in various types of tap water may give information about the concentrations of easily assimilable organic carbon compounds.

INTRODUCTION

Despite their frequent isolation, fluorescent pseudomonads usually constitute small minorities (< 1 %) of the bacterial populations of surface and tap water (Van der Kooij, 1977), silt deposits in drinking water service reservoirs (Windle Taylor, 1971-73), raw and treated sewage (Hankin and Sands, 1974) and soil (Rovira and Sands, 1971). Characterization of fluorescent pseudomonads obtained from surface water and various types of drinking water showed that these bacteria belonged to at least 45 different biotypes (Van der Kooij, 1979), many of which were similar to those defined by Stanier et al. (1966). This characterization study further revealed distinct relationships between the various water types and the occurrence of representatives of specific biotypes. Unfortunately, most characters suited for classification have no ecological significance, since they do not give information about the ability of the fluorescent pseudomonads to multiply with very low concentrations of substrates. The growth of representatives of two clearly different biotypes in tap water supplied with low concentrations of substrates was studied

to explain differences observed in the presence of these biotypes in various types of tap water as part of an investigation into the behaviour of bacteria in drinking water during distribution.

MATERIALS AND METHODS

Isolates

The isolation of bacterial strains used in this study as well as their characterization have been described previously (Van der Kooij 1977; 1979a). The following strains were used: *Pseudomonas fluorescens* P17, originating from tap water prepared from dune infiltrated river water and belonging to biotype 7.2 which corresponds to biotype C (Stanier et al, 1966), and *P.fluorescens* P500, obtained from tap water prepared from river water by physicochemical treatment and belonging to biotype 1.1, which corresponds to biotype G (Stanier et al, 1966). Both biotypes were generally observed in surface water and tap water prepared from surface water. Biotype 7.2 was also found in ground water. Both isolates are proteolytic and do not multiply at 37 °C. Isolate P17 is able to produce gaseous nitrogen from nitrate under anaerobic conditions.

Replica test

Utilization of organic compounds as sources of carbon and energy for growth was tested by the replica technique using plates of basal salts agar with separately sterilized carbon compounds in a concentration of 1 μg per liter (Van der Kooij, 1979a). The carbon compounds tested are listed in Table 1.

Growth experiments in liquid media

The growth experiments were performed in 1-liter, calibrated, glass-stoppered Erlenmeyer flasks of Pyrex glass. These flasks were cleaned with a 10 % solution of $K_2Cr_2O_7$ in concentrated H_2SO_4, followed by rinsing with hot tap water, with 10 % HNO_3 and with hot tap water again. Thereafter, they were heated overnight at 250 - 300 °C. The pipettes (1 ml) were cleaned in the same way. The cleaned flasks were filled with 600 ml of tap water originating from the municipal Dune Waterworks of The Hague, where it is prepared from dune-infiltrated river water by the addition of powdered activated carbon, followed by rapid and slow sand filtration. This water contained 3.6 mg of dissolved organic carbon and 7.3 mg of

Table 1. Compounds serving as sources of carbon and energy for growth of the fluorescent pseudomonads P17 and P500.

	Amino acids (AA)	Carboxylic acids (CA)	Carbohydrates and (poly)alcohols (CHA)	Aromatic acids (AR)
Compounds utilized by P17 and P500	L-alanine, L-valine L-leucine, L-isoleucine L-lysine, L-arginine L-aspartate, L-asparagine L-glutamate, L-glutamine L-tyrosine, L-proline	acetate, propionate, DL-lactate, pyruvate, malonate, fumarate, succinate, citrate	D-glucose, D-mannitol, glycerol	*p*-hydroxybenzoate
Compounds utilized by P17 only	DL-serine, L-threonine L-histidine, DL-tryptophan	adipate	ethanol	benzoate anthranilate
Compounds utilized by P500 only	DL-phenylalanine		D-arabinose	
Not utilized	glycine	formate, glycolate, glyoxylate, oxalate, L-tartrate	D-maltose	vanillate, phtalate, nicotinate, DL-mandelate

nitrate per liter; the pH was 7.6 (average values).

For cultures in tap water, the water was not autoclaved but heated for 2 hours at 60 °C. This treatment was chosen to preserve the organic content of the water as much as possible in its original state. The counting plates done in the course of the experiments showed that bacteria originally present in the water were effectively removed by this treatment. Solutions of single organic compounds and of nitrogen compounds were also heated at 60 °C. Unless otherwise stated, NH_4Cl was added with the carbon compounds to obtain a C/N ratio similar to that of ammonium acetate. In a few experiments the water was supplied with mixtures of amino acids (AA), carboxylic acids (CA), carbohydrates and (poly)alcohols (CHA) and aromatic acids (AR), as listed in Table 1. Stock solutions of the 4 mixtures were prepared by dissolving the individual compounds in demineralized water in equal carbon concentrations, except glutamate which was used at twice this concentration. After

neutralization with a sodium hydroxide solution, the mixtures were sterilized (120 °C, 16 min) in Pyrex glass bottles containing a screw cap with a teflon shield. Addition of NH_4Cl was omitted in the experiments with the mixtures, as it had become clear from previous experiments that N was no limiting factor.

Inoculation and determination of bacterial growth in liquid media.
Turbid suspensions of 24-h slant cultures of the isolates were used for inoculation of 50 ml of autoclaved tap water contained in 100 ml normally washed infusion bottles. The initial number of cells in the inoculated water amounted to about 100 to 500 colony-forming units (CFU) per ml. Incubation of the infusion bottles at 25 °C gave maximum colony counts (N_{max}) varying from 10^5 to 2.10^5 CFU/ml which were reached within one or two weeks. Hereafter, the colony counts decreased very slowly during a period of several months. These cultures were used for inoculating the experimental solutions to provide an initial cell density of 50 to 300 CFU/ml.

Incubation was carried out at 15 ± 0.5 °C. Growth curves were obtained by periodic determination of the number of viable cells (N_t, CFU/ml) in triplicate. This was done by spreading 0.05 ml of the culture either directly or from decimal dilutions on predried Lab-Lemco (Oxoid) agar plates. Counting was performed after 40-48 h of incubation at 25 °C. The doubling time (G in hours) of the cultures was calculated with the equation:

$$G = \log 2 \quad (t'-t)/(\log N_t' - \log N_t) \qquad (1)$$

where: $t'-t$ = the incubation time (hours) in which N_t increased to N_t'. These calculations were performed for the period in which the growth curve was linear with time in a half-logarithmic plot and in which $N_t' < 0.1$ Nmax. All experiments were performed in duplicate, unless otherwise stated.

RESULTS

Utilization of organic compounds as sources of carbon and energy
Table 1 shows that at a concentration of 1 g of substrate per liter, 24 of the tested compounds were utilized by both isolates, 8 substrates only promoted the growth of P17 and two substrates were only utilized by P500.

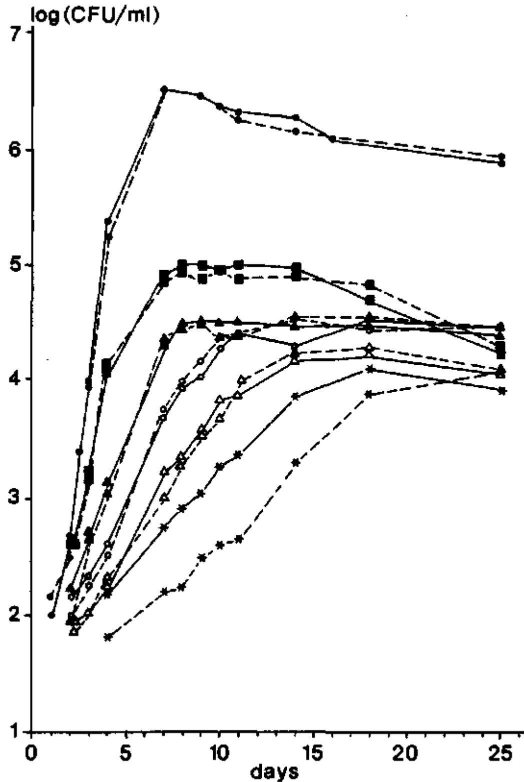

Fig. 1 Growth curves of *P.fluorescens* P500 at 15 °C ± 0.5 °C with different concentrations of acetate added to tap water: ●, 1 mg; ■, 25 µg; ▲, 10 µg; O, 5 µg; Δ, 2.5 µg of acetate C per liter; ★, blancs. Solid and broken lines indicate duplicate experiments.

Utilization of acetate and glutamate at low concentrations

At concentrations varying from 1 mg to 2.5 µg of C per liter acetate clearly enhanced the multiplication of P500 in tap water (Fig. 1). Similar observations were obtained for P500 with glutamate and for P17 with glutamate and acetate respectively (data not shown). From the maximum colony counts (N_{max}) obtained for the applied substrate concentrations (Fig. 2) the following yield values (Y) were calculated: Y (P500) = 4 x 10^9 CFU/mg acetate C and 4 x 10^9

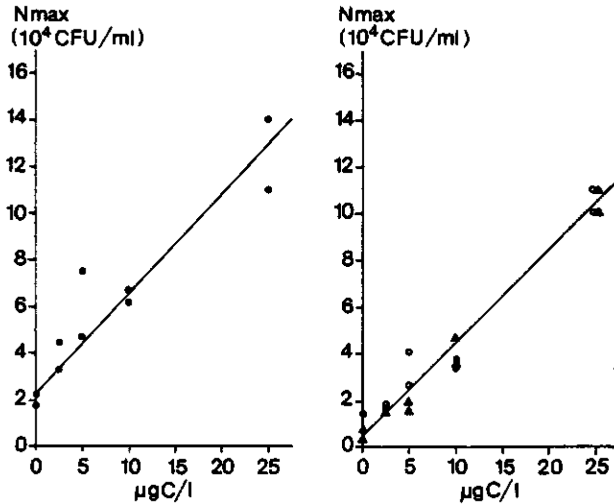

Fig. 2 Maximum colony counts of *P.fluorescens* P17 and P500 at different concentrations of acetate and glutamate added to tap water: ●, P17 on acetate, ○, P500 on acetate; ▲, P500 on glutamate.

CFU/mg of glutamate C and Y (P17) = 4.2 x 10^9 CFU/mg of acetate C. N_{max} values for P17 with glutamate were not estimated.
Using these yield data and the N_{max} values in the blanks, the natural substrate concentration (S_n) available for the two pseudomonads in the heated tap water may be expressed in equivalents of acetate C or glutamate C, giving S_n (P500) = 0.7 - 3.5 μg of acetate C or glutamate C equivalents per liter and S_n (P17) = 4.5 μg of acetate C equivalents per liter. These S_n values are only 0.1 % of the concentration of dissolved organic carbon.

In Fig. 3, the doubling times of P17 and P500 (derived from Fig. 1) are plotted against the reciprocal values of the concentrations of added substrate. The linear part of the relationship is expressed by the following adapted Lineweaver-Burk equation:

$$G = G_{min} + G_{min} \cdot K_s / \Delta S \qquad (2)$$

where G is the observed doubling time (in hours) at the concentration of substrate added ΔS (in μg of C per liter); G_{min} = minimal G.

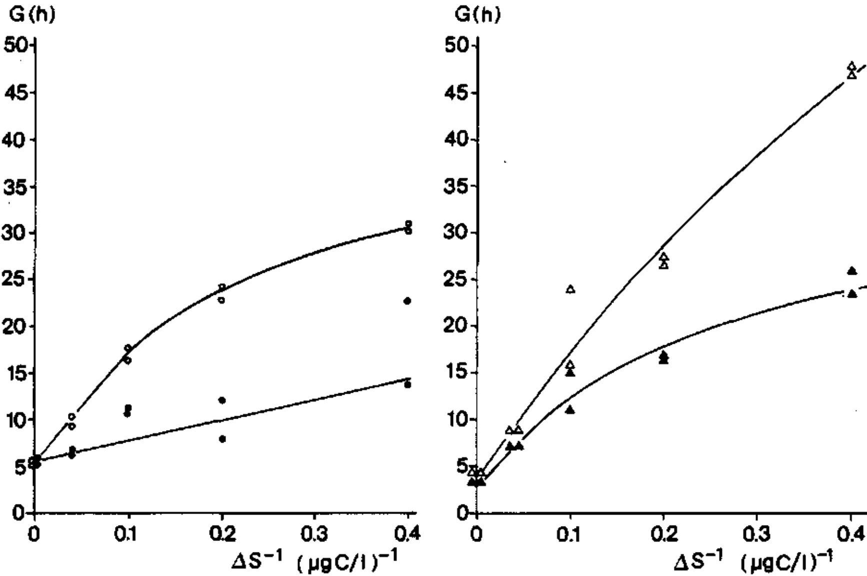

Fig. 3 Lineweaver-Burk plots of the growth of *P.fluorescens* P17 and P500 at low concentrations of acetate and glutamate added to tap water: ●, P17 on acetate; ○, P500 on acetate; ▲, P17 on glutamate; Δ, P500 on glutamate.

K_s, the saturation constant, is the substrate concentration at which $G = 2\ G_{min}$. The non-linearity of the presented curves may be explained by concurrent utilization of the natural substrates, which obviously was important when $\Delta S < 10$ μg of C/l, or when G according to eq.2 was above 20 - 30 % of G of the blanks. From the plots in Fig. 3, values for G_{min} and $G_{min} \times K_s$ were derived as presented in the equations listed in Table 2, which permitted calculations of K_s. It appears from Table 2 that P17 is better adapted (lower K_s value) to low concentrations of acetate than P500.

Using the listed equations and the G values found in the blanks, estimations could be made of the maximum natural concentrations (S_{max}) of either acetate or glutamate that might have been present

in the tap water used (Table 2). Comparison of the acetate C concentrations, calculated from the G values of P17, with S_n obtained from the N_{max} values indicates that acetate might have been at most 30 % of the substrate available for isolate P17 in the tap water.

Table 2. Growth constants of *P.fluorescens* P17 and *P.fluorescens* P500 with acetate and glutamate calculated from Fig. 3.

Isolate	Substrate	Lineweaver-Burk equation[a]	K_s		Growth in blanks	S^b_{max}
			μg C/l	μM	G(h)	μgC/l
P500	glutamate	G=3.7+130.2/ S	34.8	0.58	86.3;60.5	1.6-2.3
P17	glutamate	G=3.3+97.6/ S	29.5	0.49	43.4;27.5	2.4-4.0
P500	acetate	G=5.2+118.3/ S	22.8	0.95	48.5;45.1	2.7-3.0
P17	acetate	G=5.5+21.8/ S	4.0	0.17	22.1;21.6	1.3-1.4

[a] $G = G_{min} + G_{min} \cdot K_s/\Delta S$
[b] maximum natural concentration of the substrate involved

Utilization of various compounds at very low concentrations

Experiments with a number of substrates, representing different types of naturally occurring organic compounds of low molecular weight, revealed that the rates of growth (G^{-1}) of P17 and P500 in tap water were increased by the addition of 20 μg of substrate C per liter of all compounds tested except fumarate (Fig. 4). Fig. 5 indicates that fumarate was used by P17 after the exhaustion of S_n. P500 was unable to utilize fumarate when present in a concentration of 20 μg of C per liter. Strain P17 multiplied more rapidly with the compounds tested than isolate P500. A number of compounds promoted growth more clearly than others. The latter effect was even more pronounced at initial concentrations of 10 μg of C per liter (P17 only). P17 multiplied more rapidly at 10 μg of arginine C per liter than at 20 μg of C per liter in the form of all the other substrates tested.

The coefficients of the modified Lineweaver-Burk equations (2) for growth with the various substrates were calculated using the generation times at 1 mg of C per liter and those observed at 20 μg

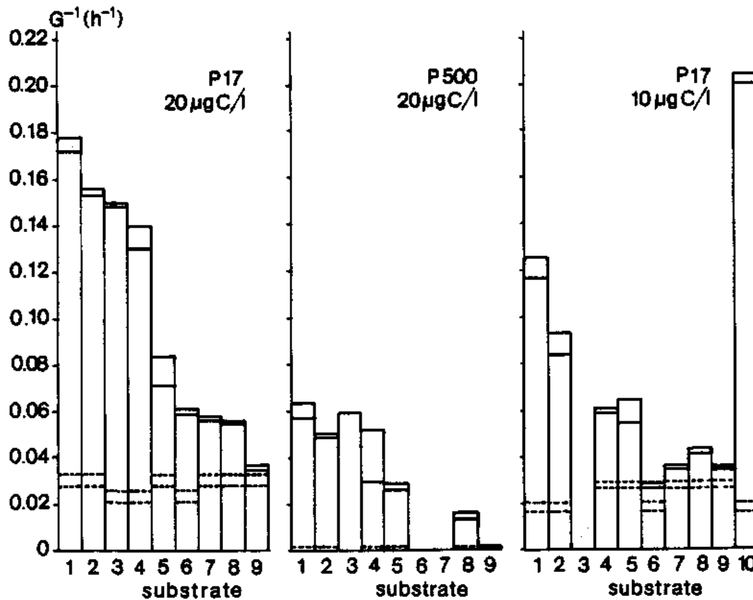

Fig. 4 Growth rates (G^{-1}) of *P.fluorescens* P17 and P500 at 15 ± 0.5 °C with 20 and 10 µg C/l of different substrates added to tap water. 1 = L-aspartate; 2, succinate; 3, acetate; 4, DL-lactate; 5, yeast extract; 6, malonate; 7, *p*-hydroxybenzoate; 8, D-glucose; 9, fumarate; 10, L-arginine. —, G^{-1} observed for growth with the added substrate; --- G^{-1} observed without substrate added; observations in duplicate. For P500 with 20 µg of acetate C/l, G^{-1} was calculated using the equation in Table 2.

of substrate C per liter (Table 3). With arginine, the generation time at 10 µg of C was used. With a few compounds, especially malonate, *p*-hydroxybenzoate and glucose, natural substrates may have significantly affected the generation times at low substrate concentrations. In these cases calculations were made with the assumption of complete preferential uptake of the added substrates. The substrate affinities of P500 were calculated assuming that this isolate has generation times similar to those of isolate P17 at 1 mg of substrate C per liter.

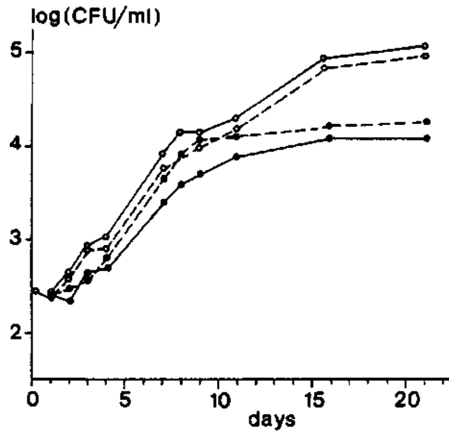

Fig. 5 Growth curves of *P.fluorescens* P17 at 15 ± 0.5 °C in tap water supplied with a low concentration of fumarate: ●, tap water without substrate added; O, tap water with 20 μg of fumarate C per liter. Solid and broken lines indicate duplicate experiments.

Table 3. Modified Lineweaver-Burk equations and K_s values of different substrates for strains P17 and P500.

Isolate	Substrate	G(h) at 1 mg C/1	Lineweaver-Burk equation[b]	K_s (μg C/1)	(μM)
P17	L-Arginine	3.8; 3.9	G=3.9+11.0/ΔS	2.8	0.04
P17	L-Aspartate	3.1; 3.3	G=3.1+51.0/ΔS	16.4	0.34
P17	Succinate	3.1; 3.1	G=3.0+68.4/ΔS	22.8	0.48
P17	DL-Lactate	3.5; 3.8	G=3.6+77.3/ΔS	21.4	0.59
P17	*p*-hydroxy-benzoate	4.4; 4.4	G=4.1+264.3/ΔS	64.4	0.76
P17	D-Glucose	4.8; 5.0	G=4.6+264.3/ΔS	57.4	0.79
P17	Malonate	11.6;14.6	G=13.0+74.4/ΔS	7.5	0.16
P500	L-Aspartate	as P17	G=2.9+274 /ΔS	94	1.9
P500	Succinate	"	G=2.8+347 /ΔS	124	2.6
P500	DL-Lactate	"	G=3.2+464 /ΔS	145	4.0
P500	D-Glucose	"	G=3.6+1341 /ΔS	372	5.2

[a] In all cases preferential uptake of the added substrate is assumed
[b] $G = Gmin + Gmin.K_s/\Delta S$

The K_s values obtained for P17 were all below 1 µM, and a few were extremely low, e.g. 0.04 µM of arginine and 0.16 µM of malonate (Table 3). The K_s of malonate C approximates the one of acetate (Table 2). The K_s values for P500 were clearly above those for P17. The slopes of the Lineweaver-Burk equation for P500 on aspartate, aspartate, succinate, lactate and glucose were all about 5 times steeper than for isolate P17 on the same compounds. This factor which was also observed with acetate, but not with glutamate (Table 2), appears to be a characteristic difference between the strains and possibly between the biotypes to which the isolates belong.

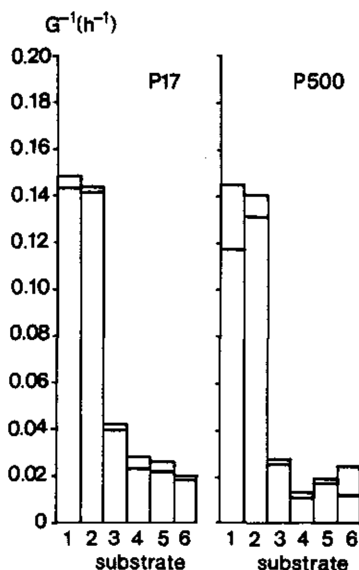

Fig. 6 Growth rates (G^{-1}) of *P.fluorescens* strains P17 and P500 at 15 ± 0.5 °C in the presence of mixtures of substrates. The concentration of the individual compounds was 1 µg C per liter, except for glutamate of which the double amount was added. 1, total mixture (46 µg of C per liter); 2, amino acids, 19 µg of C per liter; 3, carboxylic acids, 14 µg of C per liter; 4, aromatic compounds, 7 µg of C per liter; 5, carbohydrates and (poly)-alcohols, 6 µg of C per liter; 6, without substrates added; observations in duplicate.

Utilization of mixtures of substrates at very low concentrations

In natural habitats,including water, S_n is composed of low concentrations of a great variety of compounds. For this reason and to obtain rapid information about the ability of the isolates to utilize many different compounds at low concentration (1 µg of C per liter), growth experiments were performed in tap water supplied with the mixtures of 18 amino acids (AA), 6 carbohydrates and (poly) alcohols (CHA), 14 carboxylic acids (CA), 7 aromatic acids (AR) and the total mixture (TM). Fig. 6 shows that the growth rates (G^{-1}) with the amino acid mixture equalled those with the total mixture, suggesting that amino acids were preferred substrates for strains P17 and P500. Furthermore it is shown that from the other mixtures growth was most rapid with the carboxylic acids.

Using the N_{max} values observed and the numbers of compounds which may serve as a source of carbon and energy for growth (Table 1), average yields, Y_a (CFU/mg of substrate C) were calculated (Table 4). Most Y_a values of P17 exceeded or approximated the yield observed with growth on acetate. This suggests that all substrates which served as a source of carbon and energy for growth of P17 when present in 2.5 g/l were also utilized when present in 1 µg of C per liter. As compared with P17, P500 had relatively low average yields on the mixtures, which is clearly demonstrated with the total mixture of compounds. These differences might result from the inability of P500 to utilize a number of compounds at very low concentrations. Evidence for such a possibility is obtained by the inability of strain P500 to multiply in the presence of 20 µg of fumarate C per liter.

DISCUSSION

This study and previous ones (Van der Kooij et al, 1980, Chapter 5; Van der Kooij and Hijnen, 1981, Chapter 6) reveal that simple growth experiments in batch cultures provide information about bacterial growth at low substrate concentrations. In such experiments colony counts are used as a parameter for biomass concentration. From the linear relationship between maximum colony counts (N_{max}) and initial substrate concentrations as observed in this study (Fig. 2) and those mentioned above, it is concluded that this procedure is justified in a number of cases. A decrease in yield at very low concentrations as observed with an *Aeromonas*

hydrophila isolate growing on glucose (Van der Kooij et al, 1980, Chapter 5) was not found with the isolates P17 and P500 growing on acetate or glutamate (Fig. 2), probably because of the presence of the natural substrates in the tap water used. The interpretation of the generation times observed at initial substrate

Table 4. Maximum colony counts of the fluorescent pseudomonads P17 and P500 grown at 15 °C in tap water supplied with mixtures of compounds.

		strain P17			strain P500		
Mixture[a] added	Total conc.[b] (μg C/l)	Conc. of compounds used[c] (μg C/l)	N_{max} (10^4CFU/ml)	Y_a (10^9CFU/mg C)	Conc. of compounds used[c] (μg C/l)	N_{max} (10^4CFU/ml)	Y_a[d] (10^9CFU/mg C)
None	-	-	2.3; 2.3	-	-	2.9; 5.0	-
AA	19	17	15; 16	7.8	14	4.8; 8.1	3.0
CA	14	9	4.6; 5.8	3.2	8	3.8; 6.9	3.9
CHA	6	4	4.1; 4.8	5.4	4	2.5; 3.5	2.0
AR	7	3	3.5; 4.2	5.2	1	2.2; 2.2	< 1
TM	46	33	22; 23	6.1	27	6.2; 6.4	1.5

[a] AA = amino acids; CA = carboxylic acids; CHA = carbohydrates and (poly)alcohols; AR = aromatic acids; TM = total mixture

[b] All compounds added in amounts of 1 μg of C per liter, except glutamate which was added in an amount of 2 μg of C per liter.

[c] Total concentration of compounds used as sources of carbon and energy for growth when present at 1 g/l (cf. Table 1).

[d] Due to unreliable N_{max} values in the blancs, Y_a of P 500 is calculated using N_{max} with the AR mixture as reference values for growth on S_n.

concentrations below 10 μg C per liter was also complicated by the utilization of S_n (Fig. 3). These observations indicate that for growth experiments at extremely low substrate concentrations, the natural substrate concentration of the water is of critical importance. Drinking water prepared by biological treatment (e.g. slow sand filtration) seems to be most suited for this purpose.

Fluorescent pseudomonads are able to utilize a wide variety of naturally occurring compounds as sources of carbon and energy

(Den Dooren de Jong, 1926; Stanier et al, 1966; Table 1). Fumarate which was consumed at 2.5 g per liter could not be utilized by P500 when present at 20 µg of C per liter. Previous investigations revealed that an *Aeromonas hydrophila* isolate did not grow with acetate, glutamate or succinate when these compounds were present in an amount of 10 µg of C/1 though these substrates were utilized at 1 mg of C per liter (Van der Kooij et al. 1980, Chapter 5). These findings stress the importance of investigating growth responses at concentrations of a few micrograms per liter.

The significance of the naturally occurring amino acids as sources of carbon and energy for strains P17 and P500 was clearly demonstrated (Fig. 6; Table 4). Amino acids are also suitable substrates for *P. aeruginosa* (Stanier et al, 1966), which organism possesses constitutive transport systems for the uptake of these compounds at very low concentrations (Kay and Gronlund, 1969; 1971). Therefore utilization of low concentrations of amino acids seems a common character of the mesophilic and psychrotrophic fluorescent pseudomonads. This may explain why fluorescent pseudomonads occur in the bacterial populations on fresh plant debris and in rhizospheres in larger percentages than in soil itself (Rovira and Sands, 1971). Despite high-affinity uptake systems for amino acids, fluorescent pseudomonads appear to be unable to compete with many other bacteria, as may be concluded from the low percentage in which they are usually found in natural bacterial populations. Very low concentrations of dissolved free amino acids in natural environments (Burnison and Morita, 1974) combined with the presence of biodegradable compounds for which the fluorescent pseudomonads do not have saturation constants as low as other bacteria, e.g. carbohydrates which are utilized by flavobacteria (Van der Kooij and Hijnen, 1981, Chapter 6), may explain these observations.

The occurrence of representatives of specific biotypes of the fluorescent pseudomonads in specific environments as demonstrated by Sands and Rovira (1971) and Van der Kooij (1979) points to significant differences between the various psychrotrophic fluorescent pseudomonads. The distinct differences in substrate affinities as demonstrated in this study help explain that bacteria similar to strain P17 (biotype 7.2, Van der Kooij, 1979) were isolated more frequently from drinking water prepared from ground water than bacteria similar to strain P500 (biotype 1.1,

Van der Kooij, 1979). The relatively frequent occurrence of biotype 7.2 in various waters may further be related to its ability of using nitrate as a hydrogen acceptor combined with a high affinity for acetate, a compound which occurs in oxygen-depleted environments. The frequent predomination of biotype 1.1 in surface water and in tap water derived from surface water (Van der Kooij, 1979) cannot be explained with the obtained K_s values, but the similarity of the growth rates of P17 and P500 with the amino acids mixture, which is in contrast with the differing saturation constants, may be important in this respect.

Experiments with fluorescent pseudomonads, including *P. aeruginosa*, using labelled substrates have frequently revealed high affinity transport systems which are highly substrate specific, though groups of related compounds, e.g. dicarboxylic acids, aromatic amino acids or aliphatic amino acids, may be transported by one and the same system (Kay et al. 1969, 1971; Dubler et al. 1974, Stinson et al. 1976, Tsay et al. 1971, Eisenberg et al. 1974, Romano et al. 1980, Hoshino, 1979). Although transport constants (K_t, 0.1 - 1 µM) resemble K_s values obtained for P17, it remains uncertain whether K_s and K_t should have similar numerical values. Such a similarity would indicate that the transport of a substrate is the growth limiting step. The observed constant difference in K_s values of strains P17 and P500 for compounds requiring a number of totally different transport systems (Tables 2 and 3) suggests that a metabolic process was limiting the growth of P500 in the described experiments. Arguments in support of this suggestion have been given by Kay and Gronlund (1969, 1971), who observed that transport of amino acids into cells of *P. aeruginosa* rapidly declined after a few minutes as a result of saturation of the pool with the unchanged compounds. Further uptake was depending on incorporation of these amino acids from the pool into cellular proteins. The rate of C_4-acid transport into cells of *Escherichia coli* also appeared to be determined by the rate at which these substrates were metabolized (Kay and Kornberg, 1971).

The experiments reported in this paper (Table 2) revealed that the N_{max} value of a pure culture in a specific water does provide information about the concentration of compounds available to the organism as a substrate. In addition, using the observed generation time, maximum possible concentrations of specific compounds for which the coefficients of the Lineweaver-Burk equations (Tables 2

and 3) are known, may be calculated. Since P17 is able to utilize a large variety of compounds at very low concentrations, growth experiments with this organism may be valuable for the assessment of the level of easily assimilable organic carbon (AOC) in water. In recent investigations, such growth experiments are being used to study the quality of various types of drinking water and for measuring the effects of water treatment procedures on biodegradable compounds in water (Van der Kooij, 1979a).

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3. The effects of water treatment procedures on the concentration of easily assimilable organic carbon (AOC) assessed by growth experiments with a fluorescent pseudomonad

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Abstract

A strain of the species *Pseudomonas fluorescens* was grown in water samples collected from the various treatment stages of 8 treatment plants, in 7 of which drinking water was prepared from surface water. The concentrations of easily assimilable organic carbon (AOC) were calculated from the obtained maximum colony counts and the yield of the organism on acetate. AOC concentrations usually lay between 10 and 100 µg of acetate C equivalents per liter and were below 1 % of the DOC concentration in the waters examined. In water from open storage basins and in water after ozonation higher concentrations were observed. A linear relationship was obtained between AOC volume load and AOC uptake in rapid sand filters. Maximum AOC reductions of about 80 % were observed at influent AOC concentrations above 50 µg of C per liter. At AOC concentrations below 25 ug of C per liter, small reductions (< 50 %) were achieved by filtration processes.

Keywords: water treatment, drinking water, easily assimilable organic carbon, *Pseudomonas fluorescens*, ozonation, biological processes, filtration.

NOMENCLATURE

AOC	= assimilable organic carbon, µg acetate C equivalents l^{-1}
AOC_i	= AOC concentration in influent of filter, $mg.m^{-3}$
AOC_e	= AOC concentration in effluent of filter, $mg.m^{-3}$
AOC_L	= average AOC load, $mg.m^{-3}$ filter bed.h^{-1}
AOC_R	= average AOC uptake, $mg.m^{-3}$ filter bed.h^{-1}
DOC	= dissolved organic carbon, $mg.l^{-1}$
G	= generation time (mean doubling time), h
G_{min}	= minimum generation time, h
K_s	= substrate-saturation constant, µg $C.l^{-1}$
N_{max}	= maximum colony count, colony-forming units (CFU).ml^{-1}
R	= percent AOC reduction
S	= substrate concentration, µg $C.l^{-1}$
S_{ac}	= maximum apparent acetate concentration, µg $C.l^{-1}$
S_{ac}/AOC	= relative biodegradability factor
Y	= yield, $CFU.mg^{-1}$

INTRODUCTION

Most water treatment systems used for the preparation of drinking water include stages either with biological activity or with biological activity affected in a subsequent stage. The intensity of biological processes is depending on the concentration of organic and inorganic compounds in the water serving as sources of carbon and/or energy for the growth of microorganisms. Hence, the removal of such compounds by filtration through inert media is related to the biological activity in the filter. The concentration of biodegradable carbon in water is also affected by physical and chemical treatments, including coagulation/sedimentation and ozonation.

Quantitative information about the effects of the various treatments is required to improve the removal of biodegradable organic compounds either in a specific treatment or in the whole purification system. Improvements may be necessary for the prevention of either excessive or insufficient biological activity during treatment as well as to produce water with a low concentration of easily biodegradable organic carbon. Distribution of such water has been advised to prevent deterioration of drinking water quality during its transport in the distribution system as caused by regrowth of bacteria and animals (Hutchinson and Ridgway, 1977; Smalls and Greaves, 1968).

No chemical technique is available for measuring the concentration of biodegradable organic carbon. Furthermore, measurement of the biological oxygen demand is unreliable at values below a few mg/l. A bacteriological method, which is based on estimating the growth curve of a pure culture of *Pseudomonas fluorescens* in the water, was therefore developed. This technique enables the assessment of the concentration of easily assimilable organic carbon (AOC) (Van der Kooij et al, 1982b). The effects of water treatments generally applied for the preparation of drinking water from surface water have been investigated with this method in a number of treatment plants in the Netherlands.

MATERIALS AND METHODS

AOC determination

The AOC determination is based upon the estimation (in

duplicate) of the growth curve of *Pseudomonas fluorescens* strain P17 in 600 ml of the water to be tested, contained in thoroughly cleaned glass-stoppered Pyrex-glass flasks of 1 liter capacity, after heating (1 h at 60°C) and incubation at 15°C (Van der Kooij et al, 1982b). A small volume (approximately 0.5 ml) of autoclaved tap water (filtrate of the slow sand filters of the Municipal Dune Waterworks of The Hague, cf. Table 2) in which the organism was grown, was used as inoculum. The initial colony counts were usually below 1000 CFU (colony-forming units) per ml. Growth curves of the organism were derived from periodic colony counts obtained after spreading 0.05 ml of the culture or of decimal dilutions on the surface of Lab-Lemco (Oxoid) agar plates (in triplicate) and incubation at 25°C. The AOC is calculated in micrograms of acetate carbon equivalents per liter using the maximum colony counts (N_{max}, CFU/ml) and the yield of strain P17 on acetate, i.e. Y(P17)= 4.1×10^6 CFU/ug of acetate C (Van der Kooij et al. 1982b). *P. fluorescens* P17 was used for the AOC determinations because it was found that this organism is able to utilize a large number of easily assimilable organic compounds including most naturally occurring amino acids (Van der Kooij et al, 1982a, Chapter 2).

From the growth curves also generation times (G, hours) were calculated. These generation times are affected by the concentration and the biodegradability of the organic compounds utilized by the isolate. The relationship between G and the concentration of a biodegradable compound is given by the following adapted Lineweaver-Burk expression:

$$G = G_{min} + G_{min} \cdot K_s/S \quad (1)$$

in which: G_{min} = minimum generation time; S = substrate concentration in µg of C per liter and K_s, the substrate-saturation constant, equals S at wich G = 2 G_{min}. The relationship between the generation time of P17 (at 15 °C) and the acetate concentration (S_{ac} in µg of C/l) has been determined in a previous study (Van der Kooij et al. 1982a, Chapter 2) and is given by:

$$G = 5.5 + 21.8/S_{ac} \quad (2)$$

The maximum apparent acetate concentration (S_{ac}, µg of C/l) present

in the sampled water may be calculated with eq. 2 using the generation time observed with P17 cultures on AOC. When S_{ac}/AOC > 1, then compounds are present which are more easily utilized by isolate P17 than acetate. The quotient S_{ac}/AOC therefore is a relative measure of the average biodegradability of the compounds contributing to the AOC present in the water.

Measurement of dissolved organic carbon

The concentration of dissolved organic carbon (DOC) was assessed after filtration of water samples through membranes (pore size 0.45 μm) of cellulose acetate. For the analysis the Dohrmann DC-54 ultra low level organic carbon analyzer system was used.

Sampling and sampling location

Water samples (600 ml) for the AOC determination were collected in the cleaned flasks described above. They were heated on the day of sampling, inoculated and incubated at 15°C. Most of the samples were taken in the period between October 1979 and May 1980. The investigation at Weesperkarspel was repeated in April 1983. The water treatment sequences of the investigated treatment plants are presented in Table 1.

Table 1. Water treatment sequences at 8 treatment plants in the Netherlands.

1. *Leiduin* (Amsterdam Waterworks).
   Source: river Rhine.
   Treatment: coagulation and sedimentation (1); rapid sand filtration (2); transport chlorination (3); transportation; dune infiltration; collecting in Oranjekom (basin); dosage of powdered activated carbon (PAC), pH correction, aeration; rapid sand filtration; slow sand filtration; post chlorination; distribution.
   Treatments 1-3 and transportation by WRK (Water Transport Company "Rijn-Kennemerland").

2. *Weesperkarspel* (Amsterdam Waterworks).
   Sources: Water from Amsterdam Rhine Canal and water from Bethune Polder.
   Treatment: after coagulation, these waters are stored in a

reservoir (the Loenderveense Plas); rapid sand filtration; ozonation; pH correction, dosage of PAC, ferric chloride and Wisprofloc[a] (coagulant aid); coagulation and sedimentation; rapid sand filtration, slow sand filtration; post chlorination; distribution.

3. *Baanhoek* (Municipal Energy Works of Dordrecht).
   Source: river Rhine.
   Treatment: dosage of ferrous sulfate; storage in resevoir "Grote Rug"; breakpoint chlorination; dosage of ferrous sulfate, sodium hydroxide, lime and Wisprofloc[a]; coagulation; upflow filtration; downflow filtration; ozonation; mixing (see Oranjelaan).

4. *Oranjelaan* (Municipal Energy Works of Dordrecht).
   Source: ground water.
   Treatment: aeration; dosage of lime and Perfectamyl[b] (coagulant aid); coagulation/sludge blanket filtration; dry filtration; rapid filtration; mixing; (with water from *Baanhoek*); chlorination; distribution.

5. *Scheveningen* (Dune Water Works of The Hague).
   Source: river Meuse.
   Treatment: Storage in reservoir "Andelse Maas"; transport chlorination; transportation; rapid sand filtration; transport chlorination; transportation; dune infiltration; dosage of PAC and sedimentation; aeration; rapid sand filtration; slow sand filtration; distribution. Only dune infiltration etc. at Scheveningen.

6. *Andijk* (Provincial Waterworks of North Holland).
   Source: water from lake IJssel.
   Treatment: storage in reservoir; microstraining; breakpoint chlorination; dosage of ferric chloride sulfate, lime and Wisprofloc[b]; coagulation and sedimentation; dosage of sodium sulphite; rapid sand filtration; GAC filtration (2 stages); post disinfection (Cl_2 + ClO_2); microstraining; pH correction with sodium hydroxide; distribution.

7. *Kralingen* (Rotterdam Waterworks).
   Source: river Meuse.

Treatment: storage in reservoirs ("Brabantse Biesbosch") (1) transport chlorination (2); transportation (3); dosage of ferric sulphate, sodium hydroxide and Perfectamyl[a]; coagulation and sedimentation; ozonation; dosage of sodium hydroxide and Perfectamyl[a]; dual media filtration; GAC filtration; post chlorination; distribution. 1-3 by the Water Storage Corporation Brabantse Biesbosch.

8. *Zevenbergen* (Waterworks of North West Brabant).
   Source: river Meuse.
   Treatment: storage in reservoirs (see *Kralingen*); breakpoint chlorination; coagulation/flotation rapid sand filtration; GAC filtration, post chlorination; distribution.

[a] coagulant aid added only at low water temperatures (< 10 °C)
[b] coagulant aid added throughout the year

RESULTS

AOC-concentration profiles

Water sampled from the various treatment stages of 8 treatment plants, of which 7 used surface water for the preparation of drinking water, was tested by performing growth experiments with *P. fluorescens* P17. The water treatment plants at Kralingen and Weesperkarspel were investigated twice. Water temperatures were relatively low (< 10 °C) at the time of sampling. The growth curves of one of the investigations demonstrate the similarity of the bacterial growth in duplicate samples as well as the effects of various treatment procedures (Fig. 1). The AOC concentrations as calculated from the average N_{max} values of *P. fluorescens* P17 reveal that the AOC concentrations in the water types investigated were usually between 10 and 100 µg of acetate C equivalents per liter (Fig. 2). Moreover, the effects of the various treatments are clearly demonstrated.

AOC and S_{ac} concentrations, in water after open storage, after dune infiltration and in ground water

The AOC concentration of water after storage in open basins lay between 25 and 500 µg of acetate C eq./l and was usually less than a few percents of DOC. The highest AOC concentration, observed in

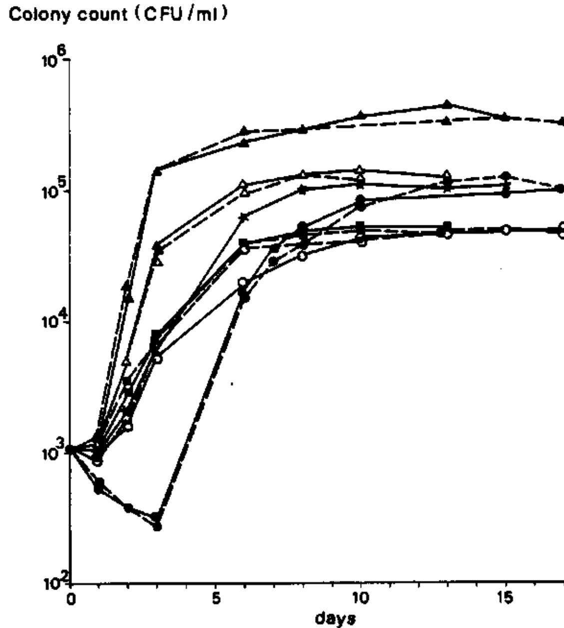

Fig. 1 Growth curves of *P. fluorescens* P17 in water sampled from various stages of the treatment plant at Kralingen (Rotterdam Waterworks). ●, after storage; O, after coagulation/sedimentation; ▲, after ozonation; Δ, after dual media filtration; ■, after GAC filtration; ★, after safety chlorination. The growth experiments were performed in duplicate (except★).

water after storage at Andijk, amounted up to 8.5 % of the DOC concentration (Table 2). Collection of dune-infiltrated river water in the Oranjekom basin at Leiduin resulted in a clear AOC increase (Fig. 2) which may be explained by the presence of algae in the basin. To which extent the AOC concentrations in the other situations either resulted from the AOC in water prior to storage or from AOC production during storage was not further investigated.

The few observations on water after dune infiltration indicate that the AOC content may be low after underground storage in the dunes. The AOC of anaerobic ground water after aeration at Oranjelaan, however, was even lower and contributed to less than

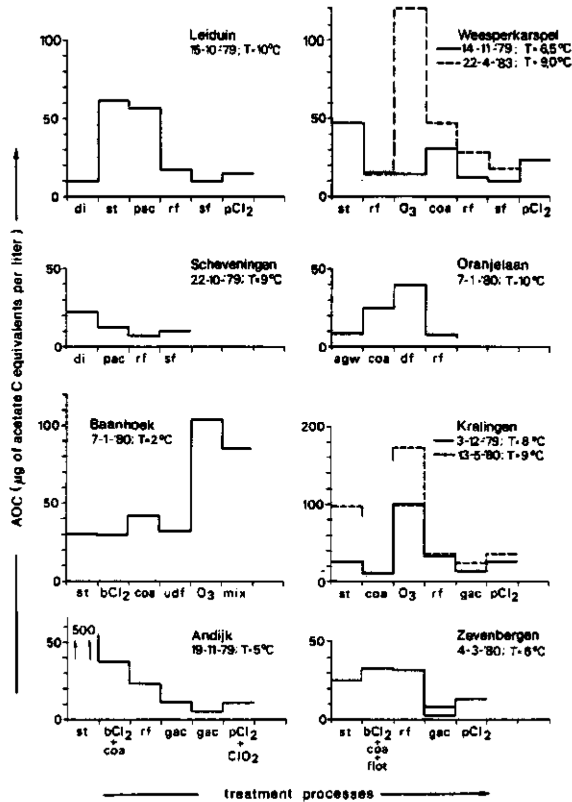

Fig. 2 AOC concentrations of water after various treatment in 8 water treatment plants in the Netherlands; di = dune infiltration; st = open storage; agw = anaerobic ground water after aeration; bCl_2 = breakpoint chlorination; pCl_2 = post chlorination; pac = dosage of powdered activated carbon; coa = coagulation usually followed by sedimentation; flot = flotation; O_3 = ozonation; rf = rapid filtration; df = downflow filtration; udf = upflow and downflow filtration; gac = granular activated carbon filtration; ClO_2 chlorine dioxide dosage; mix = mixture of ozonated water (Baanhoek) with rapid sand filtrate (Oranjelaan), after chlorination. The results shown are averages from duplicate experiments.

0.2 % of the DOC concentration. Table 2 shows that S_{ac} varied from 0.8 µg of C (in ground water) to more than 50 µg of C/l (in river water after storage). In a few cases S_{ac} could not be calculated because G was less than 5.5 h, indicating that the biodegradable compounds which contributed to AOC were more rapidly utilized by strain P17 than acetate.

Table 2. DOC, AOC and S_{ac} in ground water, in surface water after dune infiltration, and in surface water after storage in an open reservoir.

Watertype	AOC (µgC/l)[a]	DOC (mgC/l)	$\frac{AOC}{DOC}$x100%	G^{b} (h)	S_{ac}^{c} (µgC/l)	$\frac{S_{ac}}{AOC}$
Ground water						
(Oranjelaan)	7;8	4.6	0.16	35.5;28.0	0.8	0.11
Water after dune filtration						
Leiduin	10;11	3.0	0.35	17.7;18.7	1.7	0.16
Scheveningen	19;24	3.0	0.72	9.1;8.9	6.2	0.29
Water after storage in an open reservoir						
Leiduin (Oranjekom)[d]	60;62	3.2	1.9	7.8;8.0	9.1	0.15
Weesperkarspel						
(Loenerveense Plas)	48;45	7.4	0.63	5.8;6.0	54.5	1.17
Baanhoek(Grote Rug)	31	3.8	0.81	5.1	-[e]	> 1.00
Kralingen(B. Biesbosch)	24;29	3.6	0.74	12.8;12.8	3.0	0.11
Kralingen(B. Biesbosch)	97;96	4.0	2.4	6.0;5.9	48.4	0.50
Andijk(IJsselmeer)	450;500	5.6	8.5	5.2;5.4	-[e]	> 1.00
Zevenbergen						
(B. Biesbosch)	23;26	3.9	0.63	6.3;5.9	37.0	1.51

[a] In µg of acetate C equivalents per liter.
[b] Generation time of P17 at 15°C.
[c] Average value.
[d] Collecting reservoir, no storage.
[e] $G < G_{min}$ of P17 with acetate.

Table 3. DOC, AOC and S_{ac} in filtrates.

	AOC (μgC/1)[a]	DOC (mgC/1)	AOC/DOC x100%	G[b] (h)	S_{ac} (μgC/1)	S_{ac}/AOC
Rapid sand filtration						
Leiduin	15;21	3.0	0.60	6.8;6.8	16.8	0.93
Weesperkarspel (Loenen)	15;15	6.9	0.22	11.9;11.9	3.4	0.23
Weesperkarspel[c]	14;10	5.9	0.20	22.9;21.3	1.3	0.11
Weesperkarspel[c]	26;31	6.2	0.46	11.8;10.8	3.8	0.13
Baanhoek	33;33	4.0	0.83	9.2;8.1	7.0	0.21
Oranjelaan	7	4.5	0.16	26.2	1.1	0.16
Scheveningen	6;8	3.1	0.22	35.2;24.5	0.9	0.13
Andijk	26;24	3.3	0.76	15.5;21.10	1.8	0.07
Kralingen[c]	33;31	2.6	1.23	7.9;11.0	5.9	0.18
Kralingen[c]	33;36	2.6	1.32	8.3;8.3	7.8	0.23
Zevenbergen	31;33	3.3	0.97	22.3;19.8	1.4	0.04
Slow sand filtration						
Leiduin	10	2.8	0.36	44.9	0.6	0.06
Weesperkarspelc	10;10	5.6	0.18	29.2;26.0	1.0	0.10
Weesperkarspelc	15;19	5.9	0.30	22.6;16.7	1.6	0.09
Scheveningen	11;9	3.0	0.33	13.8;22.2	1.9	0.19
GAC filtration						
Andijk 1st stage	11;13	3.0	0.40	22.2;19.9	1.4	0.12
Andijk 2nd stage	6;6	2.1	0.29	29.4;31.8	0.9	0.15
Kralingen[c]	12;12	1.9	0.63	20.0;19.6	1.5	0.03
Kralingen[c]	23;26	2.5	0.98	10.4;10.9	4.2	0.17
Zevenbergen(old)[d]	8;7	2.3	0.33	19.5;23.7	1.4	0.19
Zevenbergen(new)[d]	3.1;3.6	0.9	0.37	23.8;28.3	1.1	0.32

[a] In μg of acetate C equivalents per liter.
[b] Generation time of P17 at 15° C.
[c] Investigated at two different dates (cf. Fig. 2); on the second date TOC was assessed instead of DOC.
[d] Filters after different operation periods.

Effects of filtration

The various types of filtration usually caused a definite AOC reduction (Fig. 2). With one exception, AOC in the filtrates was less than 1 % of DOC (Table 3). Most S_{ac} values were less than 2 µg of C per liter and S_{ac}/AOC values were between 0.04 and 0.32 with the exception of the rapid sand filtrate at Leiduin where a value of 0.93 was obtained. The average AOC volume load of the investigated filters (AOC_L in mg of acetate C equivalents per m^3 of filter bed per hour) was calculated with:

$$AOC_L = AOC_i \times 60/t \qquad (3)$$

where AOC_i = AOC concentration in the influent (mg/m^3) and t = empty bed contact time (in minutes). In addition, the average specific AOC reduction (AOC_R, in mg of acetate equivalents per m^3 filter bed per hour) was calculated from:

$$AOC_R = (AOC_i - AOC_e) \times 60/t \qquad (4)$$

where AOC_e = AOC concentration in the effluent (mg/m^3). The obtained AOC_L values varied from less than 1 mg per m^3 of filter bed per hour for slow sand filtration to values above 800 mg for rapid filtration of ozonated water (Table 4). Between AOC_L and AOC_R for rapid filtration a linear relationship exists (Fig. 3). This relationship is calculated as:

$$AOC_R = 0.83\ AOC_L - 15\ (n = 10;\ r = 0.997) \qquad (5)$$

Most observations on granular activated carbon (GAC) filtration were close to this relationship but AOC_R values observed with rapid filtration of chlorinated water were less than could be expected from eq. 5. Obviously, chlorination is inhibiting the biological activity during filtration through media which do not inactivate free chlorine. AOC_R values for slow sand filtration also do not fit into eq. 5, as is further demonstrated in Fig. 4, in which the percent AOC reduction (R), which equals:

$$(AOC_i - AOC_e)/AOC_i \times 100\ \% = (AOC_R/AOC_L) \times 100\ \% \qquad (6)$$

is presented in relation to the average AOC volume load *of the*

Table 4. Average AOC volume loads (AOC_L) and average specific AOC reductions (AOC_R) with rapid sand filtration, GAC filtration and slow sand filtration at various treatment plants.

	t^a (min)	AOC^b	AOC_L^c	AOC_R	AOC_R/AOC_L x100 %
Rapid sand filtration					
Leiduin	25.5	41	137	96	70
Weesperkarspel [d]	16	88	397	332	84
Weesperkarspel	15.5	18	115	70	61
Weesperkarspel(Loenen)	31	32	90	62	69
Baanhoek [e]	33.6	9	76	17	22
Oranjelaan	22	31	104	85	81
Scheveningen [d]	30	3	14	7	50
Scheveningen	30	6	25	12	48
Andijk [e]	9.2	14	254	91	36
Kralingen [d]	12	142	854	710	83
Kralingen [f]	14.7	69	414	284	68
Kralingen [f]	12.2	136	839	669	80
Zevenbergen [e]	38.4	1	52	1.6	3
Slow sand filtration					
Leiduin	315	8	3.4	1.5	45
Weesperkarspel [d]	223	-6	4.7	-1.6	-
Weesperkarspel	205	2	3.5	0.6	17
Scheveningen [d]	273	0.5	0.8	0.1	14
Scheveningen	207	-3	2.0	-0.9	-
GAC filtration					
Andijke 1st stage	15.5	11.5	91	44.5	49
Andijk 2nd stage	15.5	6	46	27	50
Kralingen [d]	11.8	16.5	137	84	61
Kralingen [f]	11.1	20	173	108	62
Kralingen [f]	10.8	10	192	56	29
Zevenbergen(old) [g]	7.2	24.5	267	204	76
Zevenbergen(new) [g]	7.2	28.5	267	242	90

[a] Empty bed contact time in minutes.
[b] Reduction of AOC concentration in µg of acetate C equivalents per liter (average values).
[c] In mg acetate C equivalents per m^3 filter bed per hour.
[d] Data from a previous investigation (Van der Kooij et al. 1982[b]).
[e] Water containing free chlorine.
[f] Investigated at two different dates (cf. Fig. 2).
[g] GAC filters after different operation periods.

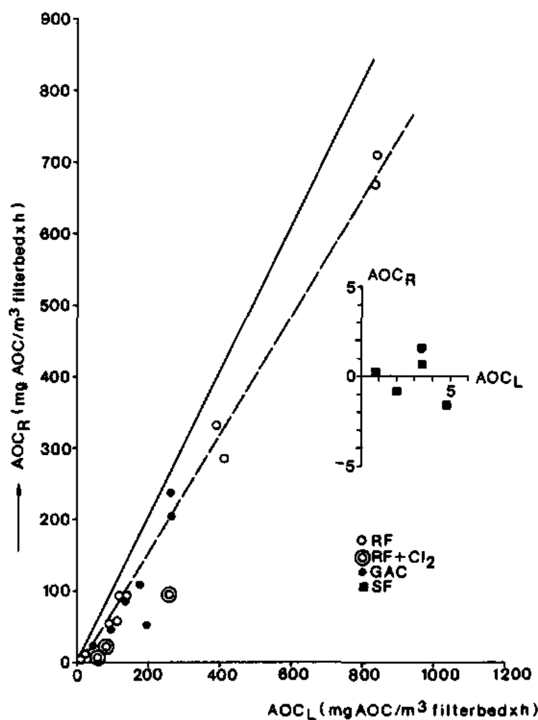

Fig. 3 Average specific AOC reductions (AOC_R) in relation to the average AOC volume loads (AOC_L) during filtration processes. RF = rapid filtration (including dual media filtration); RF + Cl_2 = rapid filtration of water after chlorination; GAC = granular activated carbon filtration; SF = slow sand filtration. The results shown are calculated using the average values of the AOC concentrations which had been assessed in duplicate.

various filters. The GAC filter at Zevenbergen with an operation period of about 5000 bed volumes caused a much larger AOC reduction than the GAC filter with an operation period of 25000 bed volumes. This suggests that adsorption processes may enhance AOC reduction. On the other hand, relatively small AOC reductions as incidentaly obtained for GAC filtration (Fig. 4), indicate that removal of easily biodegradable compounds by this treatment may sometimes be

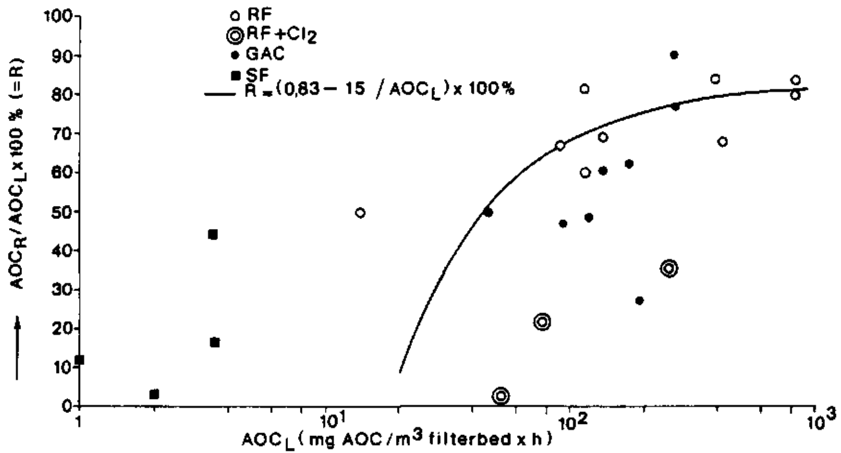

Fig. 4 AOC reduction (R) during filtration at different average AOC volume loads (AOC_L). For symbols see Fig. 3.

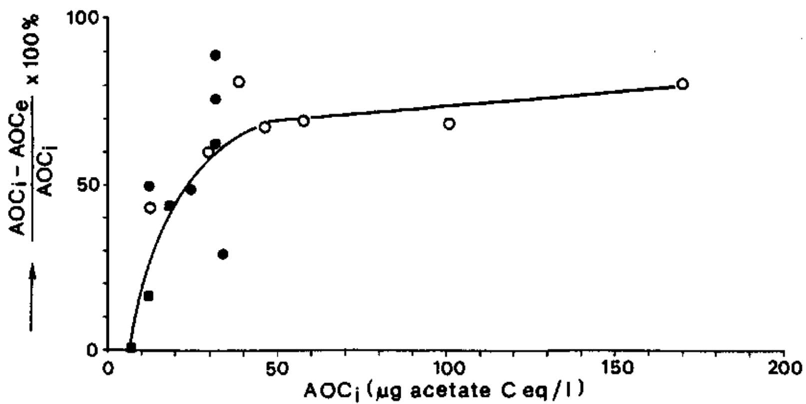

Fig. 5 AOC reduction by filtration of water with different AOC concentrations in the influent (AOC_i). For symbols see Fig. 3.

poorer than with rapid sand filtration.

The obtained AOC reductions as observed for slow sand filtration are relatively small but greater than expected with eq. 5. It seems that a significant further reduction of AOC concentrations, which are below 10 to 15 µg of acetate C eq./l, is hard to achieve with biological processes during filtration through an inert material (Fig. 5).

Effects of coagulation

Coagulation and sedimentation in combination with breakpoint chlorination gave an AOC reduction of 92 % at Andijk (Fig. 2). At Kralingen an AOC reduction of 50 % was observed after coagulation and sedimentation. The AOC concentration of ozonated water was reduced by 60 % after coagulation and sedimentation at Weesperkarspel, but without ozone dosage this treatment caused a small AOC increase. Small increases were also observed in Baanhoek and Oranjelaan (Fig. 2). In all cases studied, starch-based coagulant aids had been applied, which, if remaining in the treated water, as such do not influence AOC because isolate P17 is unable to utilize starch. Biodegradation of these compounds during the coagulation and sedimentation processes, might liberate low molecular weight compounds contributing to the AOC concentration.

Effects of ozone, chlorine (and chlorine dioxide)

Ozonation clearly increased the AOC concentration. The ozone installation at Weesperkarspel was out of operation on the first sampling date (Fig. 2). The AOC concentrations of ozonated water usually were above 100 µg of ac C eq./l and were more than 5 % of DOC (Table 5). Although AOC values had increased and relatively high S_{ac} values were obtained, compounds produced by ozonation and contributing to AOC seem to be more difficult to utilize by P17 than acetate, so that usually $S_{ac}/AOC < 1$.

Chlorination usually caused a doubling of the AOC concentration, but in most cases the average relative biodegradability was reduced (Table 6). Moreover the decrease of S_{ac} values as observed at Kralingen and Zevenbergen suggests that the biodegradability of compounds contributing to the AOC concentration is decreased by chlorination. Strain P17 did not multiply within a period of one month in the water at Andijk, sampled after dosage of chlorine and chlorine dioxide. Thereafter growth was observed with higher N_{max}

values than before disinfection. In chlorinated water, isolate P17 always multiplied directly after inoculation. Chlorine dioxide or its reaction products seem very persistent and may therefore be effective in repressing regrowth in water during distribution.

Table 5. Effect of ozonation[a] on AOC and S_{ac}

Site	AOC (μgC/l)[b]	DOC (mgC/l)	AOC/DOC x 100 %	G^b (h)	S_{ac} (μgC/l)	S_{ac}/AOC
Weesperkarspel						
before O_3	14;16	7.2	0.21	9.5;9.9	5.1	0.34
after O_3	117.124	7.1	1.7	8.7;8.3	7.2	0.06
Baanhoek						
before O_3	33;33	4.0	0.83	9.2;8.1	7.0	0.21
after O_3	116;95	3.8	2.8	5.7;6.0	61.6	0.58
Kralingen						
before O_3	12;12	3.0	0.40	13.1;12.3	3.0	0.25
after O_3	102;79	2.9	3.12	7.9;7.7	9.5	0.10
Kralingen						
before O_3[d]	-	-	-	-	-	-
after O_3	167;174	3.0	5.7	5.8;6.1	48.1	0.28

[a] Applied ozone dosage between 2-3.5 mg of O_3 per liter
[b] In μg of acetate C equivalents per liter.
[c] Generation time of P17 at 15°C.
[d] Not sampled.

DISCUSSION

The concentration of easily assimilable organic carbon as defined in this paper is related to the characteristics of the micro-organism used in the growth experiments. Isolate P17 belongs to the species *P. fluorescens*, which is known for its extreme versatility in substrate utilization (Stanier et al. 1966). Previous investigations have shown that *P. fluorescens* P17 is able to utilize a great variety of substrates present at a level of a few micrograms per liter (Van der Kooij et al, 1982a, Chapter 2). Nevertheless, a number of biodegradable compounds, including methane and starch, are not utilized by members of this species. Thus methane,

Table 6. Effect of chlorination on AOC and S_{ac}

Site		AOC (μgC/l)[a]	DOC (mgC/l)	AOC/DOC x 100 %	G^b (h)	S_{ac} (μgC/l)	S_{ac}/AOC
Leiduin							
before chlorination		10	2.8	0.36	44.9	0.5	0.05
after chlorination with 0.55 mg of Cl_2/l	f	19;16	2.7	0.65	18.8;15.5	1.9	0.11
Weesperkarspel							
before chlorination		10;10	5.6	0.18	29.2;26.0	1.0	0.10
after chlorination with 0.6 mg of Cl_2/l	f	26;22	8.3	0.29	12.7;15.2	2.6	0.11
Baanhoek							
before chlorination		31	3.8	0.82	5.1	-e	> 1
after chlorination with 3.0 mg of Cl_2/l	c	31	4.0	0.78	6.9;6.3	20.0	0.65
Andijk							
before chlorination		6;6	2.1	0.28	29.4;32.9	0.9	0.15
after chlorination with 0.03 mg of Cl_2 + 0.03 mg of ClO_2/l	f	11;12	1.5	0.77	-d	-d	-d
Kralingen							
before chlorination		12;12	1.9	0.63	20.0;19.6	1.5	0.13
after chlorination with 0.4 mg of Cl_2/l	f	26	2.1	1.23	20.7	1.4	0.05
Kralingen							
before chlorination		23;26	2.5	0.98	10.4;10.9	4.2	0.17
after chlorination with 0.4 mg Cl_2/l	f	33;36	2.1	1.64	17.6;18.5	1.7	0.05
Zevenbergen							
before chlorination		23;26	3.9	0.63	6.3;5.9	37.0	1.51
after chlorination with 2.0 mg of Cl_2/l	c	33;33	3.4	0.97	2.75;27.5	1.0	0.03
Zevenbergen							
before chlorination		7;8	2.3	0.33	19.5;23.7	1.4	0.19
after chlorination with 0.5 mg of Cl_2/l	f	13;14	2.0	0.68	79.3;82.3	0.3	0.02

[a] μg of acetate C equivalents per liter.
[b] Generation time of P17 at 15° C.
[c] breakpoint chlorination.
[d] G not measured.
[e] G < 5.5 h.
[f] Water as distributed.

as may be present in anaerobic ground water, and starch or starch-like compounds, which are used as coagulant aids, do no directly contribute to the AOC concentration as measured by the technique used in this study. Still, these compounds may promote microbial growth during treatment and distribution of the water. At present, experiments with representatives of the genus *Flavobacterium* are performed to assess the presence of starch-like compounds in water. These bacteria are effectively utilizing starch and maltodextrins at very low concentrations (Van der Kooij and Hijnen, 1981, Chapter 6; Van der Kooij and Hijnen, 1983, Chapter 7). Methane can be determined by a simple gaschromatograhic technique.

Acetate is produced by the reaction of ozone with humic acids (Ahmed and Kinney, 1950). The low S_{ac}/AOC quotients in ozonated water (cf. Table 5) suggest that acetate is only partially responsible for the AOC increase as caused by ozonation. The identity of most compounds which contributed to the observed AOC increases is not known. Ozonation also results in the production of oxalic, glyoxylic and formic acids (Ahmed and Kinney, 1950; Kuo at al. 1977), which are not utilized by P17. Recent studies reveal that the presence of these acids in ozonated water may be demonstrated by growth experiments with oxalate-utilizing bacteria.

AOC concentrations were usually less than 1 % and in a few cases even less than 0.3 % of the DOC concentration. Low molecular-weight compounds therefore constitute a very small part of the organic carbon content of the water. In many samples, the maximum apparent acetate concentration (S_{ac}) was only a small proportion of the AOC concentration. The real acetate concentrations are less than the S_{ac} values and remain unknown, as well as the identity of the other compounds contributing to the AOC values. The presence of compounds not contributing to the AOC concentration (apart from those mentioned above), but which are biodegradable by other micro-organisms occurring in water, soil or filters, cannot be excluded. The small DOC reductions (usually less than 0.5 mg of C/l) observed with rapid sand filtration and slow sand filtration indicate that either biodegradation of compounds not contributing to the AOC concentration is a slow process or that these compounds also contribute for a very minor part to the DOC values. Unfortunately, DOC determinations are not sufficiently accurate to provide reliable information about changes of a few tenths of a milligram per liter.

Many of the compounds utilized by P17 are excellent substrates

for a large variety of bacteria. Therefore, a low AOC concentration in water to be distributed is an important requirement for the prevention of bacterial regrowth. The AOC concentrations of the water produced by the various treatment plants varied with nearly one order of magnitude depending on the type of raw water and the applied treatments (Fig. 2; Tables 2 and 6). The observations are only incidental and it is not clear yet to which extent seasonal variations occur. Additional data on AOC concentrations are also required to establish the relationship between this parameter and the level of bacterial and animal growth in the water during distribution. Such a relationship, however, is sometimes complicated by the presence of chlorine (or chlorine dioxide) in practical situations. On the other hand, it may be reasoned that water whose AOC content may be reduced significantly by biological filtration is not suited for distribution. This argument would indicate that the AOC content of water for distribution should be equal or less than approximately 10 to 15 µg of acetate C eq. (Fig. 5). In most treatment plants AOC concentrations close to this value were obtained after one of the filtration steps with biological activity (Fig. 2).

The use of materials which do not release biodegradable compounds is also important in the prevention of microbiological activity in distribution systems. A variety of microbiological methods to investigate materials for this property have been described and applied (Burman et al. 1977; Schoenen et al. 1978; Ellgas and Lee, 1980). Recent experiments revealed that materials may also be tested by performing AOC determinations in water containing samples of such materials (Van der Kooij et al. 1982b).

The results presented in this paper demonstrate that assessment of AOC concentrations may be used to study individual water treatment processes in which biological processes are prevailing, e.g. filtration processes, and to quantify interrelations between physical, chemical and biological processes. Figure 3 and Table 3 reveal that the average specific AOC loads (AOC_L) of GAC filters and slow sand filters differ 10 to 100 times. This difference is reflected in the average specific AOC reductions (AOC_R) in these filters and explains the observations that numbers of microorganisms in the filtrates of GAC filters generally are much higher than those in slow sand filtrates (Van der Kooij, 1978). An indication is obtained that adsorption in a GAC filter may contribute to reduce

the AOC content. Further investigations regarding the effects of the operational period of GAC filters on AOC removal are needed to estimate the interactions between biological processes and adsorption.

The effect of chlorine on the AOC concentration may be explained by its oxidative capacity. It seems that addition of low concentrations of chlorine results in an increased concentration of compounds which may be utilized by strain P17. These compounds seem less readily biodegradable than compounds contributing to the AOC before chlorination (Table 6). Reaction of chlorine with carboxylic acids, proteins and amino acids (Murphy et al. 1975, Larson and Rockwell, 1979), which are excellent substrates for P. fluorescens P17 (chapter 2), may be responsible for the longer generation times observed in water after chlorination. In practice, stimulation of regrowth by chlorination seems possible (Windle Taylor, 1970). However, the question regarding limitation of the use of chlorine for post disinfection should first of all be answered in view of the necessity of such disinfection and of the health significance of the produced chlorinated organic compounds (Rook, 1974). The alternative use of chlorine dioxide, which seems to inhibit effectively regrowth, should be further evaluated.

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4. Growth of *Pseudomonas aeruginosa* in tap water in relation to utilization of substrates at concentrations of a few micrograms per liter

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Abstract

Five *Pseudomonas aeruginosa* strains were tested for the utilization of 47 low-molecular-weight compounds as sole sources of carbon and energy for growth at a concentration of 2.5 g per liter. Of these compounds 31 to 35 were consumed. Growth experiments in tap water at 15 °C were carried out with one particular strain (P1525), isolated from drinking water. This strain was tested for the utilization of 30 compounds supplied at a concentration of 25 µg of C per liter. The growth rate (number of generations per hour) of P1525 in this tap water was approximately 0.005 h^{-1} and with 10 compounds it was larger than 0.03 h^{-1}. An average yield of 6.2 x 10^9 CFU per mg of C was obtained from the maximum colony counts (colony-forming units per milliliter). The average yield and the maximum colony count of P1525, grown in tap water supplied with a mixture of 45 compounds, each in an amount of 1 µg of C per liter, enabled us to calculate that 28 compounds were utilized. Growth rates of two *P.aeruginosa* strains (including P1525) in various types of water at 15 °C were half of those of a fluorescent pseudomonad. The concentrations of assimilable organic carbon calculated from maximum colony counts and average yield values amounted to 0.1 - 0.7 % of the total organic carbon concentrations in 5 types of tap water. The assimilable organic carbon percentages were about 10 times larger in river water and in water after ozonation.

INTRODUCTION

The presence of *Pseudomonas aeruginosa* in surface water is generally associated with faecal pollution with raw or treated domestic sewage (2,9,29). Much attention is paid to the occurrence of the organism in tap water because of its opportunistic-pathogenic character. *P.aeruginosa* is usually not present in 100 ml volumes of piped or stored drinking water (4,7,14,22,25,30). The organism has frequently been isolated from contaminated drinking water and from drinking water in hot climates (15,20,23) but quality standards have rarely been defined (10). The ability of *P.aeruginosa* to grow in water at low concentrations of organic

substrates has been studied in relation with its presence in water used in hospitals (1,3,5,6). The present paper describes the effect of various low-molecular-weight compounds on the growth of *P.aeruginosa* in tap water supplied with very low amounts of these compounds.

MATERIALS AND METHODS

Isolates

Five strains of *P.aeruginosa* isolated from different habitats and belonging to different serological, pyocine and phage types were tested (Table 1). For comparison, a representative strain

Table 1. Type and origin of the *P.aeruginosa* strains.

Strain	Serotype	Pyocine type		Reacting phages	Origin
		active	passive		
P1525	(5)C	15C	NT	21;44;73;119x;1214++ 16;31±;F7;F8;F10.	Tap water
4A [b]	NT [c]	15C	34D	21;44;73;109;119x±	River Lek [b]
6A [b]	6	15C	24D	21++; 119x +	River Lek [b]
M14 [b]	1	NT	NT	NT	River Lek [b]
6324 [b]	L	15C	54A	21; 119x+; F8±	Wound pus [b]

NT = nontypable

[a] Serotyping, pyocine typing and phage reactions performed by J. Borst, National Institute of Public Health (RIV), Bilthoven, The Netherlands

[b] Supplied by A.H. Havelaar, RIV.

[c] NT, nontypable

(P17) of *P.fluorescens*, obtained from tap water and belonging to biotype 7.2 (26) was included in a number of experiments. A detailed description of this strain has been given previously (28).

Replica test

Colonies of the isolates grown on a peptone beef-extract agar (Lab-Lemco, Oxoid Ltd.) were replicated onto plates of mineral salts agar supplied with separately sterilized carbon compounds at a concentration of 2.5 g/l. A metal device with twelve inoculation

pins was used for replication. The mineral salts agar consisted of the basal salts solution (27), solidified with 12 g of agar per liter. The replica plates were examined for growth after 2 and 7 days of incubation at 25°C.

Growth in tap water

Growth in tap water was tested in 600 ml samples contained in rigorously-cleaned glass-stoppered Erlenmeyer flasks of Pyrex glass of 1 liter capacity (27). Drinking water originating from the Municipal Dune Waterworks of The Hague was used for measuring the effect of low concentrations of added substrates on the growth of P.aeruginosa. This water had been prepared from dune-infiltrated river (Meuse) water by the addition of powdered activated carbon, followed by rapid and slow sand filtration. It contained 3.6 mg of organic carbon (TOC) and 7.3 mg of nitrate per liter. The autochthonous bacteria were eliminated by heating the sampled water at 60 °C for 1 hour. Compounds to be tested as carbon sources were added from similarly treated, freshly prepared solutions in tap water. A nitrogen source was not added because the tap water contained sufficient nitrate.

In a few experiments the water was supplied with mixtures of substrates. The amino acids (AA) mixture included glycine, L-alanine, L-valine, L-leucine, L-isoleucine, DL-serine, L-threonine, L-lysine, L-arginine, L-asparagine, L-aspartate, L-glutamine, L-glutamate, L-tyrosine, L-proline, DL-tryptophan, L-histidine and DL-phenylalanine. The aliphatic carboxylic acids (CA) mixture included sodium formate, acetate, glycolate, glyoxylate, oxalate, propionate, DL-lactate, pyruvate, malonate, fumarate, succinate, L-tartrate, citrate and adipate. The carbohydrate (CHA) mixture included L-arabinose, D-glucose, D-maltose and the (poly)alcohols ethanol, glycerol and D-mannitol. The aromatic-acids (AR) mixture included sodium benzoate, p-hydroxybenzoate, anthranilate, vanillate, phtalate, nicotinate and DL-mandelate. Stock solutions of the mixtures were prepared by dissolving the individual compounds in tap water at a concentration of 15 mg of C/l except glutamate which was used in a double concentration. After neutralization, the mixtures were sterilized (120 °C, 16 min) in Pyrex-glass bottles.

To prepare the inoculum, a small amount of cells from a 24-h slant culture on Lab-Lemco agar was suspended in 9 ml of sterile tap

water. A volume of 0.1 ml of a decimal dilution of this suspension was added to 100-ml infusion bottles containing 50 ml of autoclaved tap water with 1 mg of glucose C per liter. The initial number of cells was less than 10^3 colony-forming units (CFU) per ml. Maximum colony counts (N_{max}) in the infusion bottles of 4 to 6 x 10^6 CFU/ml were reached within a few days of incubation at 25 °C. Thereafter, the colony counts decreased very slowly and during a period of several months these cultures were used to inoculate the Erlenmeyer flasks (initial colony counts $< 10^3$ CFU/ml). The growth of the bacteria in the experimental solutions was assessed by periodic colony counts in triplicate, using the spread plate technique on Lab-Lemco agar plates (27). The generation times (G, in hours) were calculated for the period in which growth was exponential. The growth experiments in water were conducted in duplicate at 15 °C $\pm$ 0.5 unless otherwise stated.

RESULTS

Utilization of substrates at a concentration of 2.5 g per liter

Out of 47 compounds, 31 to 35 were utilized by the *P.aeruginosa* isolates and *P.fluorescens* P17 when tested with the replica procedure (Table 2). Strain P17 and the strains of *P.aeruginosa* differed only in the utilization of a few of the substrates tested. Differences among the various *P.aeruginosa* isolates regarding the utilization of a number of aliphatic amino acids, as well as the production of fluorescent pigments and the appearance of the colonies (results not shown) confirmed the heterogeneity of the strains as revealed by serotyping, pyocine and phage typing (Table 1).

Utilization of substrates at very low concentrations

With the exception of strain 6324, the *P.aeruginosa* isolates did not grow in the tap water of the Municipal Dune Waterworks of The Hague. Strain 6324 gave a G value of approx. 60 h and an N_{max} value of approx. 2 x 10^4 CFU/ml. These values approached those of strain P17 (Table 3). Addition of a mixture of 45 different compounds (total concentration added amounted to 46 μg of C per liter) enhanced growth of all strains, including strain P17.

Further tests were done with strain P1525, isolated from drinking water (Table 1), and with strain P6324 because of its

Table 2. Utilization of substrates at a concentration of 2.5 g/liter by five *P.aeruginosa* strains and *P.fluorescens* strain P17 determined with the replica test

Substrate	Utilization by strain:[a]					
	P17	P1525	4A	6A	M14	6324
Amino acids						
Glycine	-	-	-	-	-	-
L-Alanine	+	+	+	+	+	+
L-Valine	+	+	+	(+)	+	-
L-Isoleucine	+	+	+	(+)	+	-
L-Leucine	+	+	+	-	+	-
DL-Serine	+	-	-	-	-	-
L-Threonine	(+)	-	-	-	-	-
L-Lysine	+	(+)	(+)	(+)	+	+
L-Arginine	+	+	+	+	+	+
L-Aspartate	+	+	+	+	+	+
L-Asparagine	+	+	+	+	+	+
L-Glutamate	+	+	+	+	+	+
L-Glutamine	+	+	+	+	+	+
L-Proline	+	+	+	+	+	+
L-Histidine	+	+	+	+	+	+
L-Tyrosine	+	+	+	+	+	+
DL-Phenylalanine	+	+	+	+	+	+
DL-Tryptophan	+	+	+	+	+	+
L-Citrulline	+	+	+	+	+	+
L-Ornithine	+	+	+	+	+	+
Carboxylic acids						
Formate	-	-	-	-	-	-
Acetate	+	+	+	+	+	+
Glycolate	-	-	-	-	-	-
Glyoxylate	-	-	-	-	-	-
Oxalate	-	-	-	-	-	-
Propionate	+	+	+	+	+	+
DL-Lactate	+	+	+	+	+	+
Pyruvate	+	+	+	+	+	+
Malonate	+	+	+	+	+	+
Fumarate	+	+	+	+	+	+
Succinate	+	+	+	+	+	+
L-Tartrate	-	-	-	-	-	-

Table 2. Continued

Substrate	Utilization by strain:[a]					
	P17	P1525	4A	6A	M14	6324
Citrate	+	+	+	+	+	+
Adipate	+	+	+	+	+	+
Carbohydrates and (poly)alcohols						
L-Arabinose	-	-	-	-	-	-
D-Glucose	+	+	+	+	+	+
D-Maltose	-	-	-	-	-	-
Ethanol	+	+	+	+	+	+
Glycerol	+	+	+	+	+	+
D-Mannitol	(+)	(+)	(+)	(+)	(+)	(+)
Aromatic acids						
Benzoate	+	+	+	+	+	+
p-Hydroxybenzoate	+	+	+	+	+	+
Anthranilate	+	+	+	+	+	+
Vanillate	-	+	+	+	+	+
Phtalate	-	-	-	-	-	-
Nicotinate	-	-	-	-	-	-
DL-Mandelate	-	+	+	+	+	+

[a] +, Good growth; (+), weak growth; - ,no growth.

Table 3. Growth of 5 different *P. aeruginosa* strains and *P.fluorescens* strain P17 at 15 °C in the filtrate of slow sand filters, without and with a mixture of 45 substrates[a]

		Growth[c]			
		No substrates added		Substrates added	
Strain	N_o (CFU/ml)[b]	G(h)	Nmax(CFU/ml)	G(h)	N_{max}(CFU/ml)
P17	80	42;46	$2.4x10^4$;$2.7x10^4$	≤ 9;≤ 9	$2.4x10^5$;$2.5x10^5$
P1525	170	342;264	-[d];$5.6x10^2$	12.7;11.3	$1.7x10^5$;$2.0x10^5$
4A	320	244;232	$1.4x10^3$;$6.3x10^2$	19.1;18.9	$1.4x10^5$;$1.4x10^5$
6A	180	188;1022	$8.2x10^2$;$2.9x10^2$	14.0;14.2	$1.7x10^5$;$1.8x10^5$
M14	140	192;113	$4.2x10^3$;$8.2x10^2$	21.5;20.8	$1.3x10^5$;$1.3x10^5$
6324	180	65;53	$1.7x10^4$;$2.1x10^4$	17.3;14.9	- ; -

[a] The concentration of each individual substrate was 1 µg of C per liter; glutamate was present at twice this concentration.
[b] N_o, Initial colony count (inoculum).
[c] Both values of duplicate experiments are shown.
[d] -, Not determined.

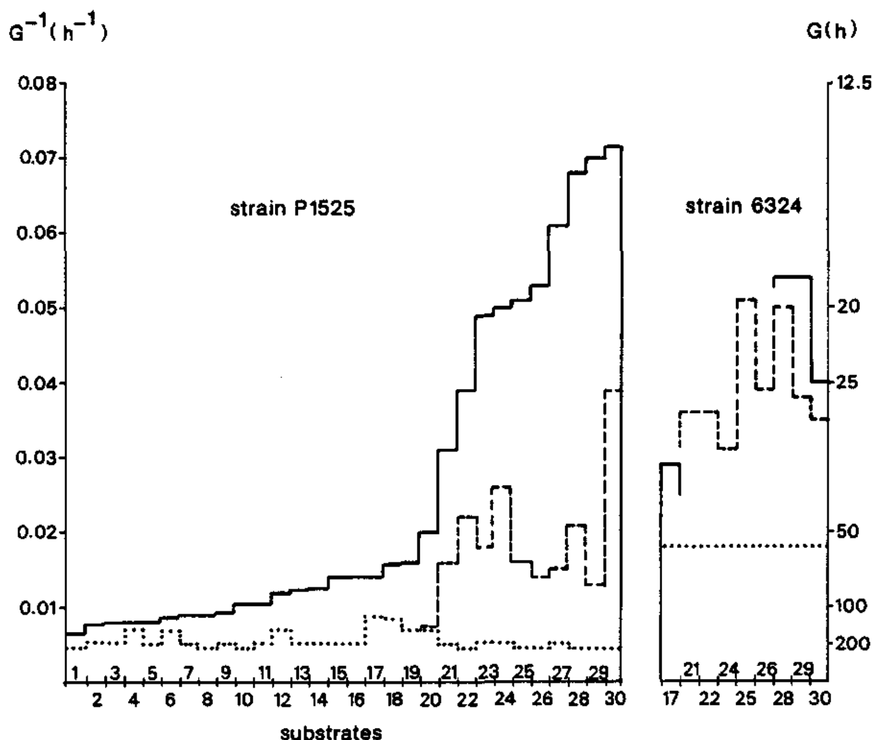

Fig. 1 Growth rates (G^{-1}, h^{-1}) of *P.aeruginosa* isolates P1525 and 6324 at 15 °C in tap water supplied with 25 and 10 μg of substrate C per liter. 1, *p*-Hydroxybenzoate; 2, L-Threonine; 3, L-Citrulline; 4, DL-Tryptophan; 5, L-Leucine; 6, DL-Phenylalanine; 7, L-Lysine; 8, DL-Serine; 9, L-Tyrosine; 10, L-Alanine; 11, L-Ornithine; 12, Glycine; 13, L-Valine; 14, L-Isoleucine; 15, Propionate; 16, Fumarate; 17, D-Glucose; 18, Glycerol; 19, Malonate; 20, Citrate; 21, Succinate; 22, Pyruvate; 23, L-Aspargine; 24, L-Proline; 25, L-Aspartate; 26, L-Glutamate; 27, L-Glutamine; 28, Acetate, 29, DL-Lactate; 30, L-Arginine. ——, 25 μg of C per liter; ----, 10 μg of C per liter; ......, blanks. Experiments not in duplicate except in blanks, for which the average values of G^{-1} are shown.

relatively rapid growth in drinking water (Table 3). A total of 30 different compounds of low-molecular-weight were tested as sources of carbon and energy for growth of strain P1525 at 25 μg of C per liter. The growth rate (G^{-1}) of strain P1525 was greater than 0.03 h^{-1} with 10 of the compounds tested (Fig.1). These compounds were also tested at 10 μg of C per liter. At this concentration G^{-1} was greater than 0.03 h^{-1} only with arginine. An average yield (Y_a) of 6.2×10^9 CFU/mg of C was calculated from the N_{max} values observed

Table 4. N_{max} values of *P.aeruginosa* P1525 grown at 15°C on different substrates added to tap water.

	N_{max} (CFU/ml)[a]	
	25 µg of C/1	10 µg of C/1
Acetate	1.1×10^5	5.7×10^4
Pyruvate	1.3×10^5	4.4×10^4
DL-Lactate	1.6×10^5	7.9×10^4
Succinate	-[b]	6.7×10^4
L-Aspartate	1.3×10^5	-
L-Asparagine	1.5×10^5	4.3×10^4
L-Glutamate	1.8×10^5	4.9×10^4
L-Glutamine	1.9×10^5	6.9×10^4
L-Proline	1.7×10^5	9.7×10^4
L-Arginine	1.6×10^5	6.6×10^4
Average yield (CFU/mgC)[c]	6.2×10^9	

[a] Single values.
[b] -, Not determined.
[c] N_{max} of the blanks was less than 10^3 CFU/ml and was therefore neglected in the calculation of the average yield (Y_a).

with these 10 substrates (Table 4). Strain P1525 grew at a very low rate in tap water supplied with amino acids (25 µg of C per liter) which were not utilized by the organism at a concentration of 2.5 g per liter (viz. glycine, serine, threonine; Table 2) and N_{max} values (< 4×10^3 CFU/ml) did not exceed those of the blanks. However, very low rates of growth were also observed at 25 µg of C per liter with

a number of amino acids which were utilized at a concentration of 2.5 g per liter. Moreover, the colony counts of strain P1525 in presence of these compounds did not exceed 10^4 CFU/ml after 15 days of incubation at 15 °C. Very low rates of growth and low colony counts also were observed with propionate, fumarate, D-glucose, glycerol and malonate.

Isolate 6324 was supplied with less compounds than P1525 (Fig. 1). The more rapid growth of this strain as compared to P1525 at 10 ug of C per liter may be explained by its ability to multiply in tap water without added substrate (Table 3).

Table 5. Growth response of *P.aeruginosa* P1525 to mixtures of different substrates added to the filtrate of slow sand filters.[a]

Mixture added (no. compounds in mixture)	Amount of supplied assimilable carbon (μg of C/1)[b]	Growth[c]	
		G (h)	N_{max} (CFU/ml)
None	-	401 ;236	$7.5x10^2$; $8.3x10^3$
AR(7)	5	270 ;256	$1.7x10^3$; $4.7x10^3$
CHA(6)	4	125 ;144	$1.2x10^3$; $9.5x10^2$
CA(14)	9	89 ; 77	$3.5x10^3$; $2.0x10^4$
AA(19)	16	21.5;21.7	$1.1x10^5$; $1.1x10^5$
TM(46)	34	16.1;18.0	$1.8x10^5$; $1.7x10^5$

[a] Incubation temperature, 15 °C.
[b] Total concentration of the compounds utilized in the replica test (cf. Table 2).
[c] Both values of duplicate measurements are shown.

Generation times of P1525 with the AA mixture were nearly equal to those with the total mixture, indicating that the amino acids were particularly growth promoting (Table 5). Of the other mixtures, only the carboxylic acids enhanced growth, but the rate of growth was very low and after 77 days N_{max} values were not yet reached. The N_{max} values shown in Table 5 reveal that compounds included in the CA, AR and CHA mixtures were more effectively utilized when incorporated in the total mixture than when present in separate mixtures.

Utilization by strain P1525 of aspartate and acetate supplied at different concentrations

A linear relationship between the N_{max} values of P1525 and the concentration of added substrate (ΔS) was obtained by growth of P1525 in tap water supplied with different amounts of either aspartate or acetate (Fig. 2). From these results, a Y value of

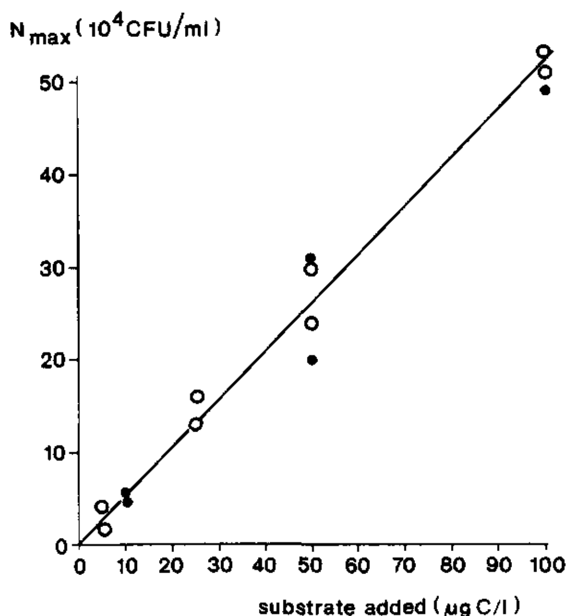

Fig. 2 Maximum colony counts of *P.aeruginosa* P1525 in tap water supplied with different amounts (µg of C per liter) of either acetate (●) or aspartate (O).

5.3 x 10^9 CFU per mg of acetate C and of aspartate C, was calculated. Plotting G against ΔS^{-1} revealed different linear relationships between G and ΔS^{-1} (Fig. 3). Such relationships may be expressed by the following Lineweaver-Burk equation:

$$G = G_{min} + (G_{min} \cdot K_s/\Delta S) \quad (1)$$

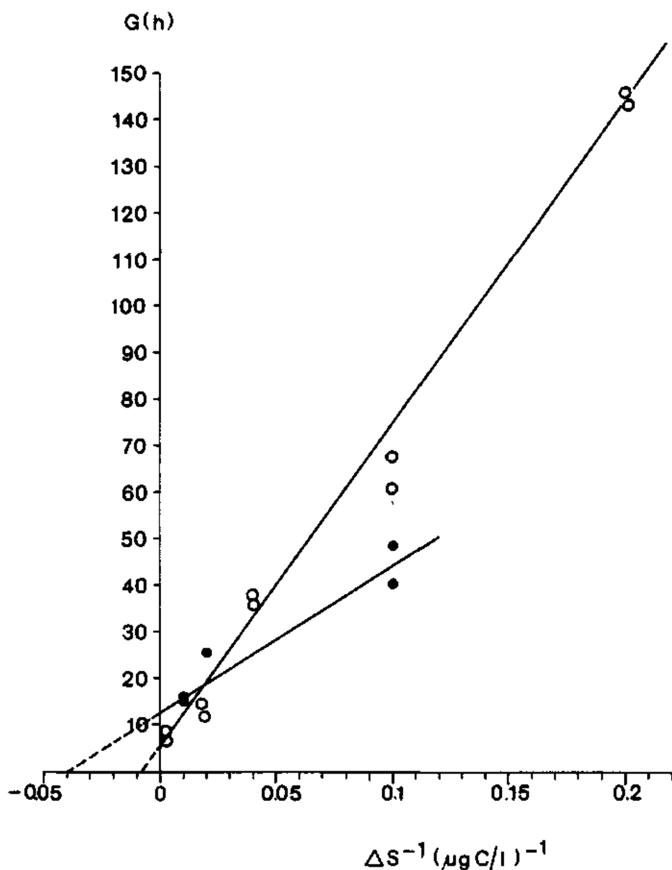

Fig. 3 Generation times of *P.aeruginosa* P1525 in tap water in relation to the reciprocal values (μg of C per liter)$^{-1}$ of different concentrations (ΔS) of added acetate (●) and aspartate (○).

in which G_{min} is minimal G and K_s is the substrate saturation constant, the substrate concentration at which G is equal to 2 G_{min}. The equations for growth with aspartate and with acetate are presented in Table 6 together with the K_s values.

Table 6. Kinetics of growth of *P. aeruginosa* P1525 with aspartate or acetate at 15°C.

Substrate	Adapted Lineweaver-Burk equation[a]	K_s	
		μg of C/l	μM
L-Aspartate	$G = 6.0+676/\Delta S$	113	2.3
Acetate	$G = 11.7+328/\Delta S$	28	1.2

[a] $G = G_{min} + G_{min} \cdot K_s/\Delta S$. This equation is described in the text.

Growth of P.aeruginosa isolates P1525 and 6324 and P.fluorescens P17 in various types of water

Table 7 gives generation times and N_{max} values of strains P1525, 6324 and P17 in a few types of tap water, in ozonated water and in river water. In these water types, NO_3 nitrogen was present in concentrations between 1.2 and 3.6 mg per liter and therefore was not growth limiting. From the average yield of strain P1525 on a number of substrates (cf. Table 4) and the N_{max} values presented it may be concluded that the concentration of assimilable organic carbon (AOC) available for P1525 varied from less than 0.1 μg of C per liter (tap water 2) to 150 μg of C per liter (ozonated water). For strain P17 a yield of 4.2 x 10^9 CFU/mg of acetate C has been obtained (28). Hence, the AOC available for strain P17 varied from 1.4 (tap water 4) to 200 (ozonated water) μg of acetate C equivalents per liter.

In general, N_{max} values of strain P1525 and 6324 did not differ much from those of strain P17 (cf. Tables 3 and 7) suggesting that the three organisms were utilizing similar substrates. This suggestion is supported by the observation that strain P17 did not grow in river water in which maximum numbers of strain P1525 had grown. Moreover, strain 6324 was unable to grow in river water in wich strain P17 had reached N_{max} (Fig. 4). In all types of water tested, strain P17 grew more rapidly than strains P1525 and 6324, the growth rate of these organisms being about half the growth rate of strain P17 (Fig. 5). Therefore, it is expected that at a temperature of 15 °C *P.aeruginosa* cannot attain large numbers

Table 7. Growth of *P. aeruginosa* P1525 and 6324 and *P.fluorescens* P17 at 15°C in four types of tap water, in ozonated water and in river water.

Water source	TOC (mg/l)	Growth of strain:					
		P17		P1525		6324	
		G(h)	N_{max} (CFU/ml)	G(h)	N_{max} (CFU/ml)	G(h)	N_{max} (CFU/ml)
Tap water 1[a]	2.1	17.9	$1.2x10^5$	47.8	$9.3x10^4$	29.1	$1.2x10^5$
		14.6	$9.9x10^4$	42.7	$9.1x10^4$	22.1	$1.2x10^5$
Tap water 2[b]	2.8	18.8	$8.4x10^4$	388	$3.7x10^2$	28.6	$9.7x10^4$
		19.5	$6.9x10^4$	155	$5.7x10^2$	35.9	$7.1x10^4$
Tap water 3[c]	5.8	8.7	$1.9x10^5$	15.9	$1.1x10^5$	26.2	$1.5x10^5$
		9.9	$1.7x10^5$	22.3	$1.3x10^5$	22.5	$1.4x10^5$
Tap water 4[d]	2.3	25.5	$5.9x10^3$	33.3	$1.1x10^3$	72.0	$4.7x10^3$
		24.2	$5.5x10^3$	39.1	$9.7x10^2$	60.1	$6.7x10^3$
Ozonated water[e]	2.9	5.3	$8.4x10^5$	7.8	$9.7x10^5$	-[g]	-
		5.1	$8.3x10^5$	8.3	$9.5x10^5$	-	-
River water [f]	3.4	4.1	$3.5x10^5$	9.3	$2.7x10^5$	8.3	$4.9x10^5$
		4.2	$3.4x10^5$	9.5	$3.1x10^5$	6.8	$4.8x10^5$

[a] Prepared from stored river (Meuse) water by coagulation/sedimentation, ozonation, dual-media filtration, activated-carbon filtration and chlorination.
[b] Prepared from pretreated and dune-infiltrated river (Lek) water by rapid sand filtration, slow sand filtration and chlorination.
[c] Prepared from polder water after precoagulation and storage in an open reservoir followed by rapid sand filtration, ozonation, coagulation/sedimentation, rapid sand filtration, slow sand filtration and chlorination.
[d] Prepared from anaerobic ground water by aeration and rapid filtration.
[e] Stored river (Meuse)water, ozonated after coagulation.
[f] From the river Lek which receives its water from the river Rhine.
[g] -, Not tested.

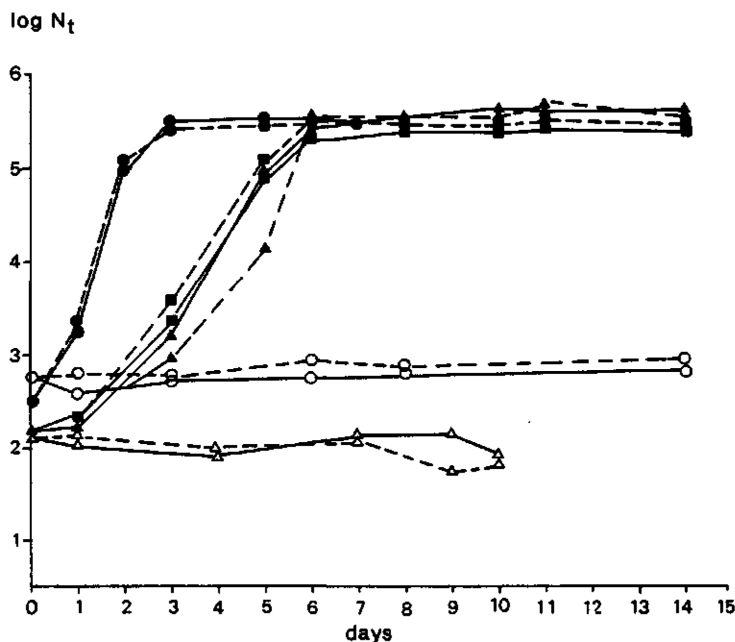

Fig. 4 Growth curves of *P.fluorescens* P17 (●), *P.aeruginosa* P1525 (■) and 6324 (▲) in pasteurized river (Lek)water at 15 °C. Growth curves of *P.fluorescens* P17 (○) in river water in which strain P1525 had reached N_{max} and growth of strain 6324 (Δ) in river Lek water in which strain P17 had reached N_{max}. Colony counts (N_t) of strain P17 growing in the presence of strain P1525 were determined by incubation of the plates at 10 °C. Colony counts of strain 6324 growing in the presence of strain P17 were determined by incubation of the plates at 37 °C. Solid and dashed lines represent duplicate experiments.

in water where bacteria such as strain P17 are present.

Competition is further demonstrated by the effect of autochthonous bacteria, added to the heated tap water, on the growth of strain 6324. Even when the water had been supplied with the total mixture of substrates (46 μg of C per liter) growth of strain 6324

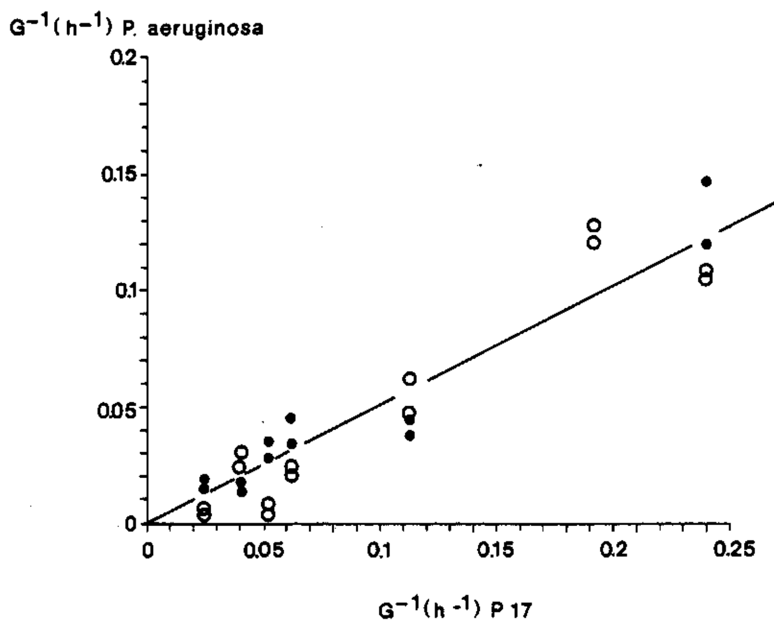

Fig. 5 Growth rates (G^{-1}, h^{-1}) at 15 °C of *P.aeruginosa* P1525 (O) and 6324 (●) in various types of water (cf. Table 7) in relation to the growth rates of *P.fluorescens* P17 in these waters.

was effectively repressed, by the more rapidly developing autochthonous bacteria. Further incubation resulted in a pronounced decrease of the colony counts of strain 6324, whereas the autochthonous bacteria remained present in larger numbers (Fig. 6). The similarity of the duplicate experiments clearly demonstrates the reproducibility of the processes which determine the growth of the various groups of bacteria.

The described experiments reveal that *P.aeruginosa* is able to grow at relatively low concentrations of substrates. In natural environments, however, the organism can not compete effectively with many aquatic bacteria, including fluorescent pseudomonads such as strain P17.

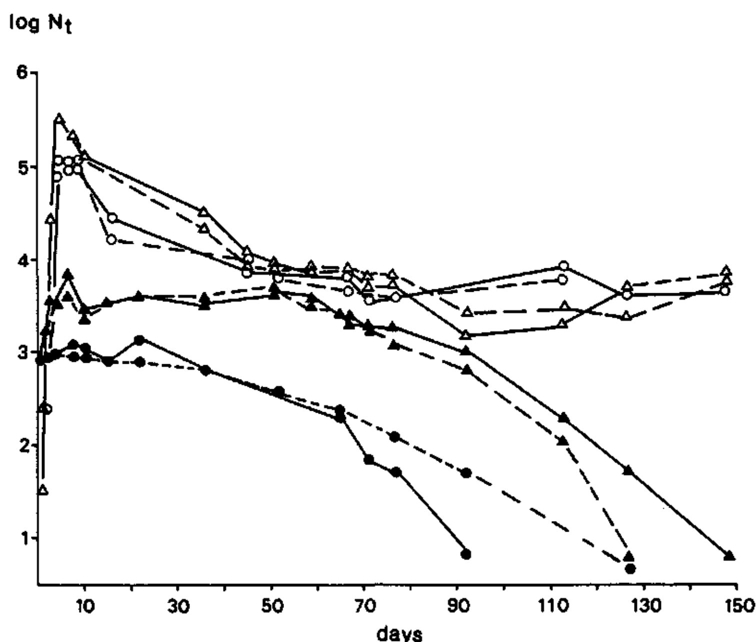

Fig. 6 Growth curves of *P.aeruginosa* strain 6324 in the presence of autochthonous bacteria in tap water and in tap water supplied with the total mixture of substrates (46 μg of C per liter); Symbols: (●) strain 6324 and (○) autochthonous bacteria in the tap water without substrates added; (▲) strain 6324 and (Δ) autochthonous bacteria in the tap water supplied with substrates. Solid and dashed lines represent duplicate experiments. For colony counts (N_t) of strain 6324 the Lab-Lemco agar plates were incubated at 37 °C; colonies of the plates at 25 °C for 10 days.

DISCUSSION

Utilization of amino acids at low concentrations by P.aeruginosa

Utilization of substrates by *P.aeruginosa* at a concentration of 2.5 g/l (Table 2) gives no information about the utilization of these substrates at a very low concentration. Similar obser-

vations have been reported for an *Aeromonas hydrophila* isolate (27). Arginine was found to be the most effective of the growth-promoting compounds tested at 10 and 25 µg of C per liter with P1525. In this respect the organism resembles *P.fluorescens* P17 (28). A preference of *P.aeruginosa* for arginine has also been reported by Kay and Gronlund (12), who found that this amino acid was rapidly taken up by the cells when present at a concentration of 1 µM. Moreover, the greatest chemotactic responses of *P.aeruginosa* have been observed with amino acids of which arginine, with a threshold of 0.7 µM, was the best attractant (18,19). Citrulline and ornithine, intermediates of the degradation of arginine by the arginine-deïminase pathway in *P.aeruginosa*, did not favour growth of strain P1525 when present at a low concentration (Fig. 1). The arginine-decarboxylase pathway seems to be the main degradation route in aerobically grown cells of *P.aeruginosa* (16). Hence, no relation may be expected between growth on arginine on the one hand and on citrulline and ornithine on the other.

The N_{max} values of strain P1525 obtained with the AA mixture (Table 5) and the Y_a value of 6.2×10^9 CFU/mg of C (Table 4) revealed that 18 µg of C, i.e. approximately all of the amino acids present in the mixture, were utilized. Obviously, many amino acids were more efficiently utilized at a concentration of 1 µg of C per liter when present in a mixture at a total concentration of 19 µg of C per liter than when present singly at a concentration of 25 µg of C per liter. The presence of constitutive transport systems in growing cells of *P.aeruginosa* for the uptake of most naturally occurring amino acids (12) may have been responsible for this phenomenon. Even amino acids which did not serve as the sole source of carbon and energy may have contributed to the production of biomass during growth with the amino acids mixture.

Many substrate-saturation constants for amino acid transport (K_t) by *P.aeruginosa* are below 1 µM (11 - 13). Assuming that G_{min} of strain P1525 with arginine is similar to G_{min} with aspartate, a K_s value of approximately 0.4 µM of arginine may be calculated using the growth rates of strain P1525 at 10 and 25 µg of arginine C per liter (Fig. 1). For aspartate a clearly higher K_s value (2.3 µM) was obtained (Table 6). To what extent K_t and K_s values are similar may be estimated with uptake experiments with radio-actively labeled substrates combined with growth experiments as described in this paper. The K_s values of strain P17 were 0.04 µM

and 0.34 μM for arginine and aspartate, respectively (28). This organism seems much better adapted to growth at low concentrations of individual amino acids than strain P1525.

Utilization of carboxylic acids, carbohydrates, (poly)alcohols and aromatic acids at low concentrations by P.aeruginosa

Low concentrations of lactate, pyruvate, succinate and particularly acetate clearly promoted growth of P.aeruginosa P1525 and 6324 (Fig. 1). Yet the K_s value of strain P1525 for acetate (1.2 μM) is high as compared to that of strain P17 (0.17 μM) for this substrate (28). Glucose and glycerol uptake by *P.aeruginosa* are dependent on inducible transport systems with a K_t value of about 8 μM (8,17,24). This value, which is 10 times above the K_s value of strain P17 for glucose (28), may explain the low growth rates of strain P1525 with glucose and glycerol each at a concentration of 25 μg of C per liter (Fig. 1). The aromatic acid *p*-hydroxybenzoate is an excellent substrate for *P.aeruginosa* (Table 2) and nearly all fluorescent pseudomonads (26) at a high concentration. At 25 μg of *p*-hydroxybenzoate C per liter, growth of strain P1525 is very slow (Fig. 1). Therefore, the K_s value of strain P1525 for this compound seems to be higher than the one obtained for strain P17 (0.76 μM) (28).

The poor growth of strain P1525 with the CA, CHA and AR mixtures (Table 5) seemed only partly due to a lack of sufficient suitable substrates in these mixtures. The difference between N_{max} values observed with the total mixture and the AA mixture indicates that about 10 compounds other than amino acids were taken up at 1 μg of C per liter during growth with the total mixture. The identity of these compounds was not further determined, but the rapid growth with a number of carboxylic acids (Fig. 1) suggests that a least a few of these compounds were involved. However, half the number of potential substrates other than the amino acids were not utilized by cells of *P.aeruginosa* growing with amino acids (total mixture).

Growth of P.aeruginosa in water without added substrates

Despite the ability of *P.aeruginosa* to utilize amino acids and a number of carboxylic acids at very low concentrations, the organism grew at a low rate in the types of tap water tested. The AOC concentration available for *P.aeruginosa* in these water types constituted only 0.1 - 0.7 % of the TOC concentration. The

identity of the utilized compounds remains unknown and it cannot be excluded that the *P.aeruginosa* strains studied are able to grow with more compounds at low concentrations than those tested in this study (Fig. 1). Yet it is clear that dissolved free amino acids and carboxylic acids as used in the described experiments are a very minor part of the organic carbon in tap water. This may be the result of the various filtrations with biological activity, viz. rapid sand filtration, activated-carbon filtration, slow sand filtration (Table 7). The AOC concentration in river water and ozonated water was 2.3 and 5.4 % of the TOC concentration, respectively. In ozonated water biodegradable low molecular weight compounds are present as a result of the effect of ozone on the large molecules of humic and fulvic acids.

The N_{max} values for tap water, obtained in this study, are 10 to 100 times below those of *P.aeruginosa* strains grown in distilled water, in inorganic salt solutions and in tap water (1,3,6). The maximum colony counts reported in those papers (10^6 to 10^7 CFU/ml) indicate that the waters examined were not really poor in organic substrates, because AOC concentrations calculated from these N_{max} values and $Y = 6.2 \times 10^9$ CFU/mg C (Table 4) varied from 0.16 to 1.6 mg of C per liter.

In most water types tested in this study, growth rates of *P.fluorescens* P17 were about twice as high as those of the *P.aeruginosa* isolates (Fig. 5). G_{min} values of strain P17 (28) were about half those of strain P1525 on acetate and aspartate at 15 °C (Table 6), possibly as a result of the mesophilic character of *P.aeruginosa*. Therefore, differences between growth rates of strain P17 and those of the *P.aeruginosa* strains in the various types of water may be due mainly to differences in G_{min} values, suggesting that the average K_s values of strains P17 and P1525 with substrates present in water are similar. Such a similarity would be in contrast with the difference between K_s values of strains P17 and P1525 as observed in this study for single substrates. This difference may be less pronounced when the organisms are growing with a number of substrates.

A large variety of *P.aeruginosa* serotypes have been observed in aquatic environments (10). To what extent specific serotypes differ in their adaptation to low substrate concentrations is not clear, but the differences observed between strains P1525 and 6324 (Fig. 1; Table 7) demonstrate some inhomogeneity of the species

in this respect. The presence of specific *P.aeruginosa* types in water may also be affected by other properties, e.g. slime production in chlorinated water (21). *P.fluorescens* P17 resembles the *P.aeruginosa* isolates in respect of their nutritional versatility (Table 1) and their ability to denitrify (26). Yet fluorescent pseudomonads belonging to the same biotype as strain P17 are found far more frequently in tapwater and in surface water than *P.aeruginosa* (26). The ability of strain P17 to grow more rapidly than *P.aeruginosa* in water at relatively low temperatures while utilizing similar substrates (Fig. 4) explains this difference. At water temperatures where G_{min} of *P.aeruginosa* is equal to or below G_{min} of the psychrotrophic fluorescent pseudomonads, the latter organisms may become replaced by *P.aeruginosa*. Indications for such a temperature effect have been presented repeatedly (10,14,20,23). The minor contribution of fluorescent pseudomonads to the bacterial flora of water (25) suggest that in these situations *P.aeruginosa* will also be only a minor component of the bacterial flora.

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5. Growth of *Aeromonas hydrophila* at low concentrations of substrates added to tap water

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Appl. Environ. Microbiol. 39 (1980): 1198-1204

Abstract

The ability of an *Aeromonas hydrophila* isolate obtained from sand filtered river water to grow at low substrate concentrations was studied in batch experiments with tap water supplied with low concentrations of substrates. Growth was assessed by colony count determinations. The isolate only multiplied in the used tap water (2 to 3 mg of dissolved organic carbon per liter) after the addition of a small amount of an assimilable carbon compound. D-Glucose especially caused growth of the organism even at initial concentrations below 10 µg of C per liter. At initial glucose concentrations below the K_s value (12 µg of C per liter), generation times and yield (colony-forming units per milligram of substrate C) were nonlinear with 1/initial glucose concentrations and initial glucose concentrations, respectively. From these observations, the maintenance coefficient m was calculated (m = 0.015 mg of glucose per mg dry wt per h at 12 °C). At initial concentrations below the K_s value of starch (73 g of C per liter), no growth was observed, but complete utilization of starch occurred in these situations after the addition of 10 µg of glucose C per liter. The results of this study show that information of ecological significance may be obtained by very simple batch experiments. Moreover, the isolate studied may be used in growth experiments to assess the maximum concentration of glucose which might be present in water, particularly tap water.

INTRODUCTION

Representatives of the species *Aeromonas hydrophila* have frequently been observed in dairy products (11), wastewater (2, 19), surface water (5, 8, 19), ground water (21), and tap water (2, 12, 22, 25). The occurrence of *A. hydrophila* in surface water may result from pollution with wastewater, in which it multiplies (19), as well as from its predominant presence in the alimentary tract of fishes (23). The ability of the organism to act as a fish pathogen (7) may also be important in this respect.

Von Wolzogen Kühr (28) demonstrated that *Pseudomonas fermentans*, which is identical to *A. hydrophila*, was

present in tap water as a result of contamination of this water by the faeces of chironomid larvae which were growing in a sand filter. In tap water, aeromonads usually constitute a minor part of the bacterial flora (25). However, the presence of these organisms is undesirable because they interfere with the determination of coliform bacteria, producing false-positive reactions (12). Moreover, *A. hydrophila* is known to be an opportunistic pathogen for humans (10, 18, 20, 26).

The aim of the present investigation was to obtain information about the growth of *A. hydrophila* at low substrate concentrations as part of an investigation on the behavior of different types of bacteria in tap water.

MATERIALS AND METHODS

Pure cultures

An aerogenic *A. hydrophila* isolate, strain 315, was obtained from the filtrate of a rapid sand filter supplied with river water. The isolation and identification procedures have been described previously (25). Two additional aerogenic *A. hydrophila* isolates, strains 578 and 666, were obtained from two types of tap water prepared from surface water.

Basal salts solution

The solution contained, per liter of demineralized water, the following: NH_4Cl, 0.5 g; $Na_2HPO_4 \cdot 12H_2O$, 0.5 g; KH_2PO_4, 2.7 mg; $K_2HPO_4 \cdot 3H_2O$, 5.3 mg; $MgSO_4 \cdot 7H_2O$, 50 mg; $CaCl_2 \cdot 2H_2O$, 50 mg; $MnSO_4 \cdot H_2O$, 5 mg; $FeSO_4 \cdot 7H_2O$, 3 mg; $ZnSO_4 \cdot 7H_2O$, 0.1 mg; $CuSO_4 \cdot 5H_2O$, 0.1 mg; $CoCl_2 \cdot 6H_2O$, 0.05 mg. The pH was 6.8 after sterilization.

Preparation of glassware and medium

The experiments were performed in 1-liter, calibrated, conical, glass-stoppered Pyrex glass flasks. These flasks were cleaned with a 10 % solution of $K_2Cr_2O_7$ in concentrated H_2SO_4, followed by rinsing with hot tap water, a 10 % HNO_3 solution, and hot tap water again. Thereafter, they were heated overnight at 250 to 300 °C. The pipettes (1 ml) were cleaned in the same way. The cleaned flasks were filled with 600 ml of tap water (deviation usually less than 3.5 %). The tap water originated from the municipal Dune Waterworks of The Hague, where it is prepared from dune-infiltrated river water

by the addition of powdered activated carbon, followed by rapid and slow sand filtration, respectively. The final water contained 2 to 5 mg of dissolved organic carbon per liter and 8 to 9 mg of NO_3^--N per liter and had a pH between 7.1 and 7.5.

The vegetative cells of the bacteria present in the sampled tap water were killed by placing the flasks in a water bath at 60 °C for 3 h. After cooling, NH_4Cl and $Na_2HPO_4 \cdot 12H_2O$, usually in concentrations of 1.16 mg of N per liter and 83 µg of P per liter, and organic compounds as sources of energy and growth, were added from separately heated solutions. The inorganic compounds were supplied to ascertain that only the organic compounds were growth limiting.

Preparation of the inoculum

After counting cell numbers in a Bürker-Türk counting chamber, a turbid suspension of a 24-h slant culture of strain 315 in sterile tap water was inoculated into a 100-ml infusion bottle containing 50 ml of sterile tap water. This suspended culture that initially contained about 5×10^4 colony-forming units (CFU) per ml was stored at 25 °C and repeatedly used for inoculating the test flasks. In some experiments strain 315 was pregrown in the basal salts solution containing D-glucose, acetate, starch, or glycerol at an initial concentration of 1 mg of substrate C per liter. Strains 578 and 666 were only pregrown in the basal salts solution with 1 mg of acetate C per liter. Cells pregrown on an added carbon source were only inoculated into the test flasks when the stationary phase (usually 2×10^6 to 3×10^6 CFU/ml at 1 mg of substrate C per liter) had been reached in the precultivation bottle. The initial concentration of cells in the test flasks usually ranged from 50 to 200 CFU/ml.

Determination of growth

After inoculation, the flasks containing 600 ml of tap water and the test compounds were incubated at 15 $\pm$ 1 °C. The growth curves were obtained by a periodic determination of the colony counts (N_t, CFU per mililiter). For this purpose, the spread plate technique was applied by plating 0.05 ml in triplicate from decimal dilutions on predried Lab-Lemco (Oxoid) agar plates. The colonies of the aeromonads were clearly visible after an incubation period of 18 to 20 h at 25 °C. The generation time G (in hours) of the colony-forming cells under the experimental conditions was calculated with the equation $G = \log (t' - t)/(\log N_t' - \log N_t)$, where t' - t =

the incubation time (in hours) during which N_t increased to N_t'. These calculations were performed for the period that the growth curve was found linear with time in a half-logarithmic plot. All experiments were performed in duplicate.

RESULTS

Growth of A. hydrophila strain 315 on different compounds at a concentration of 1 mg of substrate-C per liter

D-Glucose, DL-lactate, acetate, L-glutamate, and succinate were tested as substrates for growth (Table 1). The aeromonad grew

Table 1. Growth of *A. hydrophila* strain 315 at 15 °C in the presence of substrates added to tap water

Substrate added[a] (1 mg of C per liter)	G^b (h)		N_{max}^b ($\times 10^6$ CFU/ml)	
None	No growth	No growth	No growth	No growth
Glucose	4.1	3.9	4.2	3.8
Acetate	12.2	10.5	3.8	2.9
Glutamate	12.9	11.5	1.8	1.9
Succinate	63.9	82.4	-[c]	-
Lactate	100	100	-	-

[a] All flasks received 0.15 mg of NH_4^+-N per liter.
[b] Data from duplicate flasks are presented in corresponding sequence.
[c] -, No data.

immediately on 1 mg of glucose C per liter, whereas with acetate and glutamate, lag periods of 2 and 7 days were observed (Fig. 1). Growth on succinate and lactate was extremely slow; therefore, maximum colony counts (N_{max}) were not estimated. In tap water without added substrate no growth but a die-off of cells was observed, indicating that the organic carbon compounds originally present were not suitable substrates for the aeromonad.

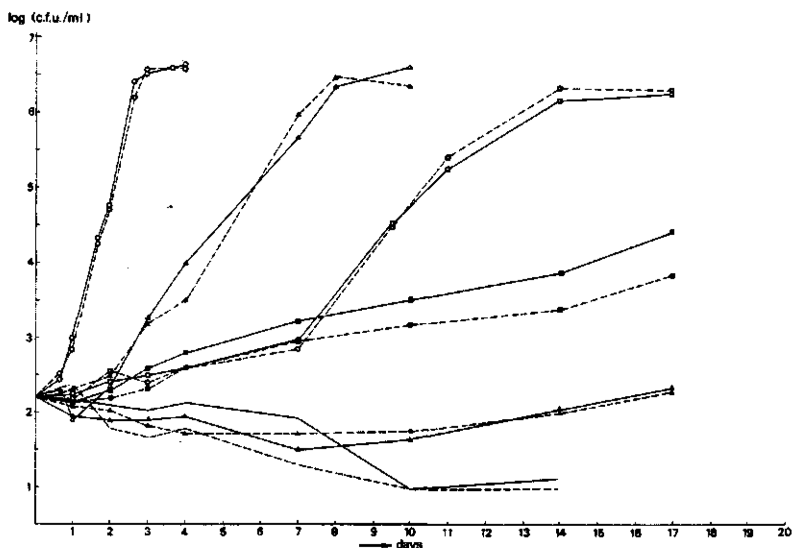

Fig. 1 Growth of *A. hydrophila* strain 315 on different compounds added to tap water at a concentration of 1 mg of substrate C per liter. Symbols: ○, glucose; △, acetate; □, glutamate; ■, succinate; ▲, lactate; lines without symbols, blancs. Solid and broken lines indicate duplicate experiments.

Table 2. Growth of *A. hydrophila* strain 315 at 12 °C on glucose added to tap water (series B)[a]

Substrate added (µg of C per liter)	G^b (h)		$N_{max}{}^b$ (CFU/ml)	
None	No growth	No growth	No growth	No growth
2.5	72.4	75.1	4.7×10^3	4.7×10^3
5.0	32.7	33.4	1.2×10^4	1.1×10^4
10.0	14.9	16.5	3.5×10^4	3.6×10^4
25	9.4	12.0	1.1×10^5	9.3×10^4
1,000	6.9	7.5	3.3×10^6	3.3×10^6

[a] Inoculum derived from a flask from series A initially containing 100 µg of glucose C per liter.
[b] Data from duplicate flasks are presented in corresponding sequence

Utilization of glucose at low concentrations

Two series of experiments (A and B) with glucose revealed that growth of the isolate was caused even by the addition of 2.5 µg of glucose C per liter of tap water (Table 2). The relationship between N_{max} and initial glucose concentration (S) was linear when S was ≥ 10 µg of glucose C per liter (Fig. 2). From this linear relationship, a yield of 3.7 x 10^9 CFU/mg of glucose C was calculated. During the experiments of series B, the incubation temperature was 12 °C, resulting in a prolonged G at S = 1 mg of glucose-C per liter, as compared with earlier results (Table 1) when the temperature was 15 °C.

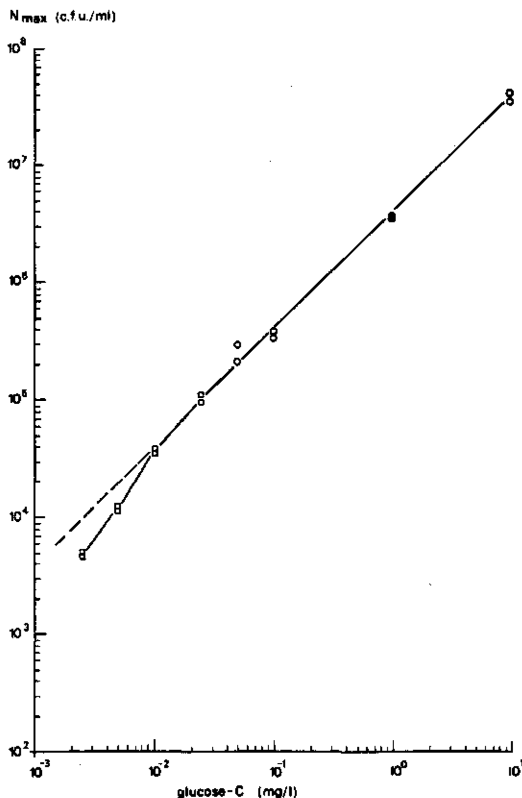

Fig. 2 The observed N_{max} in relation to the amount of glucose added to tap water. Symbols: O, series A; □, series B.

As the utilization of glucose may have been influenced by the precultivation carbon source, an experiment on glucose (10 µg of C per liter) was conducted with cells of strain 315 pregrown in the basal salts solution with 1 mg of acetate C per liter. The organism was found to grow rapidly with a lag phase less than 24 h, suggesting that its glucose uptake is controlled by a constitutive enzyme system with a high substrate affinity.

Two other representatives of *A. hydrophila*, strains 578 and 666, obtained from tap water were tested for their ability to grow at low glucose concentrations. During an incubation period of 11 days in tap water without glucose, N_t of both isolates decreased. The addition of 10 µg of glucose C resulted in distinct growth with N_{max} and Gs identical to those found with strain 315 in a similar situation.

Utilization of starch at low concentrations

A. hydrophila may produce extracellular enzymes for the hydrolysis of proteins, blood cells, starch, chitin, and lipids. However, information is lacking about the effect of low concentrations of macromolecular compounds. Experiments with strain 315 revealed that added starch only produced growth when S was $\geqslant$ 100 µg of starch C per liter of tap water. Still no increase of N_t was observed at initial concentrations of 10 and 25 µg of starch C per liter after an incubation period of 21 days. On day 21, the inoculum in the bottles without growth was raised with glucose-grown cells to about 1,600 CFU/ml. After another 15 days of incubation, no increase of cells was again observed. Then, 10 µg of glucose C per liter was added to the flasks, immediately resulting in growth.

The results of this experiment (Table 3) show that a linear relationship existed between the observed N_{max} values and the starch-C concentrations of 100 µg per liter and above. The obviously complete utilization of 100 µg of starch C per liter reveals that strain 315 is able to utilize this compounds at very low concentrations. This conclusion is supported by the N_{max} values of the starch-containing bottles supplied with 10 µg of glucose, which approximated the sum of the values that could have been expected by the complete use of both starch and glucose.

Table 3. Growth of *A. hydrophila* strain 315 at 15 °C on starch added to tap water [a]

Substrate C added (per liter)		G^c (h)		N_{max} [c] (CFU/ml)	
Starch	Glucose [b]				
None	None	No growth	No growth	No growth	No growth
10 µg	None	No growth	No growth	No growth	No growth
10 µg	10 µg	12.4	13.9	6.1×10^4	6.1×10^4
25 µg	None	No growth	No growth	No growth	No growth
25 µg	10 µg	9.8	10.3	1.4×10^5	1.4×10^5
100 µg	None	6.4	6.1	3.9×10^5	4.4×10^5
1 mg	None	3.3	3.7	3.0×10^6	3.5×10^6
10 mg	None	3.8	3.3	3.2×10^7	3.2×10^7

[a] Inoculum derived from a flask from series A initially containing 100 µg of glucose C per liter.
[b] Glucose was added after an incubation period of 36 days.
[c] Data from duplicate flasks are presented in corresponding sequence

Table 4. Growth of *A. hydrophila* strain 315 at 12 °C on acetate added to tap water [a]

Substrate added (mg of C per liter)	G^b (h)		N_{max} (CFU/ml)	
None	No growth	No growth	No growth	No growth
0.025	No growth	No growth	No growth	No growth
0.1	21.7	21.9	3.5×10^5	– [c]
1.0	16.5	15.8	3.0×10^6	2.8×10^6
10.0	14.8	16.5	2.9×10^7	2.8×10^7

[a] Inoculum from infusion bottle
[b] Data from duplicate flasks are presented in corresponding sequence
[c] –, No data.

Utilization of acetate and some other compounds at low concentrations

Strain 315 was able to use acetate C at concentrations of 100 µg of C per liter or above (Table 4). Maximum growth rate already occurred at 1 mg of acetate C per liter. The effect on the growth of the isolate was also tested with succinate, L-glutamate, yeast extract, L-arabinose, D-mannitol, D-glucosamine, and gluconate at initial concentrations below 1 mg of substrate C per liter of tap water. Neither succinate nor glutamate caused growth when added at an initial concentration of 10 µg of C per liter. An additional supply of 10 µg of glucose C per liter resulted in rapid growth, but the N_{max} values indicated that only glucose was utilized.

Strain 315 also did not multiply with yeast extract, D-glucosamine, and gluconate at concentrations of 10 µg of substrate C per liter. Growth occurred in the presence of glycerol C at a concentration of 1 mg/liter, but not at concentrations of 10 and 20 µg/liter. On 10 µg of mannitol C per liter, no growth was observed within 14 days, but slow and immediate multiplication (G = 68 h) occurred with 20 µg. In an experiment with 10 µg, 20 µg, and 1 mg of arabinose-C per liter with cells from a flask with 20 µg of mannitol C per liter in which N_{max} had been reached, growth was observed after a lag period of about 24 h. The G values were 6.1 at 1 mg of substrate C per liter, 27.3 h at 20 µg of substrate C per liter, and 36.4 h at 10 µg of substrate C per liter. The results of these experiments indicate that only a few specific carbohydrates could produce growth of the aeromonad at initial substrate concentrations below 100 µg of C per liter.

DISCUSSION

In this study, N_t was used to determine the G value as well as to quantify the amount of biomass. From the linear relationship observed between S and N_{max} when S was $\geq$ 10 µg of glucose C per liter and on starch and acetate when S was $\geq$ 100 µg of substrate C per liter, it may be concluded that the use of N_t to quantify biomass and to estimate G in the described experiments is justified. With low numbers of cells (< 100 CFU/ml) the applied method is not accurate, but this was not important in these cases.

The G values of *A. hydrophila* strain 315 observed at different concentrations of a number of substrates may be used to

calculate the substrate concentration (K_s), which produces half the maximum growth rate. For this purpose a slightly adapted Lineweaver-Burk modification of the Monod equation was used:

$$G = G_{min} + (G_{min})(K_s)(1/S) \qquad (1)$$

in which G is given in hours at substrate concentration S, and G_{min} is equal to minimal G. K_s is the substrate concentration at which $G = 2 \times G_{min}$. The relationship between G and the reciprocal value of the S (Fig. 3) is linear at S above 10 µg/liter. Calculation of this function produced the equation:

$$G = 7.17 + (0.086)(1/S) \qquad (2)$$

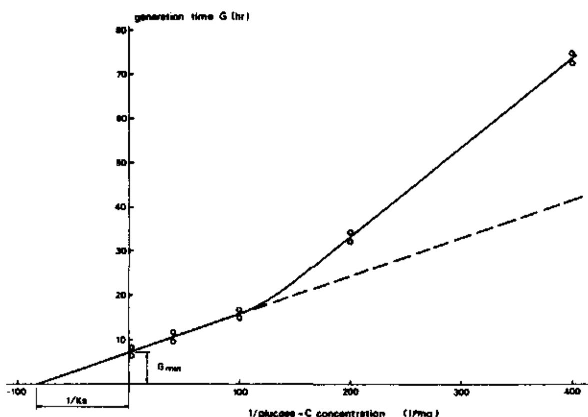

Fig. 3 Lineweaver-Burk plot for the growth of *A. hydrophila* strain 315 on glucose added to tap water. Symbols: —, observed relationship; -----, calculated linear relationship.

According to equation (2), the K_s value of strain 315 equals 12 µg of glucose C per liter. Similar calculations with the results of the experiments with starch, acetate, and arabinose gave K_s values of 73, 40, and 38 µg of C per liter, respectively. At S below 10 µg of C per liter, nonlinear relationships were observed between N_{max} and S (Fig. 2) and between G and 1/S (Fig. 3). The differences between the observed Gs (G_{obs}) and those following from equation 2 (G_{calc}) may be expressed by the equation:

$$t_e^{-1} = G_{calc}^{-1} - G_{obs}^{-1} \quad (3)$$

in which t_e = the mean half-life assigned to endogenous metabolism. When S = 2.5 μg of glucose C per liter, G_{calc} = 41.4 h and t_e = 95.7 h. When S = 5 μg of glucose C per liter, G_{calc} = 24.3 h and t_e = 93.5 h. Obviously, the t_e values were similar at the two glucose concentrations, and strain 315 had a half-life of nearly 4 days in these situations.

From the t_e, the specific endogenous metabolism rate μ_e may be calculated with $\mu_e = 0.693/t_e$. This produces $\mu_e = 7.33 \times 10^{-3}$ h^{-1}. According to Pirt (17), the maintenance coefficient m (milligrams of substrate per milligram dry weight per h) can be calculated from $m = \mu_e \times Y_G$ (Y_G = yield corrected for endogenous use of substrate, grams dry weight per gram of substrate). Although Y_G cannot be estimated directly, it will not differ much from yields measured in batch cultures at high initial substrate concentrations. Previous experiments revealed that these yields varied between 0.43 to 0.53 g (dry wt)/g of glucose for some fluorescent pseudomonads and an aeromonad. The m of strain 315 therefore approximates 0.015 mg of glucose per mg (dry wt) per h. This value is lower than those reported by Pirt (17) for *Aerobacter aerogenes* (m = 0.076 on glycerol at 37 °C) and *Aerobacter cloacae* (m = 0.094 on glucose at 37 °C) and those reported by Palumbo and Witter (16) for *Pseudomonas fluorescens* (m = 0.20 on glucose at 20 °C). The difference with the values reported by Pirt (17) might be explained by the low temperature (12 °C instead of 37 °C) at which strain 315 was grown on glucose.

The experiments with glucose, arabinose, and mannitol revealed that strain 315 multiplied on these compounds even at initial substrate concentrations below K_s. On acetate and on starch no growth occurred at corresponding concentrations. However, the observations presented in Tables 3 and 4 indicate that these compounds could be used at low concentrations once growth had started. Enzyme production probably was not induced in nongrowing cells at starch C concentrations below the K_s value but occurred at such concentrations when the cells were multiplying as a result of added glucose.

The determined K_s value of strain 315 on glucose is very low as compared with substrate saturation constants for transport or growth of other bacteria for this substrate, e.g., *Pseudomonas*

aeruginosa, 0.5 to 0.8 mg of glucose C per liter (3, 6, 14); *P. fluorescens*, 72 μg of glucose C per liter (13); and *Escherichia coli*, 72 μg of glucose C per liter (27). This suggests that the aeromonad may compete effectively with these bacteria if glucose would be growth limiting. As both drinking water isolates and strain 315, which was of surface-water origin, multiplied at very low glucose concentrations, it is suggested that this property may be rather common among representatives of the species *A. hydrophila*. The minor contribution of the aeromonads to the bacterial populations of drinking water (22, 25) and surface water (5, 8, 19) in which glucose concentrations are in the range of a few micrograms per liter (29), may be explained by the presence of many other bacteria having glucose transport systems with higher affinity than the aeromonads (9, 15, 24) and the inability of the aeromonad studied to multiply at low concentrations of acetate, lactate, succinate, and glutamate.

The organic carbon compounds originally present in the used tap water (dissolved organic carbon = 2 to 3 mg/liter) were not used as sources for carbon and energy of the *A. hydrophila* strains tested. With equation 2 the maximum glucose concentration at which strain 315 does not grow can be calculated. In this situation G_{calc} equals t_e, which is 94.6 h, and S = 0.98 μg of glucose C per liter. Strain 315 was unable to maintain itself in the tap water used; therefore, the glucose concentration in this water was always below 1 μg of C per liter. This calculation demonstrates that growth experiments as described may be used to measure the concentration of substrate available for a test strain in drinking water or in water from different treatment stages to assess treatment efficiency regarding substrate removal. For this purpose, representatives of specific species, particularly *P. aeruginosa*, which multiplies in clean water without a carbon source added (1, 4), may be used. With the N_{max} values the concentration of assimilable compounds originally present may be expressed in equivalents of a substrate on which the yield (CFU per milligram of substrate C) is known. In addition, G values may be used to calculate the maximum concentration of certain substrates that might be present in water. For such calculations G_{min} and K_s values should be known.

ACKNOWLEDGMENTS

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6. Utilization of low concentrations of starch by a *Flavobacterium* species isolated from tap water

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Appl. Environ. Microbiol. 41 (1981): 216-221

Abstract

Experiments in well-cleaned glass flasks revealed that addition of starch in concentrations of 10 and 25 µg of substrate C per liter to the filtrate of slow sand filters stimulated the development of a yellow-pigmented bacterium which was identified as a *Flavobacterium* species. The isolate was able to multiply in tap water without substrates added, but addition of starch and glucose in amounts as low as 1 µg of substrate C per liter clearly enhanced growth. The substrate affinities of the *Flavobacterium* for these compounds were 3.9 µg of starch C and 3.3 µg of glucose C per liter. The results of this study indicate that microorganisms which rapidly utilize starch at a level of a few micrograms per liter commonly occur in water.

INTRODUCTION

The ability of bacteria of aquatic habitats to utilize organic compounds at concentrations of a few micrograms per liter has frequently been demonstrated, particularly with low-molecular-weight compounds, such as glucose (9, 13, 23, 24, 26, 29), acetate (1, 19, 29), amino acids (5, 6, 8), some other organic acids (16), and synthetic organic chemicals (4). However, the utilization of biopolymers, such as proteins and polysaccharides, that can be taken up only after hydrolysis by extracellular enzymes has hardly been studied at such low concentrations.

Starch and starch-based compounds are being used as coagulant aids in water purification processes (15). Therefore, these compounds, when remaining at low concentrations in the final product, might contribute to the multiplication of microorganisms in tap water during distribution. For these reasons starch was selected to study the ability of bacteria to utilize high-molecular-weight compounds at the level of a few micrograms per liter.

MATERIALS AND METHODS

Glassware

The growth experiments were performed in calibrated, conical,

glass-stoppered, Pyrex glass bottles with a volume of 1 liter. These bottles as well as the pipettes used were thoroughly cleaned as described previously (26).

Water

The cleaned bottles were filled with 600 ml of tap water produced by the Municipal Dune Waterworks of The Hague, The Netherlands. This tap water is prepared from dune-infiltrated river water by the successive steps of addition of powdered activated carbon, rapid sand filtration, and slow sand filtration. The dissolved organic carbon content of the final product was about 3.6 mg of C per liter; the pH was approximately 7.5.

Growth experiments

Growth experiments with the autochthonous bacterial population of the filtrate of the slow sand filters were performed by incubating the bottles at 15 $\pm$ 0.5 °C directly after sampling. For pure culture studies, the vegetative cells of the autochthonous bacteria were killed by heating the bottles with sampled water in a water bath at 60 °C for 1 h followed by incubation at 60 °C for 2 h. After cooling, an organic compound from a freshly prepared, separately heated solution was added as a source of carbon and energy. Subsequently the bottles were inoculated with an initial number of cells varying from 100 to 500 colony-forming units (CFU) per ml from a starving culture grown in tap water supplied with an assimilable carbon compound.

Growth of the autochthonous bacteria and of the pure culture was measured by colony counts. For this purpose, the spread plate technique was applied by inoculating (in triplicate) predried Lab-Lemco (Oxoid) agar plates with 0.05 ml from decimal dilutions of the water. These plates were incubated at 25 °C until numbers of colonies on the plates no longer increased, i.e., 10 days for counts of autochtonous bacteria. With the obtained colony counts (N_t, in CFU per milliliter), the mean doubling times (G values, in hours) under the different experimental conditions were calculated by the equation:

$$G = \log 2 \cdot t/(\log N_{t+\Delta t} - \log N_t) \qquad (1)$$

where Δt is the incubation period (hours) in which N_t increased to

$N_{t + \Delta t}$. These calculations were only performed for that part of the growth curve during which the initial substrate concentration was not significantly reduced by the produced number of cells, i.e., when the colony counts were less than 10 % of the maxmimum colony count (N_{max}, in CFU per milliliter).

Characterization procedures

Isolates were characterized by the following tests: Gram stain, oxidase test (12), oxidation-fermentation test with glucose (11), arginine deiminase test (22), test for NO_2^- or N_2 production from NO_3^- (18), and urease activity (20). In addition, the isolates were tested for the ability to hydrolyze proteins (casein, gelatin), starch, chitin, and Tween-80, using a standard agar medium either made turbid with casein of chitin or containing 2.5 g of gelatin, starch, or Tween-80 per liter. The media and procedures used for these tests have been described previously (25).

RESULTS

Isolation and characterization of a bacterium utilizing starch at low concentrations

Starch was added in concentrations (added substrate ΔS) of 10 and 25 µg of C per liter to duplicate bottles containing 600 ml of freshly sampled filtrate from slow sand filters. Two bottles received no starch. The development of the bacteria in these bottles, stored at 15 °C, revealed that both N_{max} and G of the bacteria present in the water were strongly influenced by the low amounts of starch added (Fig. 1). Moreover, it was observed that in the logarithmic as well as in the stationary phases of growth, the population of all four bottles with added starch consisted almost completely of bacteria that formed similar yellow-pigmented colonies requiring 72 h of incubation before being visible. The bottles without starch contained bacteria which formed nonpigmented, transparent colonies.

Seven isolates of the yellow-pigmented bacteria of the starch-supplied cultures were obtained. The characterization procedures confirmed that one type of organism had become dominant, and from the properties of the organism (Table 1) it was concluded that it belonged to the genus *Flavobacterium*.

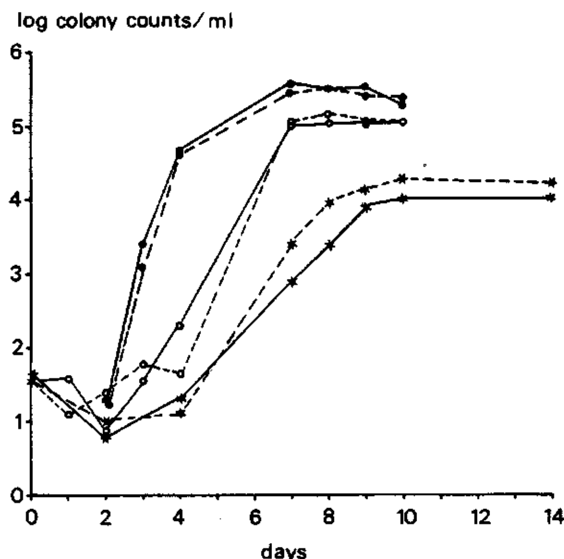

Fig. 1 Growth of bacteria present in the filtrate of slow and filters without added starch (★) or with 10 (○) or 25 (●) µg of starch C added per liter. Solid and broken lines indicate determinations.

Growth on starch

To test the ability of the isolated organism to utilize starch at low concentrations, cells of one of the isolates (strain 166) grown on a Lab-Lemco agar slant were added (N_o = 600 CFU/ml) to a bottle with heated tap water supplied with 5 µg of starch C per liter and to two bottles containing this heated tap water without added substrate. Growth of the organism in the presence of starch (N_{max} = 1.1 x 10^5 CFU/ml, G = approximately 8 h) was much stronger than in the bottles without substrate added (N_{max} = 3.2 x 10^4 CFU/ml, G = 30 to 38 h). These observations confirmed that the organism was able to utilize starch at the level of micrograms per liter.

Strain 166 was also grown in tap water supplied with starch at different concentrations (ΔS). The growth curves (Fig. 2) and the

Table 1. Some properties of the starch-utilizing *Flavobacterium* sp.

Property	Isolate characteristic
Shape of cells	Rods
Gram stain	-
Motility	-
Pigment of colony	Yellow
Oxidase	+
Acid from glucose:	
Oxidation	-
Fermentation	-
Arginine deiminase	-
Urease	-
NO_2^- from NO_3^-	-
N_2 from NO_3^-	-
Casein hydrolysis	+
Gelatin hydrolysis	+
Starch hydrolysis	+
Chitin hydrolysis	-
Growth at 37 °C	-

calculated mean doubling times (Table 2) clearly demonstrate that even addition of starch at initial concentrations below 5 μg of C per liter stimulated growth remarkably. The relationship between the N_{max} values observed and the different initial concentrations of starch (Fig. 3) revealed that at low concentrations of this substrate, the organism yielded 2.0×10^{10} CFU/mg of starch C. Using this value and the N_{max} of the blank, the natural substrate concentration (S_n) may be expressed in micrograms of starch C equivalents per liter. Thus, at an N_{max} of 2.7×10^4 CFU/ml, $S_n = 1.4$ μg of starch C equivalents per liter.

The survival ability of the isolate after exhaustion of the added substrate was studied by measuring the number of viable cells in bottles originally containing 100 μg of starch C per liter during prolonged incubation at 15 °C. From the results obtained (Fig. 4) it is clear that the colony counts decreased rapidly; i.e., a 99 % decrease was obtained after about 42 to 58 days of incubation.

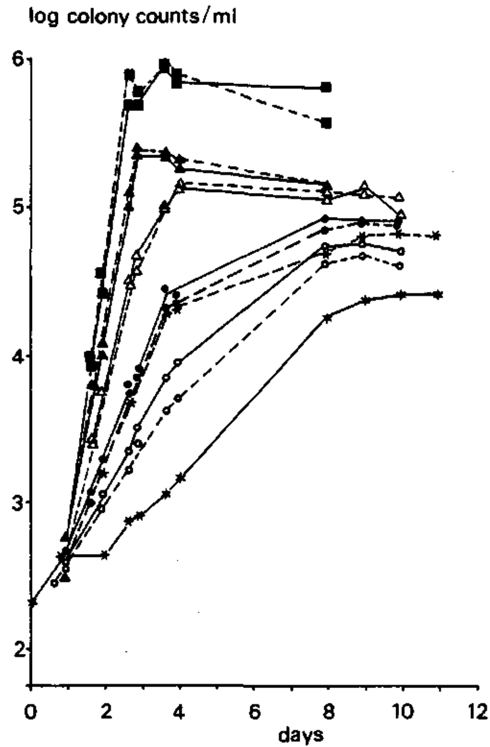

Fig. 2 - Growth of *Flavobacterium* strain 166 in the filtrate of slow sand filters without added starch (★) or with 1 (○), 3 (●), 5 (△), 10 (▲), or 100 (■) µg of starch C added per liter. Solid and broken lines indicate duplicate determinations.

Table 2. Growth (at 15 ± 0.5 °C) of isolate 166 in tap water supplied with starch[a]

Starch added (μg of C/liter)	Growth[b]			
	G(h)		N_{max} (CFU/ml)	
100	4.0	3.8	9.2×10^5	9.1×10^5
10	5.3	4.9	2.3×10^5	2.3×10^5
5	6.8	6.5	1.4×10^5	1.4×10^5
3	9.9	9.5	8.4×10^4	7.9×10^4
1	15.0	17.8	5.6×10^4	4.7×10^4
0	27.5	12.3[c]	2.7×10^4	6.6×10^4[c]

[a] Starving cells grown at an initial concentration of 5 μg of starch C per liter were used as inoculum.
[b] Data from duplicate flasks are presented in each pair of columns.
[c] Aberrant results, which were not used for calculations.

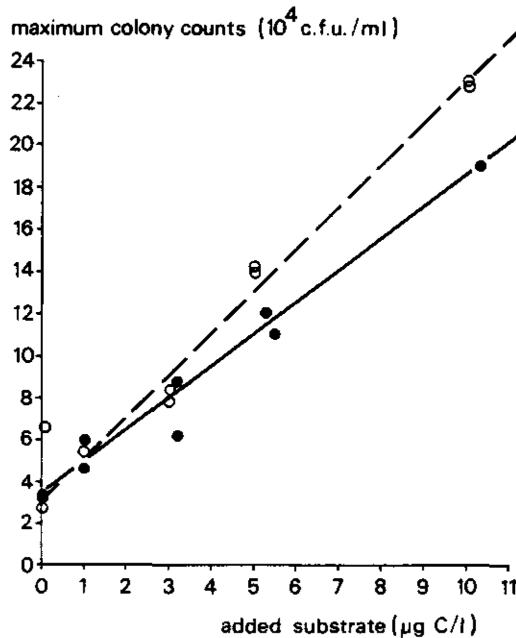

Fig. 3 Maximum colony counts of *Flavobacterium* strain 166 in relation to the amounts of starch C (O) and glucose C (●) added to the filtrate of slow sand filters.

Growth on glucose and some other substrates

Growth of isolate 166 was assessed in bottles with tap water to which D-glucose had been added in different concentrations. The results of this experiment are shown in Table 3, whereas the relationship between the N_{max} and ΔS values is presented in Fig. 3. Comparison of the observed mean doubling times at different glucose concentrations (Table 3) with those observed at different starch concentrations shows that the organism grew more rapidly in the presence of starch. The yield of strain 166 on glucose at low concentrations ($\Delta S \leqslant 10$ µg of glucose C per liter) was 1.6×10^{10} CFU/mg of glucose C; thus, S_n expressed in glucose C equivalents was 2.0 µg of glucose C per liter.

Table 3. Growth (at 15 ± 0.5 °C) of isolate 166 in tap water supplied with glucose[a]

Glucose added (µg of C/liter)	Growth[b]			
	G (h)		N_{max} (CFU/ml)	
100	5.0	4.8	4.4×10^5	4.7×10^5
10.3	6.1	6.5	1.9×10^5	1.9×10^5
5.4	9.5	8.9	1.2×10^5	1.1×10^5
3.2	11.2	11.7	8.7×10^4	6.1×10^4
1	14.0	14.4	4.6×10^4	5.9×10^4
0	28.2	30.5	3.2×10^4	3.3×10^4

[a] Starving cells grown at an initial concentration of 100 g of starch C per liter were used as inoculum.

[b] Data from duplicate flasks are presented in each pair of columns.

As it is unlikely that glucose or starch contributed significantly to the S_n of the tap water used, a number of compounds with relatively low molecular weights which might have contributed to the S_n were tested at low concentrations for their growth-stimulating properties for strain 166. These compounds were: acetate, DL-lactate, pyruvate, succinate, L-aspartate, L-glutamate, and L-alanine. Glycerol, maltose, and yeast extract were also tested.

Growth of isolate 166 was stimulated strongly only by maltose and, to a much lesser extent, acetate (Table 4). The other substances tested did not affect the G or N_{max} values.

DISCUSSION

The above described experiments reveal that biodegradation of certain compounds at levels of ecological significance, as well as the microorganisms responsible for this degradation, may be studied by very simple experiments, i.e., without the use of radioactive substrates and without laborious continuous culture experiments. However, such experiments cannot be performed with water in which the S_n enables the autochthonous microorganisms or the pure culture to multiply to high colony counts or in which the N_o (autochtonous bacteria) is high as compared with the N_{max} that may be expected on a ΔS of a few micrograms. The application of the above-described method may also be limited by the presence or growth in the water of microorganisms which utilize the added substrate, but which do not grow on a solid medium.

The experiments also showed that an organism, tentatively identified as a *Flavobacterium* sp., utilized starch at low concentrations at least as rapidly as it did low concentrations of the monomer glucose. None of the species of the genera *Flavobacterium* and *Cytophaga* described in Bergey's Manual (27) and by Hayes (10) were similar to the isolated bacterium. However, the ability to utilize starch as a source of carbon and energy seems to be a common property of bacteria belonging to these genera. Moreover, *Flavobacterium* spp. have been found to dominate in activated sludge fed with starch (J.M.A. Janssen, Ph.D. Thesis, Agricultural University, Wageningen, The Netherlands, 1979). Therefore, the ability of the isolate studied to grow at concentrations of a few micrograms of either starch, glucose, or maltose per liter of water may contribute to explaining the widespread occurrence of bacteria that form yellow-pigmented colonies in surface water (2, 21), ground water (28), and tap water (3, 7, 14, 17).

The yields (CFU per milligram of substrate C) of *Flavobacterium* strain 166 on glucose and on starch, respectively, were approximately five times larger than the yields of an *Aeromonas hydrophila* strain on these compounds (26), suggesting

that cells of the *Flavobacterium* isolate were five times smaller than those of the aeromonad. Calculation, based on the assumptions that 50 % of the substrate C is assimilated and that dry weight is 20 % of total weight, reveals that the volume of the *Flavobacterium* cells grown on starch and on glucose approximated 0.25 to 0.3 μm^3, which is very small. This small cell volume is expected to be advantageous at low substrate concentrations.

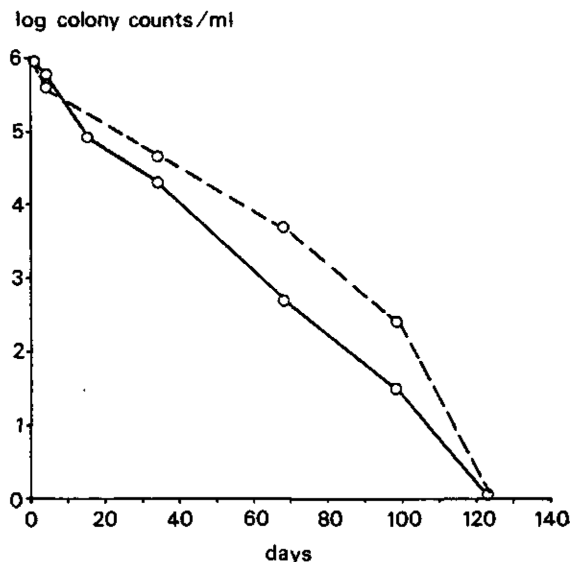

Fig. 4 Colony counts of *Flavobacterium* strain 166 during prolonged incubation at 15 ± 0.5 °C (values of duplicate flasks).

The N_{max} values of isolate 166 observed in the experiments with starch or glucose in tap water resulted from the utilization of both the added substrate and some unknown compounds present in the filtrate of slow sand filters (Fig. 3). These unknown compounds (S_n available to isolate 166) amounted only to a few micrograms of carbon per liter, which was about 0.1 % of the dissolved organic carbon content of the water. The nature of the unknown compounds utilized by the organism was not elucidated, although it may be concluded from Table 4 that alanine, aspartate, glutamate, lactate,

pyruvate, and succinate did not contribute to S_n. Nevertheless, these compounds may have been present in the filtrate used.

The G values of isolate 166 observed in the experiments with different concentrations of starch and glucose may be used to obtain the substrate saturation constants (K_s, micrograms of C per liter) of the isolate for these compounds. For this purpose, the following

Table 4. Growth (at 15 ± 0.5 °C) of isolate 166 in tap water supplied with different organic carbon compounds[a]

Substrate added	Concn (µg of C/liter)	Lag period (days)	G(h)	N_{max} (CFU/ml)
None		8	29.6	4.9×10^4
None		8	19.3	5.4×10^4
Glycerol	5.5	2.5	22.5	4.3×10^4
Maltose	5	1.5	5.8	9.6×10^4
L-Alanine	5	10	25.9	5.2×10^4
L-Aspartate	5	9	24.1	5.0×10^4
L-Glutamate	5	9	24.0	5.4×10^4
Yeast extract	4.2	1.5	23.0	5.1×10^4
Acetate	5	3.5	21.3	1.4×10^5
DL-Lactate	8.2	8	22.7	4.9×10^4
Pyruvate	5	9	23.9	6.8×10^4
Succinate	5	8.5	24.2	4.3×10^4

[a] Starving cells grown at an initial concentration of 100 µg of starch C per liter were used as inoculum

slightly adapted Lineweaver-Burk modification of the Monod equation was used:

$$G = G_{min} + (G_{min} \cdot K_s)(1/S) \quad (2)$$

In this equation G and G_{min} are the real and the minimum mean generation times (hours), respectively, S is the concentration of the growth-limiting substrate, and K_s is the substrate saturation constant. The use of equation 2 is complicated by the fact that in

the experiments described above, S is composed of a known amount of the added compound (Δ S) and unknown amounts of other utilized compounds (S_n). The pronounced and immediate stimulation of growth as observed after the addition of starch or glucose suggested that these compounds were utilized preferentially. Plotting the observed G values (cf. Tables 2 and 3) against the reciprocal values of ΔS shows that in the starch experiment the G values were linearly

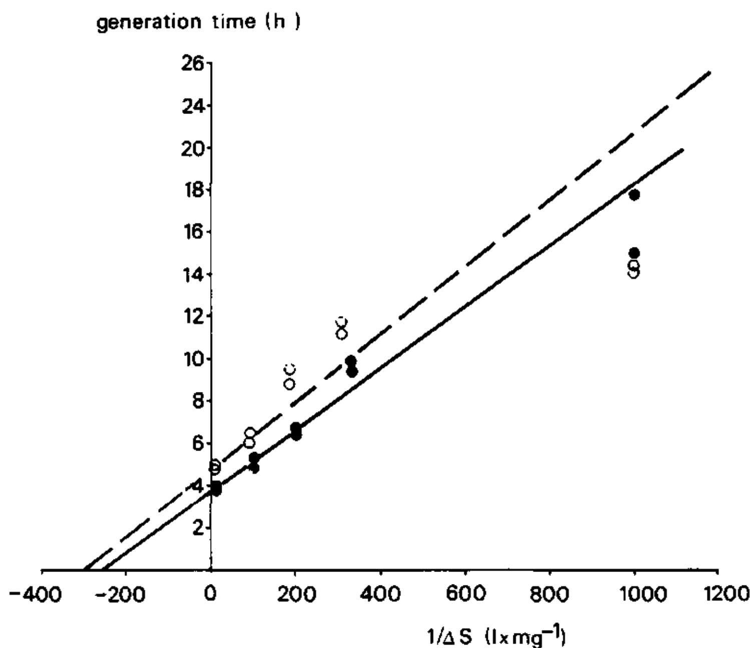

Fig. 5 Lineweaver-Burk plots of *Flavobacterium* strain 166 on starch (●) or glucose (O) added to the filtrate of slow sand filters.

related with 1/ΔS when S was $\geqslant$ 5 µg of starch C per liter (Fig. 5). Calculation of this relationship gave:

$$G = 3.7 + (14.5)(1/\Delta S) \quad (3)$$

thus, G_{max} was 3.7 h and K_s was 3.9 µg of starch C per liter. In the

experiment with glucose, deviating generation times were observed when ΔS was $\leqslant 5$ µg of glucose C per liter. Calculation of the linear relationship shown in Fig. 5 gave:

$$G = 4.7 + (15.5)(1/\Delta S) \quad (4)$$

thus, G_{min} was 4.7 h and K_s was 3.3 µg of glucose C per liter.

The calculated linear functions of equations 3 and 4 represent the kinetic behavior of isolate 166 towards starch and glucose when sequential uptake of substrates occurred (i.e., the added substrates were preferentially used) and when S_n did not contain significant amounts of either starch or glucose. The mechanisms responsible for the sequential uptake would have been most effective at the highest ΔS values. As the water used in the experiments was prepared without the addition of starch or starchlike compounds in any treatment stage and previous experiments with water from the same location revealed that the natural glucose concentration was extremely low (26), the contribution of naturally occurring starch and glucose to S_n is assumed to have been negligible, at least at the ΔS values used to calculate linear functions. Hence, equations 3 and 4 indeed represent the kinetic behavior of isolate 166 towards starch and glucose.

The use of substrate for maintenance processes did not clearly influence the yield of the *Flavobacterium* on the added substrates (cf. Fig. 3), as was observed previously (26) for an *A. hydrophila* strain when $S < K_s$. The ability of isolate 166 to use natural substrates in addition to the added substrates is thought to be responsible for this difference in behavior. This uptake of natural substrates may also explain the positive deviations from the linear functions at a ΔS of 1.0 µg of C per liter (Fig. 5).

K_s values as found in the present study have frequently been reported for glucose. By using radioactive substrates and pure cultures, transport constants (K_t) of between 2 and 6 µg of glucose C per kiter were obtained by Wright and Hobbie (29) for a *Pseudomonas* sp. obtained from lake water; by Hamilton et al. (9) for gramnegative, oxidase-positive marine isolates; and by Vaccaro and Jannasch (24) for *Achromobacter aquamarinus (Alcaligenes aquamarinus)*. However, these transport constants, which had been estimated at 5 to 6 °C, were all considerably higher at 15 °C.

Nevertheless, in nature many microorganisms must have very low K_t values for glucose, as may be concluded from the $K_t + S_n$ values of glucose in various water types, including seawater and freshwaters, being usually as low as a few micrograms of glucose C per liter (13, 23, 24, 29).

A previous investigation (26) showed that growing cells of an *A. hydrophila* isolate were able to use starch at concentrations of a few micrograms per liter. However, bacteria with a low K_s for starch, as described herein, seem to be scarcely studied. Still, the presence of the described *Flavobacterium* sp. in tap water, produced without the use of starch or starchlike compounds in any of the treatment stages, indicates that such bacteria are common components of the bacterial flora of water.

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7. Nutritional versatility of a starch-utilizing *Flavobacterium* at low substrate concentrations

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Abstract

A starch-utilizing yellow-pigmented bacterium, isolated from tap water, was tested for the utilization of 64 natural compounds at a concentration of 1 g/liter by measuring colony growth on agar media. Only 12 carbohydrates and glycerol promoted growth. Growth experiments with the organism in pasteurized tap water supplied with mixtures of substrates at concentrations of 1 or 10 µg of C of each substrate per liter, followed by separate experiments with a number of carbohydrates at 10 µg of C per liter showed that of these 64 natural compounds only sucrose, maltose, raffinose, starch, and glycerol promoted growth at very low concentrations. Also maltotriose, -tetraose, -pentaose, -hexaose, and stachyose, which were not included in the mixtures, enhanced growth, and generation times of 3 to 5 h at 10 µg of C per liter were observed. The organism, which was tentatively identified as a *Flavobacterium* species, thus appeared to be highly specialized in the utilization of glycerol and a number of oligo- and polysaccharides at very low concentrations.

INTRODUCTION

Yellow-pigmented bacteria, usually belonging to the genus *Flavobacterium*, are commonly part of the bacterial flora of waters poor in organic substrates i.e. distilled water (7), ground water (23) and drinking water (2, 10, 15). Adaptation of these organisms to low concentrations of substrates is indicated by their multiplication in drinking water (1, 12, 14). More specific information has been presented by Van der Kooij and Hijnen (20) who isolated a *Flavobacterium* species with very low substrate-saturation constants for starch and glucose (3.9 and 3.3 µg of C, respectively) which predominated in samples of stored slow sand filtrate supplied with starch (10 and 25 µg of C per liter). In additional experiments with starch added in low concentrations to various types of drinking water, yellow-pigmented bacteria also predominated. The nutritional versatility of one of these bacteria was examined to clarify its dependence upon starch or starch-like compounds for growth as well as its taxonomic position. For this

purpose a large variety of naturally occurring amino acids (AA), carboxylic acids (CA), aromatic acids (AR) and carbohydrates and (poly)alcohols (CHA) were tested as sources of carbon and energy for growth, both at high (grams per liter) and very low concentrations (micrograms per liter).

MATERIALS AND METHODS

Bacterial strain

The organism (strain S12) was isolated from tap water (Municipal Dune Waterworks of The Hague) prepared from pretreated dune-infiltrated Meuse river water treated by addition of powdered activated carbon, rapid sand filtration and slow sand filtration. Treated water contained average concentrations of 3.6 mg of dissolved organic carbon per liter and 0.12 mmol of nitrate. After storage at 15 °C of 600 ml of this water supplied with 100 ug of starch C per liter, the predominating organism which formed small non-pigmented colonies on Lab Lemco (Oxoid Ltd.) agar (LLA) plates incubated at 25 °C was isolated. A similar procedure has been described previously (20).

Media and substrates

LLA contained 5 g of peptone, 3 g of Lab Lemco beef extract and 12 g of agar (Oxoid nr. 3) in 1 liter of demineralized water. The final pH was 7.4. Mineral salts agar (MSA) consisted of 0.5 g NH_4Cl, 0.5 g of $Na_2HPO_4 \cdot 12H_2O$ and 12 g of purified agar in 1 liter of demineralized water. The final pH was 6.9.

Substrates tested as sources of carbon and energy for growth at 1 g per liter included 18 AA, 18 CA, 11 AR and 18 CHA which are listed in Table 1. The compounds were present in sterile solutions of either 1 or 10 % (wt/vol). Moreover, with the listed compounds four different mixtures (i.e. the AA mixture, the CHA mixture, the CA mixture and the AR mixture) were prepared by dissolving equal amounts of carbon of the various substrates in demineralized water. After neutralization with 0.1 N NaOH, the mixtures were autoclaved. For use of individual substrates at low concentrations (micrograms per liter) small volumes of freshly prepared solutions, which were heated at 60 °C for 0.5 h, were added to the experimental bottles. Melibiose, melezitose, trehalose, stachyose, maltotriose, maltotetraose, maltopentaose and maltohexaose were only tested at a

low concentration. The latter three compounds were kindly supplied by H. Hokse of the Potato Processing Research Institute (TNO), Groningen (The Netherlands).

Growth on agar media

Low numbers (10 to 30) of viable cells grown in tap water supplied with a very low amount of substrate were spread on predried plates (9 cm) of either LLA or MSA with 1 g of substrate per liter. After inoculation, the plates were incubated at 25 °C and during a period of about 2 weeks diameters of 4 to 5 colonies on each plate (experiments done in duplicate) were measured periodically using a Wild M7S binocular microscope.

Growth in water

The technique applied to assess the growth of a pure culture in water supplied with various substrates at low concentrations has been described in previous communications (20, 21). The water used for the experiments described in this paper originated from the pumping station Tull and 't Waal (Midden-Nederland Waterworks), where it is prepared from anaerobic ground water by aeration and rapid sand filtration. The final product contained 2.3 mg of dissolved organic carbon per liter and 0.01 mmol of nitrate; the pH was 7.7 (after heat treatment).

Characterization procedures

The following tests were used to characterize the organism: Gram stain, oxidase test (6), oxidation-fermentation test with glucose (5), arginine-deiminase test (18),tests for NO_2^- or N2 production from NO_3^- (16) and urease activity (17). In addition, the isolate was tested for hydrolysis of proteins (casein, gelatin), starch, chitin and Tween-80, using a standard agar medium either made turbid with casein or chitin or containing 2.5 g of gelatin, starch or Tween-80 per liter. All media were incubated at 25 °C. The media and procedures for these tests have been described previously (19).

RESULTS

Utilization of substrates present at a concentration of 1 g per liter

Strain S12 multiplied slowly on LLA and after 48 hours of

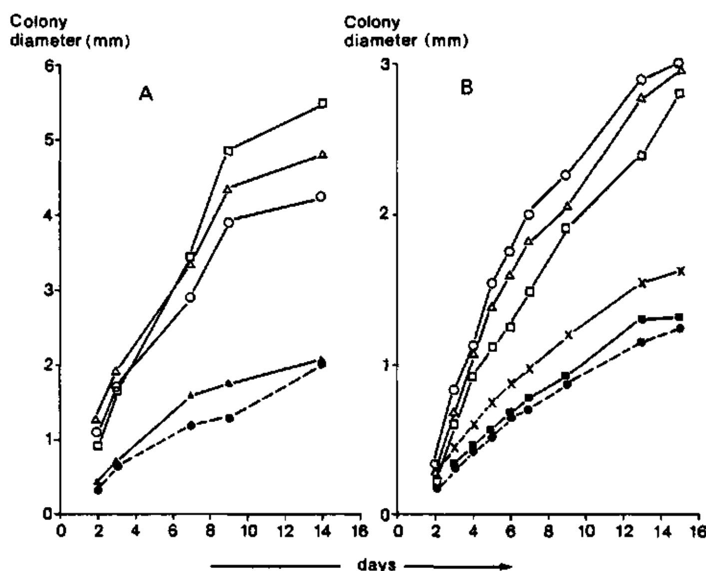

Fig. 1 The increase of the diameter of colonies of strain S12 during growth at 25 °C on Lab-Lemco agar (A) and Mineral Salts Agar (B), supplied with glucose (○), maltose (△), starch (□), mannitol (▲), succinate (■) and pyruvate (X) at a concentration of 1 g of substrate per liter; ●, shows blanks.

incubation, very small circular convex nonpigmented colonies became visible. Colony size increased upon further incubation but pigmentation did not appear. With 12 out of 18 CHA added to LLA, growth of the colonies was clearly enhanced (Fig. 1A; not all data shown). These colonies rapidly increasing in size, and those on the agars supplied with lactose and inositol, respectively, which did not promote growth, were brightly yellow coloured. On MSA similar growth-favoring effects were observed with CHA (Fig. 1B). The radial growth rates of the colonies on LLA and MSA supplied with CHA had equal maximum values of 8-11 µm/h, but the colonies on MSA remained smaller (Fig. 1). The effects on the size (R^2) of the colonies of all the substrates tested at 1 g per liter are presented in Table 1. From the presented results it can be concluded that AA, CA and AR

Table 1. Effects of 64 different substrates on the relative colony size of *Flavobacterium* sp. strain S12 on agar media[a]

CHA	R_s^2/R_b^2(LLA)	R_s^2/R_b^2(MSA)	AA	R_s^2/R_b^2(MSA)	CA	R_s^2/R_b^2(MSA)	AR	R_s^2/R_b^2(MSA)
L-Arabinose	5.6	5.4	Glycine	0.4	Formate	-[b]	Benzoate	-
D-Xylose	4.5	4.5	L-Alanine	0.6	Acetate	-	*p*-Hydroxy-	
D-Glucose	4.5	5.3(6.4)[c]	L-Valine	0.8	Glyoxylate	-	benzoate	1.1
D-Mannose	4.2	2.7	DL-Serine	0.4	Oxalate	-	Salicylate	-
D-Galactose	4.8	3.3	L-Threonine	0.6	Glycolate	1.1	Gallate	-
L-Rhamnose	5.2	2.4	L-Leucine	0.8	Propionate	-	Anthranilate	-
Fructose	7.2	3.4	L-Isoleucine	0.7	Pyruvate	1.8	Vanillate	-
Sucrose	7.2	2.6	L-Histidine	0.6	DL-Lactate	-	Phtalate	0.7[e]
Cellobiose	0.9	0.7	L-Arginine	1.1	Malonate	1.4	Ferulate	-
Lactose	1.2	1.3(1.2)[c]	L-Aspartate	1.0	β-OH-Butyrate	1.3	Nicotinate	0.5[e]
Maltose	5.8	5.3(6.1)[c]	L-Asparagine	1.2	Fumarate	1.3	*p*-Hydroxy-	
Raffinose	5.9	4.0	L-Glutamate	1.3	Succinate	1.1	phenylacetate	0.4[e]
Starch	7.6	6.4	L-Glutamine	0.7	Malonate	-	DL-Mandelate	0.5[e]
Gluconate	1.2	1.1	L-Proline	1.0	L-Tartrate	1.5		
Ethanol	1.3	1.7	L-Lysine	0.9	Valerate	NT[e]		
Glycerol	7.8	1.8	L-Tyrosine	1.1	α-Ketoglutarate	1.1		
Mannitol	0.7	1.0	DL-Tryptophan	1.3	Citrate	-		
Inositol	0.9	0.9	DL-Phenylalanine	0.6	Adipate	1.2		

[a] Relative colony size is R_s^2/R_b^2 in which R_s is radius of colony grown on agar supplied with a specific substrate and R_b is radius of colony on agar without this substrate (blank). Lab-Lemco agar (LLA) and Mineral Salts agar (MSA) are used as blanks. Incubation period is 14 to 15 days.

[b] -, No visible colonies developed within 14 days of incubation.

[c] Repeated experiment.

[d] Number of colonies on the agar supplied with the substrate was about 50 % of the number of colonies on the agar without the substrate.

[e] NT, not tested.

were either not utilized, growth retarding, or lethal when tested at a concentration of 1 g per liter.

Utilization of substrates at low concentrations

As compared with growth of the blanks, growth of strain S12 was

Table 2. Growth of *Flavobacterium* sp. strain S12 at 15 ± 0.5°C in tap water supplied with mixtures of naturally occurring substrates in a concentration of 1 µg (A) and 10 µg (B) of C per liter.[a]

Mixture added	Total concentration of added substrate (µg of C/liter)[b]		Generation time (h)		N_{max} (CFU/ml)	
	A	B	A	B[e]	A	B[e]
None 1[d]	-	-	36.7	NG	$1.5x10^4$	350
None 2[d]	-	-	42.6	NG	$1.0x10^4$	300
AA (1)	18.1	180	55.6	NG	$5.0x10^3$	140
AA (2)	18.3	179	46.0	NG	$9.1x10^3$	160
CA (1)	18.9	180	46.0	NG	$1.2x10^4$	110
CA (2)	17.1	186	38.3	NG	$6.2x10^3$	120
AR (1)	10.2	106	48.8	NG	$1.5x10^4$	100
AR (2)	11.4	111	32.6	NG	$1.3x10^4$	110
CHA (1)	17.7	178	10.0	3.2	$1.1x10^5$	$8.3x10^5$
CHA (2)	18.6	174	10.1	3.4	$7.3x10^4$	$8.8x10^5$
TM (1)	63.7	665	11.8	3.5	$6.5x10^4$	$9.1x10^5$
TM (2)	68.3	634	10.4	3.5	$8.8x10^4$	$7.4x10^5$

[a] Starving cells grown at an initial concentration of 100 ug of starch C per liter were used as inoculum (110 to 150 CFU/ml).

[b] Total concentration after correction for the water volume deviating from 600 ml.

[c] Within an incubation period of 8 days; N_{max} values with CHA and TM were reached within 4 to 6 days.

[d] 1 and 2; Duplicate flasks.

[e] NG; No growth (no systematic increase of the initial colony counts) within 8 days of incubation.

clearly enhanced by the addition of the CHA mixture, and by the addition of all mixtures together, i.e. the total mixture (TM) but not by the AA, CA and AR mixtures (Table 2). The maximum colony count (N_{max}) values of the TM and CHA mixtures in experiment B were approximately 10 times higher than the N_{max} values of these mixtures in experiment A. Thus, increasing the concentration of substrate did not increase the ability of strain S12 to utilize substrates. Moreover, the similarity of the N_{max} values of the TM and CHA mixtures as observed in both experiments indicates that AA, CA and AR were not assimilated by the cells growing on the CHA.

The components of the CHA mixture, which were utilized by strain S12 at a low concentration, were identified by separate tests at a concentration of 10 µg of C per liter of those compounds which favored growth at 1 g per liter. Only 5 substrates, namely sucrose, maltose, raffinose, starch, and glycerol, were utilized for growth. (Fig. 2; not all negative data shown). The N_{max} values of strain S12 obtained for the utilized substrates varied from 1.1 x 10^5 CFU/ml (maltose) to 2.5 x 10^5 CFU/ml (sucrose) and the generation times (G) varied from 4.5 h (sucrose) to 12 h (maltose). These observations explain the N_{max} values presented in Table 2. In addition to the sugars included in the CHA mixture, melibiose, melezitose, trehalose, stachyose, maltotriose, maltotetraose, maltopentaose and maltohexaose were tested at 10 µg of C per liter. Only with the five latter compounds was a rapid growth (generation time varied from 3 to 5 h) observed, with N_{max} values of about 2 x 10^5 CFU/ml.

Characterization of strain S12

In addition to the substrates which could serve as sources of carbon and energy for growth of strain S12 several additional properties of this bacterium have been determined (Table 3). The organism was identified as a non-proteolytic *Flavobacterium* species. In mineral salts medium (indentical to MSA without agar) containing 100 mg of starch C per liter, the maximum level of growth (measured as extinction at 450 nm) was not influenced by replacement of NH_4-N with an equivalent amount of NO_3-N. Therefore, both nitrogen compounds may serve as a source of nitrogen, and it is concluded that the organism does not require specific growth factors.

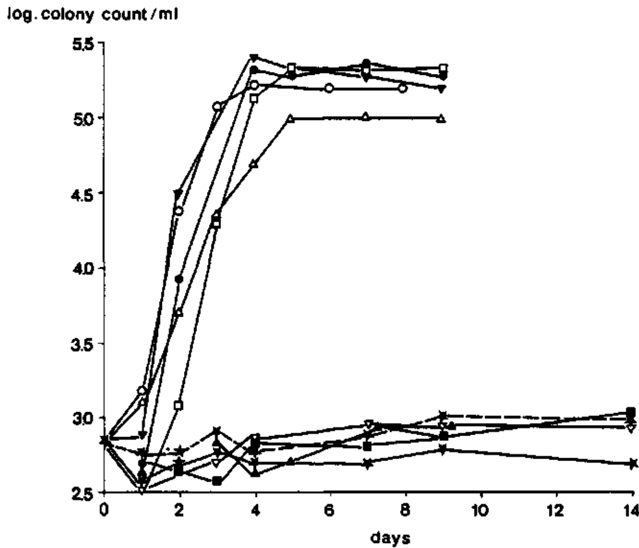

Fig. 2 Growth curves of strain S12 at 15 ± 0.5 °C in tap water supplied with glucose (▲), maltose (△), raffinose (●), starch (○), lactose (■), glycerol (□), sucrose (▼) or rhamnose (▽) at a concentration of 10 μg of C per liter. These were all single growth experiments, except those with the blanks (★), which were done in duplicate.

DISCUSSION

Significance of characterization procedures

Procedures to characterize bacteria usually include tests for the production of acids from carbohydrates in peptone media under aerobic and anaerobic conditions.The *Flavobacterium* sp. strain S12 studied in this paper and *Flavobacterium* sp. strain 166 described previously (21) did not produce acids from glucose in the oxidation-fermentation test (5). However, glucose as well as a number of other carbohydrates and polyalcohols appeared to be suitable substrates for growth (Table 1), but the radial growth

rates of the colonies of strain S12 are very low as compared to those of *Escherichia coli* (8) and *Pseudomonas fluorescens* (13) growing on MSA with glucose. Hence a technique more sensitive than simple replica plating had to be used to demonstrate the utilization of these and other substrates added to MSA. It is

Table 3. Some additional properties of *Flavobacterium* sp. strain S12

Property	Isolate characteristic
Shape of cells	Rods (1.5 - 2 x 0.5 μm)[a]
Motility	-
Gram stain	-
Oxidase	+
Acid from glucose:	
Oxidation	-
Fermentation	-
Arginine deiminase	-
Urease	-
NO_2^- from NO_3^-	-
N_2 from NO_3^-	-
Casein hydrolysis	-
Gelatin hydrolysis	-
Starch hydrolysis	+
Chitin hydrolysis	-
Tween-80 hydrolysis	-
Growth at 37 °C	-

[a] Cells grown at 25 °C in Mineral Salts Medium (without agar) with 1 g of starch per liter. Cells on Lab-Lemco agar had similar dimensions but after prolonged incubation (> 1 week) elongated forms (25 to 40 μm) without clearly visible septa were observed.

possible that the substrate concentration of 1 g/l is unfavourable for *Flavobacterium* sp., strain S12 which grows rapidly in water at very low concentrations of substrates.

Isolate S12 fits well into the description of the genus *Flavobacterium* as given by Holmes and Owen (1979) but none of the

species of this genus as described in Bergey's Manual of Determinative Bacteriology (22) includes a combination of properties as observed with *Flavobacterium* sp. strain S12, which is amylolytic and non-proteolytic (Table 3). The organism also does not resemble groups of *Flavobacterium*-like bacteria defined by McMeekin et al (9) or Hayes (3), in which the amylolytic bacteria were also proteolytic. These observations once more indicate the incomplete description of species belonging to the genus *Flavobacterium*. Carbohydrates and (poly)alcohols are suitable substrates to differentiate between bacteria belonging to a specific genus (16, 19). Therefore it is suggested to differentiate between *Flavobacterium* species on the basis of utilization of these compounds and not on acid production. Additional significant information may be obtained from tests on the production of extracellular enzymes (proteases, amylases, etc.).

The experiments described in this paper reveal that apart from a taxonomic value, tests for growth at high concentrations of carbohydrates and other substrates only have limited ecological significance. Compounds which were not utilized at high concentrations also did not stimulate growth at low concentrations (1 and 10 µg of C/l). However, a number of compounds which clearly favoured growth at 1 g per liter gave negatieve results at low concentrations. Unfortunately, tests to measure growth with substrates present at low concentrations are time consuming. The results presented in this study demonstrate that this disadvantage may be minimized by combining tests at high concentrations with those with mixtures of substrates at low concentrations.

Growth of strain S12 at very low substrate concentrations

At concentrations equal to or below 10 µg of C per liter *Flavobacterium* sp. strain S12 only utilized a number of oligosaccharides, the polysaccharides included in starch (amylose and amylopectin), and glycerol. The monosaccharides of which these carbohydrates consisted (glucose, galactose and fructose), as well as arabinose, xylose, mannose and rhamnose, did not favor growth at these concentrations, although they were utilized at a concentration of 1 g per liter (Table 1). Obviously, the molecules of oligo- and polysaccharides were more efficiently transported into the cells at very low concentrations than those of the monosaccharides. This finding may reveal the specific character of *Flavobacterium*

sp. strain S12. *Flavobacterium* sp. strain 166 studied previously (20) was also found to grow more rapidly with starch than with glucose suggesting that this property is not uncommon amongst the amylolytic species of the genus *Flavobacterium*. Moreover, the isolation of yellow-pigmented bacteria from tap water enriched with very low concentrations of starch suggests that a high affinity for starch is typical for species of bacteria belonging to the genus *Flavobacterium*.

Flavobacterium sp. strain S12 did not grow in the slow sand filtrate from which it had been isolated (data not shown) despite its high affinities for glycerol and a number of carbohydrates. *Flavobacterium* sp. strain 166, which was isolated from the same source, however multiplied in this water possibly as result of its ability to utilize carboxylic acids in addition to carbohydrates (20). Growth of strain S12 was occasionally observed in the tap water prepared from anaerobic groundwater as is demonstrated by the differences in growth of the blanks with water of the same source collected at different times (Table 2).

The described properties of the isolate suggest that if growth occurs, it is the result of the presence of maltose- and starch-like compounds in the water. Generally, such compounds may originate from natural sources including glycogen and polysaccharides derived from bacteria and algae, but they may also have been introduced into the water during treatment. This applies particularly to starch-based compounds which are frequently used as coagulant aids in water treatment processes to prepare drinking water from surface water (11). The presence of small amounts of such compounds in drinking water may promote biological processes in the distribution system. Chemical methods for the assessment of these compounds occurring at low concentrations (< 1 mg/l) are lacking. For these reasons growth experiments with strain S12 may be used to obtain information about the presence of maltose- and starch-like compounds in tap water and in water at various treatment stages.

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8. Determination of the concentration of maltose- and starch-like compounds in drinking water by growth measurements with a well-defined strain of a *Flavobacterium* species

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Abstract

The growth kinetics of the *Flavobacterium* sp. strain S12, specialized in the utilization of glycerol and a number of oligo-and polysaccharides (8) were determined in experiments at 15 °C in pasteurized tap water supplied with very low amounts of substrates. Substrate saturation constants (K_s) for the growth on maltotriose, maltotetraose, maltopentaose and maltohexaose were 0.03 μM or less, and below those for glucose (1.7 μM) and maltose (0.17 μM). K_s values for starch, amylose and amylopectine were 13.7, 31, and 10.8 μg of C per liter, respectively. A yield of 2.3×10^7 colony-forming units per μg of C on the oligo- and polysaccharides was calculated from the linear relationships observed between maximum colony counts in pasteurized tap water and the concentrations (all below 25 μg of C/l) of supplied compounds. The maximum colony counts of strain S12 grown in various types of raw water and tap water revealed that raw water contained only a few micrograms of maltose- and starch-like compounds per liter; in tap water the concentrations were all below 1 μg of C, and usually below 0.1 μg of C per liter. The application of starch-based coagulant aids gave increased concentrations of maltose- and starch-like compounds in the water during treatment, but these concentrations were greatly reduced by coagulation and sedimentation, rapid sand filtration and slow sand filtration.

INTRODUCTION

Polysaccharides consisting of glucose units with 1,4-α and 1,6-α linkages are normal constituents of animals, higher plants, algae and bacteria and are therefore present in surface water. Starch-based polyelectrolytes are used as coagulant or filter aids and as sludge conditioners (1). Starch-like compounds either originating from the raw water source or from their use in treatment processes may remain present in drinking water, thus contributing to an undesirable growth of bacteria in distribution systems.

Bacteria belonging to species of the genus *Flavobacterium* are able to utilize starch and a number of maltose-like oligo-saccharides at concentrations below 10 μg of C per liter (6, 8). The

growth constants of one particular strain were determined. Subsequently, growth of this organism was measured in water sampled from various treatment stages and in drinking water to determine the concentration of maltose- and starch-like (MSL) compounds in these water types.

MATERIALS AND METHODS

Organism

Flavobacterium sp. strain S12 had been isolated from tap water (slow sand filtrate) incubated at 15 °C after enrichment with 100 μg of starch C/l. Of 28 carbohydrates tested the following were utilized as sole source of carbon and energy for growth when present at very low concentrations ($\leq$ 10 μg of C/l): sucrose, maltose, raffinose, maltotriose, maltotetraose, maltopentaose, maltohexaose, stachyose, and the polysaccharides amylose and amylopectin as present in starch. None of 18 amino acids, 18 carboxylic acids and 11 aromatic acids promoted growth when present either at very low concentrations or at a high concentration (2.5 g/l). Of the 4 alcohols tested, only glycerol was used for growth. Simple nitrogen compounds (NH_4^+, NO_3^-) were suitable N sources and no vitamins are required for the growth of strain S12 (8).

Growth experiments

Growth experiments with strain S12 in various types of water, or in tap water supplied with very low amounts of carbon compounds, were conducted in heat-treated (0.5 h at 60 °C) samples of 600 ml in thoroughly cleaned Erlenmeyer flasks (1 l) of Pyrex glass. These flasks were incubated at 15 $\pm$ 0.5 °C (no shaking). The cleaning procedure is described in a previous communication (6). Solutions of carbon compounds and NH_4 Cl were prepared in tap water contained in culture tubes and also heated at 60 °C for 0.5 h before their addition to the water samples. Ammonium N was supplied in a ratio to added C of 1 : 8. The water samples were inoculated with a low number of colony-forming units (CFU) of strain S12 per ml. For this purpose, the organism was grown in tap water supplied with a low amount ($\leq$ 0.1 mg of C/l) of a suitable carbon compound (usually maltose or maltotetraose). These cultures were used for inoculation when numbers of cells were declining as a result of starvation. The initial colony count (N_o) usually was between 100 and 500 CFU/ml.

Growth curves of the organism under various experimental conditions were obtained by determination of the colony counts at regular intervals. The streak-plate technique on Lab-Lemco agar (Oxoid Ltd.) described previously (6) was used for this purpose. All growth experiments were done in duplicate unless otherwise stated. From the growth curves, values for the maximum colony counts (N_{max}, CFU/ml) and for the generation time (G, in hours) were obtained. The latter were calculated by linear regression analysis of the curve in the exponential phase of growth.

The effect of very low concentrations of substrates on G and N_{max} of strain S12 was tested in drinking water originating from pumping station Tull and 't Waal (Midden Nederland Waterworks). This TW water is prepared from anaerobic ground water by aeration followed by rapid sand filtration. The final product contained 2.3 mg of dissolved organic carbon per liter and 0.01 mM of nitrate; the pH was 7.7 after heat treatment.

Chemicals

Maltotetraose, maltopentaose and maltohexaose were kindly supplied by H. Hokse, Potato Processing Research Institute (TNO), Groningen, The Netherlands. The starch-based coagulant aids Wisprofloc P (a cationogenic polymer), Wisprofloc 20 (a non-ionogenic polymer) and Perfectamyl (an anionogenic polymer) were obtained from waterworks using these compounds in water treatment. These aids were produced from potato starch by AVEBE, Veendam, The Netherlands.

RESULTS

Kinetics of growth of strain S12 with very low concentrations of carbohydrates

To assess the concentration of MSL compounds in water by growth experiments with *Flavobacterium* sp. strain S12, data were needed to determine the growth constants (yield, substrate saturation constant, minimum generation time) of the organism for a number of such compounds. Growth experiments with the organism in tap water supplied with various low amounts of selected carbohydrates were therefore conducted. Glucose added to TW water in various concentrations (ΔS; 0, 10, 25, 100, 250 μg of C per liter) clearly affected

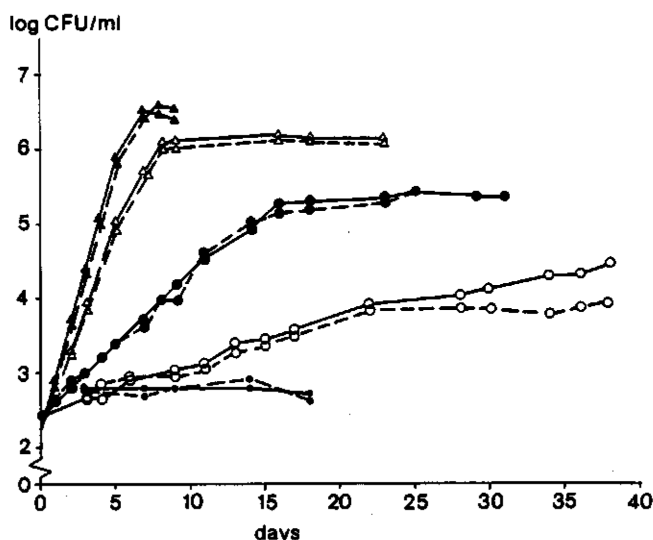

Fig. 1 Growth curves of strain S12 at 15 °C in pasteurized tap water (TW) without substrate added (•) and in the presence of glucose in concentrations of 10 (O); 25 (●); 100 (Δ) and 250 (▲) μg of C per liter. Solid and dashed lines represent duplicate experiments.

the rate of growth (G) and the maximum level of growth (N_{max}) of strain S12 (Fig. 1). N_{max} was reached after 8 days of incubation at an initial concentration of 250 μg of C per liter. At a concentration of 10 μg of C/l, growth was very slow (G = 97-131 h) and N_{max} (0.9-3.9 x 10^4 CFU/ml) was only reached after about 45 days of incubation. The growth curves, shown in Fig. 1, demonstrate the reproducibility of the experiments.

Strain S12 was also grown in the presence of various concentrations of maltose, maltotriose, maltotetraose, maltopentaose, maltohexaose, soluble starch, amylose and amylopectin. Fig. 2 shows the N_{max} values observed over the range of 0 to 25 μg of C/liter. The slopes of the linear parts of these curves were calculated and gave the yield values (Y, CFU/μg of C) of strain S12 for the various compounds. The Y value for glucose was calculated from N_{max} values obtained at 100 and 250 μg of C per liter (Fig. 1). Most Y values

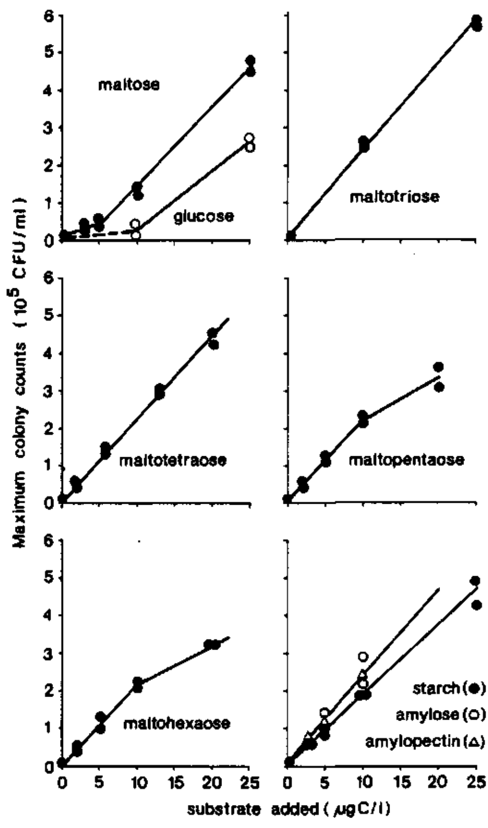

Fig. 2 Maximum colony counts of strain S12 observed in pasteurized tap water (TW) incubated at 15 °C in relation to the concentrations of various added compounds.

were equal to or close to 2.3 x 10^7 CFU/μg of C (Table 1).

The G values of strain S12 calculated from the slopes of the growth curves in the exponential growth phase were plotted against the reciprocal values of ΔS for the various substrates (Figs. 3 and 4). The excellent applicability of the Lineweaver-Burk plot for the various experimental conditions is clearly demonstrated by these

Table 1. Growth constants of *Flavobacterium* sp. strain S12 as calculated from growth experiments at 15 °C in pasteurized tap water (TW) supplied with various substrates.

Substrate	Yield (Y) (CFU/μg of C)[a]	G_{min} (h)	K_s (μg of C/l)	Substrate affinity $(G_{min} \cdot K_s)^{-1}$
Glucose[b]	1.8×10^7	6.2	121	1.3×10^{-3}
Maltose	2.0×10^7	2.8	24	1.5×10^{-2}
Maltotriose	2.3×10^7	2.5	5.5	7.2×10^{-2}
Maltotetraose	2.3×10^7	2.1	7.9	6.0×10^{-2}
Maltopentaose	2.3×10^7	2.2	6.2	7.4×10^{-2}
Maltohexaose	2.2×10^7	2.1	6.2	7.8×10^{-2}
Amylose	2.1×10^7	1.7	31	1.7×10^{-2}
Amylopectin	2.3×10^7	2.1	10.8	4.4×10^{-2}
Starch	2.0×10^7	2.2	13.7	3.3×10^{-2}

a Calculated from the linear relationships between N_{max} and ΔS (see Fig. 2)

b Results based on data from growth measurements at concentrations between 100 and 250 μg of C/l.

figures. The slopes and intercepts of the plots were obtained by linear regression analysis using the following equation:

$$G = G_{min} + G_{min} \cdot K_s/\Delta S$$

in which G is observed generation time (h) and G_{min} is minimum generation time; K_s is the substrate saturation constant which equals the substrate concentration (ΔS) at which $G = 2\ G_{min}$.

The growth constants for the various substrates (Table 1) reveal that at low concentrations, strain S12 is particularly adapted to growth on maltotriose, maltotetraose, maltopentaose and maltohexaose. For these substrates the highest substrate affinities $(G_{min} \times K_s)^{-1}$ were obtained, viz. $6.0 - 7.8 \times 10^{-2}$ l/h.μgC. Furthermore, strain S12 multiplied much more rapidly at low concentrations of polysaccharides (amylose, amylopectin and starch) than at low concentrations of glucose and maltose. The organism grew very poorly in the blanks; the N_{max} values were always below

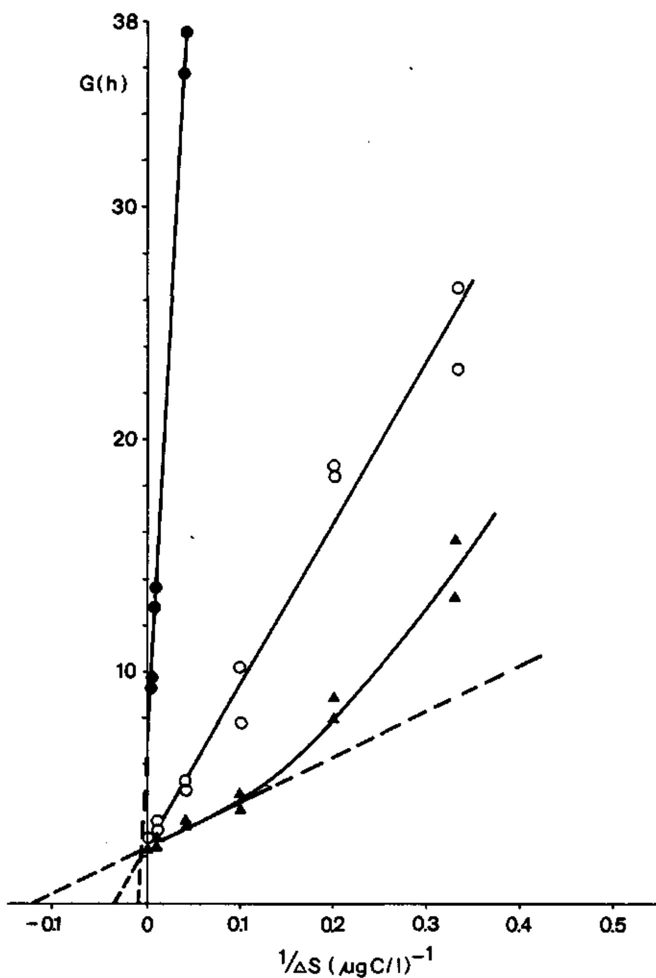

Fig. 3 Lineweaver-Burk plots for strain S12 grown at 15 °C in pasteurized tap water (TW) supplied with various amounts of glucose (●); maltose (○) and starch (▲); G = generation time; ΔS = concentration of added substrate.

1.7 x 10^4 CFU/ml (occasionally below 10^3 CFU/ml) and G values above 40 h (occasionally above 300 h). Plotting of these G values against

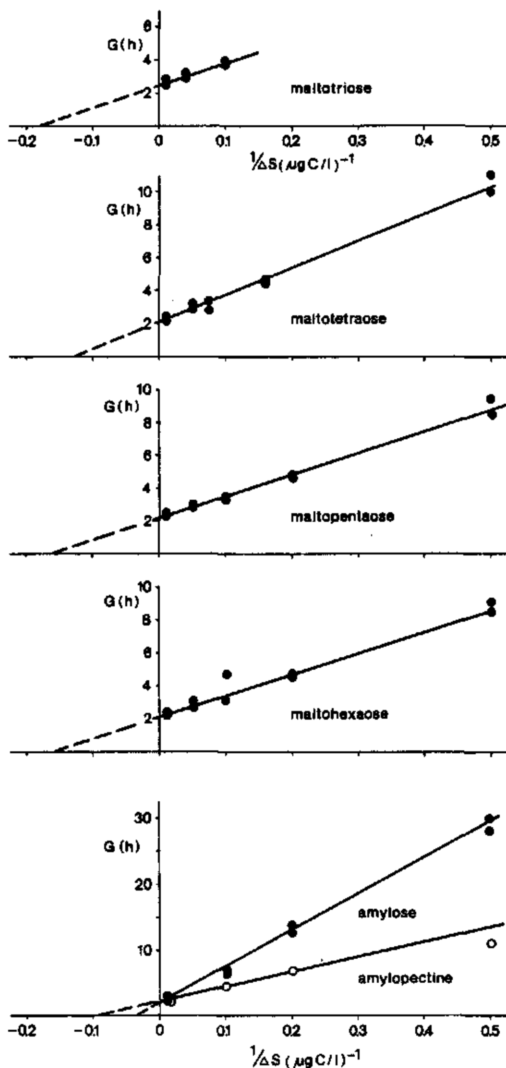

Fig. 4 Lineweaver-Burk plots for strain S12 grown at 15 °C in pasteurized tap water (TW) supplied with various amounts of oligo- and polysaccharides; G = generation time; ΔS = concentration of added substrate.

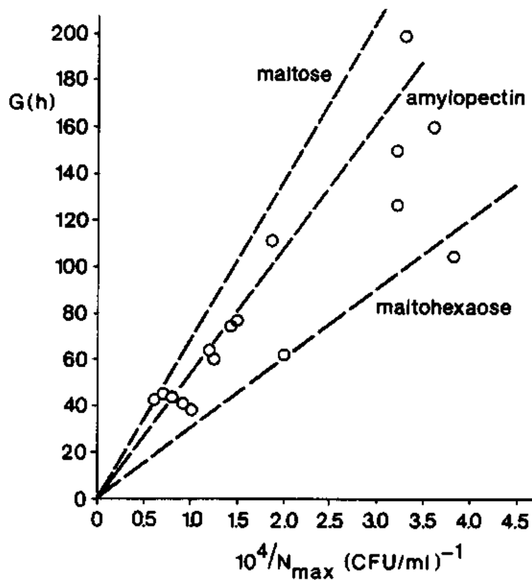

Fig. 5 Relationship between the generation time (G) of strain S12 and reciprocal values of the maximum colony counts (N_{max}) as obtained in growth experiments in tap water prepared from anaerobic ground water (TW). The lines for maltose, amylopectin and maltohexaose were calculated using the growth constants obtained for these compounds (see Table 1).

the reciprocal values of the corresponding N_{max} gives a curve which is comparable with the Lineweaver-Burk plot. A clear relationship existed between values of G and N_{max}^{-1} of the blanks (Fig. 5). To know what type of carbon compound is responsible for the growth of the organism in the blanks (TW), calculated lines for maltose, amylopectin and maltohexaose are given in Fig. 5. Most of the

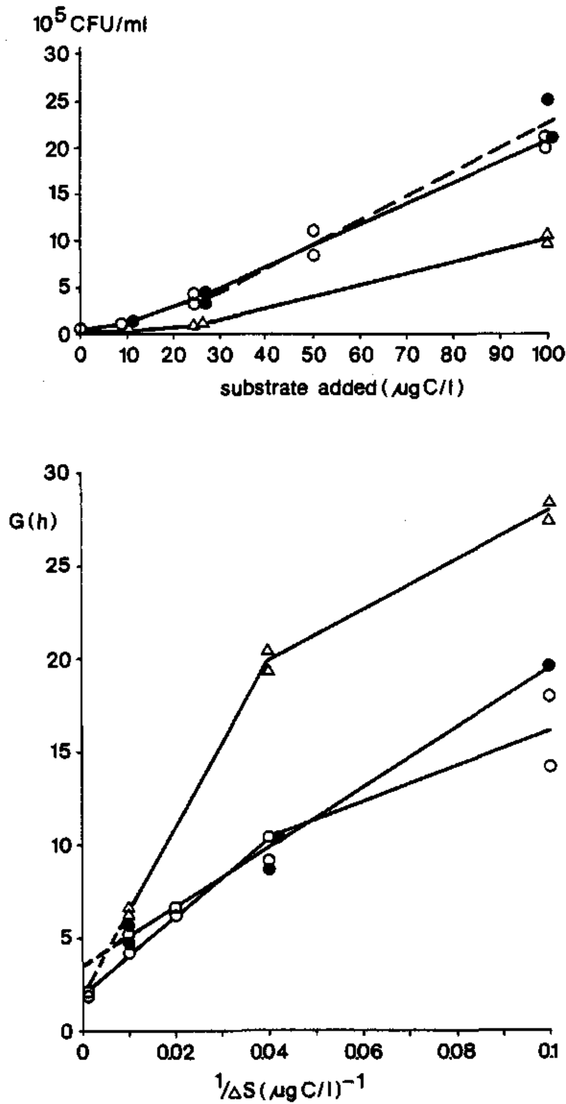

Fig. 6 Kinetics of the growth of strain S12 at 15 °C in pasteurized tap water (TW) supplied with the starch-based coagulant aids Wisprofloc 20 (○), Perfectamyl (●) and Wisprofloc P (Δ).

combinations of values for G and N_{max}^{-1} observed in the tap water without added carbon compounds were close to those obtained for amylopectin, suggesting that very low concentrations (< 1 µg of C/l) of compounds resembling amylopectin were present in the blanks.

Kinetics of growth at low concentrations of starch-based coagulant aids

Three starch-based coagulant aids were tested for their growth-promoting effect on strain S12 at concentrations which were equal to or below 1000 µg of C/l. The N_{max} values of strain S12 observed with identical concentrations of Wisprofloc 20 and Perfectamyl were quite similar but lower values were obtained for growth on Wisprofloc P. (Fig. 6). The relationships between ΔS and N_{max} were non-linear at low ΔS values. Interpretation of the Lineweaver-Burk plots is complicated by their non-linearity at ΔS values below 25 µg of C per liter (Fig. 6). The substrate affinities of strain S12 for the starch-based coagulant aids (Table 2) were 5 to 10 times lower than those for amylopectin and amylose (Table 1).

Table 2. Growth constants of *Flavobacterium* sp. strain S12 for three starch-based coagulant aids as determined by growth experiments at 15 °C in pasteurized tap water (TW).

Compound	Yield (Y)[a] (CFU/µg of C)	G_{min} (h)	K_s (µg of C/l)	Substrate affinity $(G_{min} \cdot K_s)^{-1}$
Perfectamyl	2.0×10^7	3.2	51	6.2×10^{-3}
Wisprofloc 20	2.0×10^7	1.8	133	4.1×10^{-3}
Wisprofloc P	1.0×10^7	1.4	290	2.2×10^{-3}

[a] Calculated from the linear part of the relationship between N_{max} and S (see Fig. 6).

Growth of strain S12 in water sampled from various stages of treatment systems

The growth kinetics of strain S12 for maltose-and starch-like (MSL) compounds, in combination with its specialization in these compounds, indicate that the organism is suited for the estimation of low concentrations of these compounds in water. To obtain

information about the concentration of MSL compounds in various types of water, strain S12 was grown in (a) samples of drinking water prepared from dune-infiltrated river water (final treatment step was slow sand filtration); (b) samples of drinking water prepared from aerobic ground water without further treatment; (c) water (including drinking water) sampled from various treatment systems in the period that no starch-based coagulant aids were applied and (d) water sampled when starch-based coagulant aids were applied. Table 3 shows that strain S12 multiplied not at all or very

Table 3. Growth of *Flavobacterium* sp. strain S12 at 15 °C in various types of drinking water prepared without the use of starch-based coagulant aids

Location	Source	Water Treatment[a]	Generation time G(h)	Maximum colony count (CFU/ml)
1	River water	B,C,J,C,A,F,E,J,K	N.G.[b]	500
2	River water	G,C,A,E,F,J,K	N.G.	500
3	Polder water	G,B,J,D,G,J,K	N.G.	500
4	River water	B,C,G,D,H,I	N.G.	700
5	Ground water	Aerobic water, no treatment	D.[c]	D.
6	Ground water	Aerobic water, no treatment	D	D.

[a] A, underground storage (dunes); B, storage in open reservoirs; C, chlorination; D, ozonation; E, aeration; F, dosage of powdered activated carbon; G, coagulation and sedimentation; H, dual media filtration; I, granular activated carbon filtration; J, rapid sand filtration; K, slow sand filtration.

[b] N.G. = no growth; no doubling of the initial colony count within an incubation period of 2 to 3 weeks ($G > 300-500$ h).

[c] D = die-off (slowly).

slowly in various types of drinking water (a, b). From the very low N_{max} values it is concluded that the concentrations of MSL compounds were far below 1 µg of C/l. Even in samples of raw or partly treated

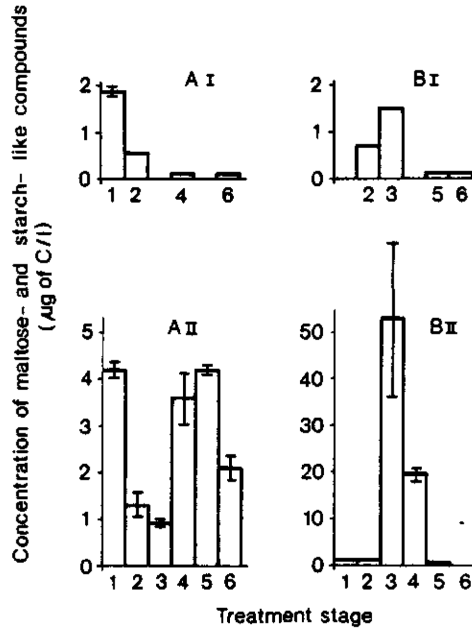

Fig. 7 The concentration of maltose- and starch-like (MSL) compounds as determined by growth experiments with strain S12 in water samples derived from various treatment stages of waterworks A and B in a summer period (I) and a winter period (II). During the winter Perfectamyl was dosed at waterworks A and Wisprofloc at waterworks B. Treatment stages of waterworks A: 1, river Meuse water after storage in open reservoirs; 2, coagulation and sedimentation (without a coagulant aid); 3, ozonation (2 mg/l); 4, dosage of Perfectamyl (0.2 mg/l) in winter; 5, dual media filtration; 6, granular activated carbon filtration. Waterworks B: 1, ground water after storage in an open reservoir and rapid sand filtration; 2, ozonation (2 mg/l); 3, dosage of Wisprofloc (3.0 mg/l) in winter; 4, coagulation and sedimentation 5, rapid sand filtration; 6, slow sand filtration. Average values and values of duplicate measurements are shown.

water, concentrations of such compounds usually do not seem to exceed a few μg of C/l (Figs. 7 AI and BI). Coagulation and sedimentation (A2, B4) caused clear reductions (60-70 %) of the concentration of the MSL compounds at locations A and B. The concentration of these compounds was clearly affected by a dosage of starch-based compounds causing the presence of such compounds (2 μg of C/l) in drinking water produced at location A (Fig. 7). A combination of rapid and slow sand filtration as applied at location B (B5, B6) removed the MSL compounds to a non-detectable level (N_{max} < 10^3 CFU/ml) in the slow sand filtrate BII,6. Ozonation (A3; B2) had a negligible effect on the concentration of the MSL compounds.

The concentration of MSL compounds as determined with growth measurements of strain S12 in water sampled directly after the addition of starch-based compounds were only 5-6 % of the added amount of these compounds. In a laboratory experiment, in which practical conditions were simulated, also a very low recovery (6 %) was observed (data not shown). Chemicals as used in the coagulation process ($FeCl_3$, lime, powdered activated carbon), seem to bind a large part of the added starch-based compounds, preventing their utilization by strain S12 in the growth experiments.

DISCUSSION

Growth characteristics of strain S12

The specific character of strain S12 regarding its utilization of a number of oligosaccharides as had been observed in a previous investigation (8), was clearly confirmed by the results of the experiments described in this paper. When expressed as μM, the K_s values of strain S12 for the maltodextrins (0.014-0.027 μM) were about 10 times below the K_s value observed for maltose (0.17 μM) which in turn was 10 times lower than K_s for glucose (1.7 μM). Comparison of these values with either K_s values or substrate saturation constants for transport (K_t) observed in other bacteria is limited by the few data available for maltodextrins. K_t values of *Escherichia coli* for maltose (1 μM) and maltotriose (2 μM) as reported by Szmelcman et al. (4) are clearly higher than the K_s values found for strain S12, which is not astonishing. In contrast, the K_s value for growth of *E.coli* on glucose, observed in batch experiments, varies between 0.3 μM (5) and 1 μM (10) and is clearly below the K_s of strain S12 for this substrate. For galac-

tose, which did not promote growth of strain S12 when present at a concentration of 10 µg of C per liter (8), *E.coli* has a low K_s value (0.49 µM) (2).

Strain S12 did not hydrolyse maltotriose before uptake as may be concluded from the large difference between K_s values for this compound and for glucose and maltose, respectively. For the same reason, it is likely that the other maltodextrins tested were also transported into the cells without extracellular hydrolysis. However, extracellular hydrolysis of the polysaccharides amylose and amylopectin is necessary to allow growth on these substrates. To attain this result, the enzymes can be excreted by the cells or they are attached in some way to the cell surface. The ability of strain S12 to excrete starch-hydrolyzing enzymes has been demonstrated by the clear zone appearing around colonies growing on starch-containing agar plates (8). It is unkown if at low substrate concentrations, the starch-decomposing enzymes function in a different way (J.M.A. Janssen, Ph.D. thesis, Agricultural University, Wageningen, The Netherlands, 1979). Growth kinetics of strain S12 obtained for amylose and amylopectin reveal that these compounds also were not hydrolysed into glucose and maltose before uptake, but most likely maltodextrins were formed. The more rapid growth of strain S12 with amylopectine than with amylose when present at low concentrations demonstrates an efficient adaptation to the hydrolysis of this branched polymer which is structurally related to glycogen. The latter compound is widespread in nature as a storage compound in bacteria, algae and plants. The mechanism for the rapid growth on this branched polymer was not elucidated.

The starch-based coagulant aids were much less readily utilized by strain S12 than amylopectin and amylose. Structural changes in the polysaccharides contained in these compound, as induced by various chemical processes seem responsible for this difference.

The K_s values and substrate affinities $(G_{min} \times K_s)^{-1}$ of strain S12 for maltotriose, maltotetraose, maltopentaose and maltohexaose have remarkably similar values when expressing concentrations in µg of C per liter. This similarity suggests that either the transport constants decrease proportionally with increasing size of the molecules or that a metabolic process is determining the observed substrate affinities. It is obvious that the low substrate affinities for glucose and maltose are not the result of a metabolic process, because the above-mentioned substrates also provide glucose

molecules as source of carbon and energy. An indication that a metabolic process may determine the rate of growth at very low concentrations of substrates was obtained in a previous study by growth experiments with two different strains of *Pseudomonas fluorescens*. A constant difference in substrate affinities between these two strains was observed for a number of substrates requiring different transport systems (7). The highest $(G_{min}.K_s)^{-1}$ values observed for strain S12 (7.8×10^{-2} 1/μg C.h) are very close to the highest substrate affinity obtained for *P. fluorescens* strain P17 (approx. 9×10^{-2} 1/μg C.h) for growth on arginine and the value observed in an other *Flavobacterium* sp. (approx. 6.5×10^{-2} 1/μg C.h) for growth on glucose and starch. Further research is required to show if significantly higher substrate affinities for single substrates exist in nature.

Plotting of G values against the reciprocals of N_{max} resembles the construction of the Lineweaver-Burk plot, and enables the characterization of the growth-promoting properties of a specific water sample for a specific organism without knowledge about the substrate concentration. Fig. 5 suggests that the water tested did not contain substrates which could promote growth more rapidly than maltohexaose. When mixtures of compounds are present in the water, no clear relationship is expected to exist between G values and reciprocals of N_{max}. In this situation, the generation time in the exponential phase of growth is particularly affected by compounds for which the organism has the greatest affinities, whereas N_{max} is the result of the utilization of all growth promoting compounds. For this reason, the relationship between G values and reciprocals of N_{max} cannot simply be used as a means to identify the compound responsible for growth. Still it is possible that glycogen-like compounds were indeed responsible for the growth of *Flavobacterium* strain S12 in a number of water samples.

The shape of the curve representing the relationship between N_{max} and the applied substrate concentrations (Figs. 2 and 6) is related to the type of compound applied. N_{max} values were lower than expected on a linear basis with concentrations of glucose and maltose below 20-30 % of K_s. This effect was not observed with amylose but starch-based coagulant aids also gave lower N_{max} values than expected. These deviations might either be due to (1) relatively great proportion of substrate being utilized for maintenance at very low growth rates, (2) inability to utilize the

compound at very low concentration, or (3) lesser viability of the cells at very low growth rates. The lower than expected N_{max} values at maltopentaose and maltohexaose concentrations above 10 μg of C per liter might be the result of a larger size of cells growing at a nearly maximum rate.

Growth of strain S12 in various types of water

The results presented in this paper show that growth measurements with a selected bacterium may give information about the concentration of a specific group of compounds in water. The concentrations of maltodextrins and starch-like compounds calculated from the N_{max} values of strain S12 are maximum values because growth may also have been promoted by the presence of other substrates of unknown identity including glycerol. On the other hand, the real concentration of MSL compounds may significantly be above the calculated value when adsorption onto solids prevents their utilization by strain S12. Such an adsorption, however, was not observed in the experiments with tap water supplied with various compounds (Fig. 2).

The growth experiments with a few types of water used for the preparation of drinking water revealed that the concentrations of MSL compounds were only a few micrograms of C per liter. In most of the tap water samples, these concentrations were far below 1 μg of C/l (Table 3 and Fig. 7). Ozonation, which is causing a clear increase of the concentration of carboxylic acids, including oxalic acid (9), hardly affected the concentration of MSL compounds. Coagulation and sedimentation processes (without a dosage of starch-based compound), rapid sand filtration and slow sand filtration, resulted in a substantial decrease of these concentrations. Concentrations of MSL compounds were increased by the addition of starch-based products. Most of the coagulant aids seem to be removed in the coagulation and sedimentation process.

MSL compounds introduced into water may promote biological activity in filters removing these substances. When they remain in the tap water, it results in bacterial regrowth in distribution systems. Under such situations, particularly growth of *Flavobacterium* sp. may be enhanced. This genus includes bacteria which seem specialized in growth at very low concentrations of oligo- and polysaccharides consisting of glucose with 1-4α- and 1-6 α-glucosidic linkages. However, the common occurrence of yellow pigmented

bacteria in water samples in various treatment stages and in drinking water (3), is not necessarily related to the use of starch-based coagulant aids, since (i) these bacteria are able to grow on various other compounds and (ii) MSL compounds may be present in natural waters at higher levels than reported in this paper.

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9. Substrate utilization by an oxalate-consuming *Spirillum* species in relation to its growth in ozonated water

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Abstract

The nutritional versatility of a vibrio-shaped oxalate-utilizing isolate, strain NOX, obtained from tap water supplied with low concentrations of formate, glyoxylate and oxalate, was determined by growth experiments with low-molecular-weight carbon compounds at high (grams per liter) and very low (micrograms per liter) concentrations. The organism, which was identified as a *Spirillum* species, appeared to be specialized in the utilization of a number of carboxylic acids. Yields of 2.9 x 10^6 CFU)/μg of oxalate C and 1.2 x 10^7 CFU/μg of acetate C were obtained from growth experiments in tap water supplied with various low amounts of either oxalate or acetate. A substrate saturation constant of 0.64 μM of oxalate C was calculated for strain NOX from the relationship between growth rate and concentration of added oxalate. Maximum colony counts of strain NOX grown in ozonated water (dosages of 2.0-3.2 mg of O_3 per liter) were 15 to 20 times larger than the maximum colony counts of strain NOX grown in water prior to ozonation. Based on the nutritional requirements of strain NOX, it was concluded that carboxylic acids were produced by ozonation. Oxalate concentrations were calculated from the maximum colony counts of strain NOX grown in samples of ozonated water in which a non-oxalate utilizing strain of *Pseudomonas fluorescens* had already reached maximum growth. The oxalate concentrations obtained by this procedure ranged from 130 to 220 μg of C/l.

INTRODUCTION

Ozone is applied in water treatment for disinfection, as well as for the reduction of color, taste and odor, organic micropollutants and for reducing the content of organic matter of the water (13). Ozonation of water containing organic compounds, which are resistant to biodegradation, e.g. fulvic and humic acids, results in the formation of a number of easily biodegradable carboxylic acids, including formic, glyoxylic, oxalic, pyruvic and acetic acids (1, 5, 11). Acetate and oxalate are particularly resistant to further oxidation by ozone (7). An increase in the concentration of biodegradable compounds caused by ozonation has also been demon-

strated for the effluent of a wastewater treatment plant by long-term BOD tests (14) and in the preparation of drinking water by growth experiments with a pure culture of *Pseudomonas fluorescens* (22). When ozonation in water treatment is followed by rapid sand filtration, the biodegradable compounds formed are removed by bacterial activity in the filter (22). However, when ozonated water is distributed without further treatment, growth of bacteria may occur in the distribution system (3, 4, 15) resulting in deterioration of water quality.

Measurement of the biodegradable compounds formed by ozonation by using chemical methods is not only complicated by the diversity and the nature of these compounds but also by their low concentrations. Therefore an attempt was made to develop a microbiological method to quantify the presence of the easily biodegradable carboxylic acids, including oxalic acid. The nutritional versatility of an oxalate-utilizing bacterium which was selected for this purpose was determined. Moreover, the growth of the organism in tap water supplied with very low amounts of oxalate and acetate and in ozonated water was studied.

MATERIALS AND METHODS

Strains

Strain NOX was isolated from the filtrate of slow sand filters of the Municipal Dune Waterworks of The Hague. Six hundred milliliters of this water was incubated at 15 °C after addition of a mixture of formate, glyoxylate and oxalate, each at a concentration of 25 μg of C per liter. The development of the bacterial flora was investigated by periodic colony counts on Lab-Lemco Agar (LLA) plates, and the organism which formed the predominating colony type was isolated.

P. fluorescens P17 was isolated from tap water prepared from dune-infiltrated river water. A detailed description of the organism has been given in a previous communication (23).

Media

Mineral salts agar (MSA) was prepared by adding 0.5 g of NH_4Cl, 0.5 g of $Na_2HPO_4 \cdot 12H_2O$ and 12 g of purified agar per liter to the basal salts solution described previously (18). LLA consisted of 3 g of Lab-Lemco (Oxoid Ltd.) beef extract, 5 g of peptone and 12 g of

agar (Oxoid no. 3) per liter of demineralized water.

Characterization procedures

The following tests were used for characterization of the isolate: Gram stain, oxidase test (9), oxidation-fermentation test with glucose (6), arginine dihydrolase test (17), urease activity (16) and phosphatase activity (8). Production of NO_2^- and N_2 from NO_3 was tested in tap water supplied with 1 g of KNO_3, 1 g of DL-lactate and 5 g of yeast extract per liter. Media and procedures of tests for hydrolysis of proteins (casein, gelatin), starch, chitin, and Tween-80 have been described previously (18). Cultivation was done at 25 °C.

Colony growth test

This technique was used to determine the utilization of substrates as sources of carbon and energy for growth at a high concentration. The compounds tested were present in autoclaved solutions of either 1 or 10 % (wt/vol). About 100 colony-forming cells of the organism, cultivated at 25 °C in autoclaved tap water supplied with 1 mg of acetate C/liter, were spread over the surface of plates with MSA containing 1 g of the substrate to be tested per liter. The plates were incubated at 25 °C, and during a period of about 2 weeks the diameters of four or five randomly selected colonies on each plate were measured periodically with a Wild M7S binocular microscope. A previous study (19) revealed that when a substrate is utilized then $R_s^2/R_b^2 > 2$; R_s = radius of colony grown on agar containing the substrate, and R_b = radius of colony grown on agar without substrate (blank).

Growth at very low substrate concentrations

The utilization of substrates at very low concentrations was tested by growth experiments in tap water from The Hague (TH) and tap water from Tull and 't Waal (TW). TH was prepared from dune-infiltrated river Meuse water by the Municipal Waterworks of The Hague. Treatment of water abstracted from the dunes successively included dosage of powdered activated carbon, aeration, rapid sand filtration and slow sand filtration. TH contained 3.4 mg of organic carbon and 7.1 mg of NO_3^- per liter; the pH was 7.7. TW was prepared from anaerobic ground water using aeration followed by rapid sand filtration. It contained 2.3 mg of organic carbon and 0.6 mg of NO_3^-

per liter; the pH was 7.7.

Growth of a pure culture at very low substrate concentrations was investigated in tap water supplied either with mixtures of compounds or with the compound to be tested. The experiments were conducted in 600 ml of water contained in 1 liter-Pyrex glass flasks. These flasks and the pipettes that came in contact with tap water were thoroughly cleaned as previously described (29). Bacteria originally present in the tap water were eliminated by heating the water samples to 60 °C for 30 minutes. Solutions of individual substrates were freshly prepared in tap water and heated in the same way. Mixtures of substrates were added from stock solutions which had been prepared as previously described (20). The following mixtures, containing the compounds listed in Table 2, were present: a mixture of 18 amino acids (AA), a mixture of 14 carboxylic acids, a mixture of 6 carbohydrates and alcohols and a mixture of 7 aromatic acids.

The experimental solutions were inoculated with usually less than 10^3 colony-forming units (CFU) per ml and subsequently incubated at 15 ± 0.5 °C. These cells were either precultured in autoclaved tap water supplied with a low concentration ($\leq$ 1 mg of C per liter) of acetate or derived from an experimental culture in tap water. These cultures were only used when the maximum number of cells (N_{max}, CFU/ml) had been reached. Growth of the organism in water was estimated by periodic colony counts on plates of LLA (in triplicate). The experiments were continued until the N_{max} values were reached. The generation time (G, in hours) of the population in the exponential-growth phase, was calculated by linear regression analysis of the log values of the colony counts in this phase. Growth experiments were carried out in duplicate unless otherwise stated. N_{max} values obtained in duplicate growth experiments with strain NOX deviated 10 % or less from each other in 90 % of the experiments.

Estimation of growth kinetics

By analogy with Michaelis-Menten enzyme kinetics, the following relationship between growth rate (number of generations per hour, G^{-1}) and the concentration of the growth-limiting substrate (S) may be defined:

$$1/G = (1/G_{min}) \times S/(K_s + S) \qquad (1)$$

G and G_{min} represent the observed and minimum generation time, respectively, and K_s, the substrate-saturation constant, is equal to S when G = 2 G_{min}. Equation (1) may be rearranged into the following form (the Lineweaver-Burk plot):

$$G = G_{min} + (G_{min} \cdot K_s/S) \quad (2)$$

G is measured at various concentrations of substrate (ΔS) added to the tap water. If the concentration of natural substrates (S_n) is very low, (no or no significant growth of the blank), then values for G_{min} and K_s may be calculated using equation (2) in which S = ΔS. When significant growth occurs in the blanks, S in equation (2) is replaced by $S = \Delta S + S_n$. In this situation, no linear relationship exists between $1/\Delta S$ and G, unless the added substrate is preferentially utilized. When ΔS and S_n are utilized concurrently, values for G_{min}, K_s and S_n may be obtained by measuring G values at various concentrations of ΔS and calculation of the best fitting curve using equation (2) in which $S = \Delta S + S_n$. In this case S_n, the apparent natural substrate concentration, is obtained as the amount of substrate equivalents of the added substrate. With concurrent utilization of ΔS and S_n) however, another linear function may be derived from equation (2):

$$G_{min} / (G - G_{min}) = (S_n / K_s) + (\Delta S / K_s) \quad (3)$$

The G_{min} value may be obtained from experiments with a high concentration of ΔS, where G is very close to G_{min}. A concurrent uptake of S_n and ΔS is particularly expected when ΔS values are close to S_n values. In this situation, the largest differences between G and G_{min} are obtained.

RESULTS

Nutritional and morphological characteristics of strain NOX at high substrate concentrations

Strain NOX gave negative reactions in most of the tests applied to determine its physiological characteristics (Table 1). From these results and the morphological properties of the organism, particularly its vibrio shape (Fig. 1), it is concluded that strain NOX represents a species of the genus *Spirillum* as described by Krieg

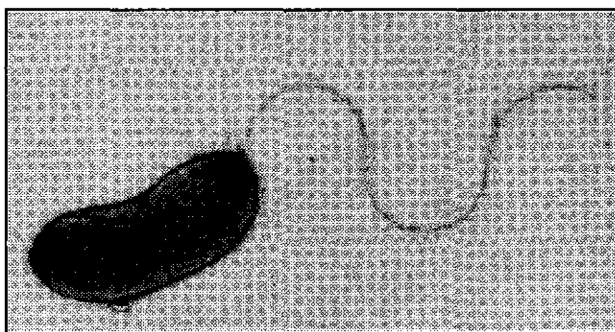

Fig. 1 Morphology of strain NOX, grown in yeast-extract (0.8 % (wt/vol)) medium. Cell length = 1.3 µm.

(10).

Sixty-five low-molecular-weight compounds were tested as sole sources of carbon and energy for strain NOX by using the colony-growth test. Only with 11 carboxylic acids and with the amino acids glycine and alanine were the size (R_s^2; R_s^2 is radius of colonies grown on MSA supplied with a substrate) of the colonies at least two times larger than the size (R_b^2) of the colonies on MSA without substrate (Table 2). A number of aromatic acids were toxic for strain NOX under the conditions used.

Nutritional versatility at very low concentrations of substrates

Growth experiments in TH supplied with mixtures of various carbon compounds, each present at a concentration of 1 µg of C/l, were conducted for a rapid screening of the nutritional versatility of strain NOX at a very low concentration of substrates; the obtained N_{max} values and growth rates revealed that strain NOX was specialized in the utilization of carboxylic acids (Fig. 2). The utilization of carboxylic acids at very low concentrations was further investigated in separate tests by measuring the growth of strain NOX in tap water supplied with carboxylic acids, each at a concentration of 10 µg of C per liter. The obtained N_{max} values (Table 3) demonstrate that formate, glyoxylate, glycollate, propionate, pyruvate, lactate, malonate, malate, fumarate and succinate clearly promoted growth (in addition to acetate and oxalate;

Table 1. Nutritional and morphological characteristics of strain NOX

Property	Isolate characteristic
Oxidase	+
Acid from glucose	
aerobic	-
anaerobic	-
Arginine dihydrolase	-
NO_2^- from NO_3^-	-
N_2 from NO_3^-	-
Urease	-
Phosphatase	-
Hydrolysis of casein	-
gelatin	-
starch	-
chitin	-
Tween-80	-
Growth at 37 °C	-
Cell size[a]	1-2x0.5 m
Motility[a]	+
Flagella insertion(cf.Fig.1)	polar
Pigmentation of colonies[b]	-

[a] Cells grown in denitrification medium (cf. Materials and Methods

[b] Grown on LLA at 25 °C.

see below).

Based on the results shown in Table 2 and the growth on dicarboxylic acids, four amino acids were selected for the test at 10 µg of C/l. Growth of strain NOX was most clearly promoted by aspartate, but the increase of N_{max} was much less than with the dicarboxylic acids when present at this concentration (Table 3).

Kinetics of the growth on oxalate and acetate at very low concentrations

Strain NOX was grown in tap water supplied with various concentrations (ΔS) of either oxalate or acetate to determine the rela-

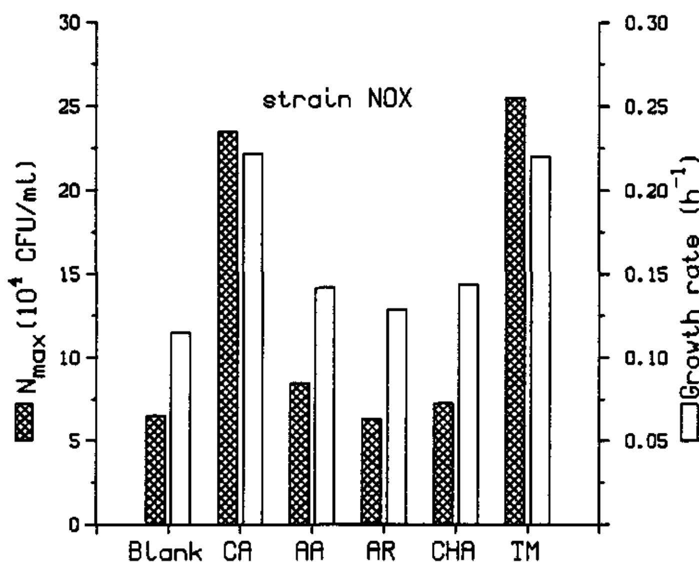

Fig. 2 Effect of various mixtures of substrates on the maximum colony count (N_{max}) and the growth rate of strain NOX in tap water at 15 °C. The compounds in the mixtures (see Table 2) were each present at a concentration of 1 μg of C per liter, except glutamate which was present at twice this concentration. The total mixture (TM) included all compounds present in the other mixtures. The presented results are average values of duplicate experiments. Abbreviations: CA, carboxylic acids; AA, amino acids; AR, aromatic acids; CHA, carbohydrates and alcohols.

tionship between ΔS and N_{max} values, and to determine the growth kinetics of the organism for these substrates which are main products of ozonation. The growth curves observed in TW supplied with oxalate demonstrated that the N_{max} values and the rate of growth (slope of the curves) depended on ΔS (Fig. 3). The growth curves observed in TW supplied with acetate also demonstrated an effect of S on N_{max}, but the initial rate of growth was not promoted by the presence of acetate (Fig. 4; not all growth curves are shown). The duplicate growth curves shown indicate that the

Table 2. Sources of carbon and energy for strain NOX at a concentration of 1 g per liter determined with the colony growth test at 25 °C.

Carbohydrates and alcohols(CHA)	Growth[a]	Amino acids (AA)	Growth[a]	Carboxylic acids (CA)	Growth[a]	Aromatic acids (AR)	Growth[a]
L-Arabinose	–	Glycine	+	Formate	–	Benzoate	–[b]
D-Xylose[c]	–	L-Alanine	+	Acetate	+	*p*-OH-	
D-Glucose	–	L-Valine	–	Glyoxylate	(+)	Benzoate	–
D-Mannose[c]	–	L-Isoleucine	–	Glycolate	+	Salycilate[c]	–[b]
D-Galactose[c]	–	L-Leucine	–	Oxalate	+	Gallate[c]	–[b]
L-Rhamnose[c]	–	DL-Serine	–	Propionate	+	Anthranilate	–[b]
D-Fructose[c]	–	L-Threonine	–	DL-Lactate	+	Vanillate	–
D-Gluconate[c]	–	L-Lysine	–	Pyruvate	–	Phtalate	–
Sucrose[c]	–	L-Arginine	–	Malonate	+	Ferulate[c]	–[b]
Cellobiose[c]	–	L-Aspartate	–	Fumarate	+	Nicotinate	–
Maltose	–	L-Asparaginate	–	Succinate	+	*p*-OH-Phenylacetate[c]	–
Lactose[c]	–	L-Glutamate	–	β-OH Butyrate[c]	–	DL-Mandelate	–
Raffinose[c]	–	L-Glutaminate	–	Malate[c]	(+)		
Starch[c]	–	L-Tyrosine	–	Maleate[c]	–		
Ethanol	–	L-Proline	–	L-Tartrate	–		
Glycerol	–	DL-Tryptophan	–	Valerate[c]	–[b]		
D-Mannitol	–	L-Histidine	–	α-Ketoglutarate[c]	+		
Inositol[c]	–	DL-Penylalanine	–	Citrate	–		
				Adipate	–		

[a] Symbols: +, relative colony size $R_s^2/R_b^2 \geq 3.0$ (R_s is radius of colony grown on MSA with a specific substrate and R_b is radius of colony grown on MSA without this substrate); (+), $R_s^2/R_b^2 = 2$; –, $R_s^2/R_b^2 \leq 1.3$.

[b] No visible colonies developed on the plates within 14 days of incubation.

[c] Not included in the CHA, AA, CA and AR mixtures, respectively.

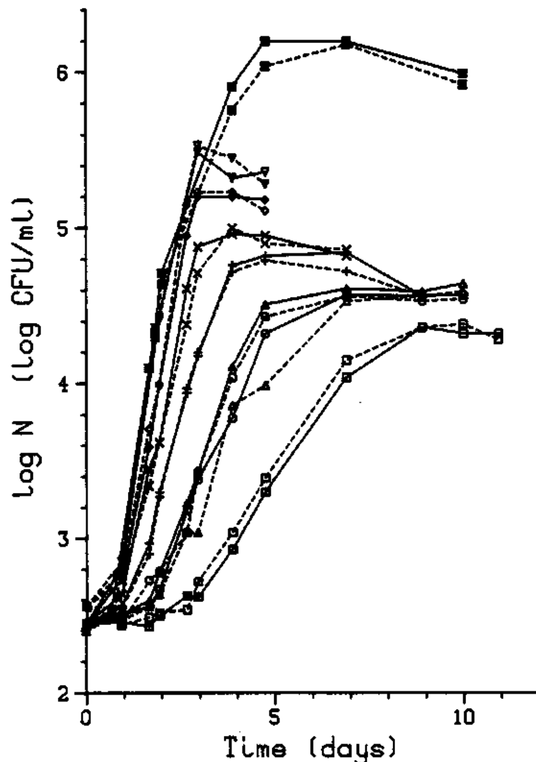

Fig. 3 Growth curves of strain NOX at 15 °C in tap water without added oxalate (□) and with 3 (○), 5 (Δ), 10 (+), 20 (x), 50 (◇), 100 (▽) or 500 (⊠) µg of oxalate C added per liter. Solid broken lines indicate duplicate experiments.

applied technique gives highly reproducible results.

The linear relationships observed between the N_{max} values of strain NOX and the various concentrations of oxalate and acetate added to the tap water (Fig. 5) demonstrate that these compounds (i) were growth limiting and (ii) were utilized at very low concentrations. The relationships enabled calculation of the yield (Y) values expressed in CFU per microgram of added C. Y on acetate was about four times than on oxalate (Table 4), which was due to the low nutritional value of oxalate. The natural substrate concentration S_n available for the organism can be calculated from the N_{max} values

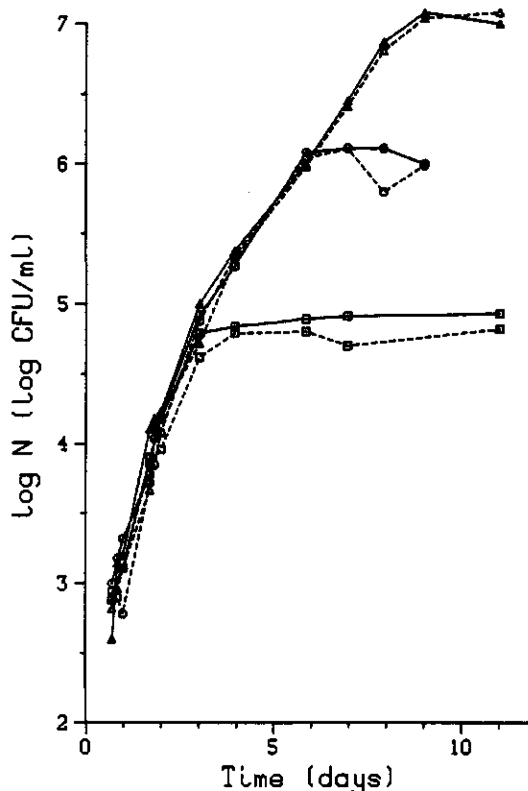

Fig. 4 Growth curves of strain NOX at 15 °C in tap water without added acetate (□) and with 100 (O) or 1000 (Δ) µg of acetate C added per liter. Solid and broken lines indicate duplicate experiments.

of the blanks and the obtained Y values. The average N_{max} values of the blanks were 2.4 x 10^4 CFU/ ml (TW) and 7.5 x 10^4 CFU/ml (TH) in the respective experiments. The S_n values thus were either 2 (in TW) or 6 (in TH) µg of acetate-C equivalents per liter or 8 (in TW) or 26 (in TH) µg of oxalate-C equivalents per liter.

A plot of the generation time (G) of strain NOX versus ΔS^{-1} of oxalate (see equation 2) gave a non-linear function (Fig. 6). Growth of the blanks indicated that this nonlinearity was due to the utilization of substrates present in the tap water (S_n). A linear

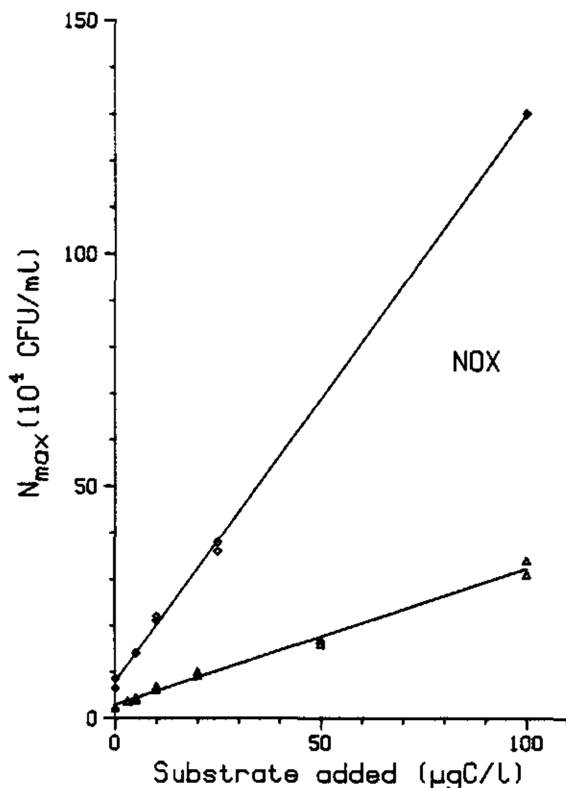

Fig. 5 Relationship between the concentrations of oxalate (Δ) or acetate (◇) and the maximum colony counts (N_{max}) of strain NOX grown at 15 °C in tap water supplied with these compounds.

relationship, however existed between G and ΔS^{-1} when ΔS was greater than or equal to 50 μg/l (Fig. 6), and with these results values of G_{min} and K_s for oxalate were calculated (Table 4). Using G at 1 mg of oxalate C per liter (G = 4.1 h) as the G_{min} value enabled linearization according to equation 3 (Fig. 7), an alternative calculation of K_s(17.8 μg of oxalate C/l) and the calculation of S_n (5.7 μg of oxalate-C equivalents per liter). Calculation of the best fitting curve (Fig. 6) with equation 2, in which $S = \Delta S + S_n$,

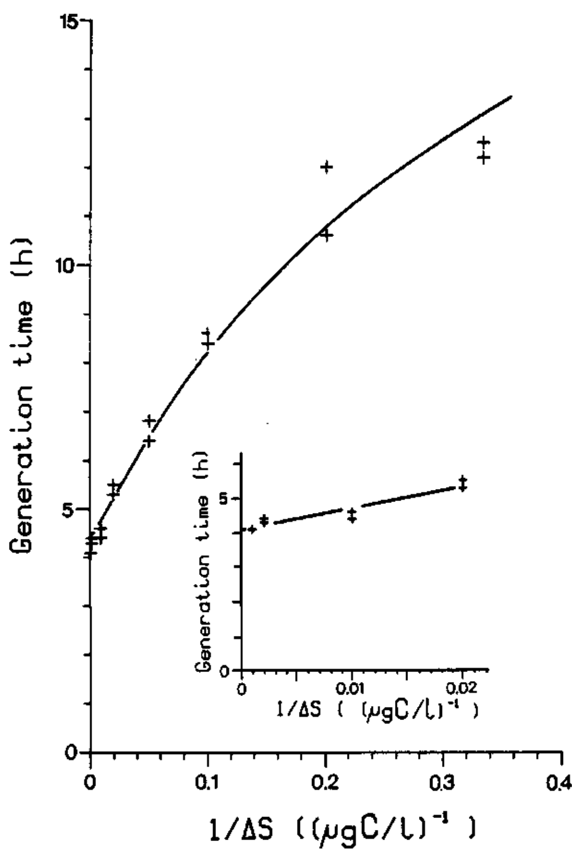

Fig. 6 Lineweaver-Burk plot of strain NOX for growth with oxalate.

gave the following results: G_{min} = 4.3 h; K_s = 11.5 µg of oxalate C per liter and S_n = 2.6 µg of oxalate-C equivalents per liter. The S_n values obtained by the calculation of the growth kinetics are below the S_n value (8 µg of oxalate C equivalents per liter) calculated from N_{max} and Y.

The similarity between generation times in the presence of acetate and those of the blank made calculation of growth kinetics for acetate impossible. A G value of 17.6 h was calculated for strain NOX in the presence of 1 mg of acetate C per liter from the

Table 3. Maximum colony counts (N_{max}) of strain NOX grown at 15 °C in tap water (TW) supplied with various carboxylic and amino acids at a concentration of 10 μg of C per liter.

Compound added	N_{max} [a] (CFU/ml)	Compound added	N_{max} [a] (CFU/ml)
None	$3.9x10^4$	Succinate	$2.0x10^5$
Formate	$1.9x10^5$	Malate	$9.2x10^4$
Glyoxylate	$1.3x10^5$	Maleate	$4.3x10^4$
Glycolate	$1.1x10^5$	Valerate	$4.4x10^4$
Propionate	$2.4x10^5$	α-ketoglutarate	$6.0x10^4$
Pyruvate	$1.7x10^5$	Adipate	$4.8x10^4$
DL-lactate	$2.5x10^5$	Citrate	$4.5x10^4$
Malonate	$1.0x10^5$	Glycine	$4.4x10^4$
β-OH-butyrate	$6.6x10^4$	L-Alanine	$4.2x10^4$
L-Tartrate	$4.2x10^4$	L-Aspartate	$7.5x10^4$
Fumarate	$1.9x10^5$	L-Glutamate	$5.0x10^4$

[a] Single values, except for the blank which is the average of duplicate experiments.

slope of the growth curve at colony counts above the N_{max} values observed for the blanks (Fig. 4).

Growth of strain NOX and P.fluorescens strain P17 in ozonated water

Increased N_{max} values and growth rates as caused by ozonation applied in the water treatment systems of the Rotterdam Waterworks (at Kralingen) and the Amsterdam Waterworks (at Weesperkarspel) and in a pilot plant demonstrated increases in the concentrations of substrates which can be utilized by strains NOX and P17 (Table 5). From the N_{max} values of strain NOX grown in water sampled before and after ozonation, and the properties of this isolate, it is concluded that the concentration of carboxylic acids was increased 15 to 20 times due to the effect of ozone on the organic carbon present in the water. Strain P17 is unable to utilize oxalate (20). The increases of N_{max} values for this organism therefore indicate that ozonation also resulted in increased concentrations of other compounds. The concentrations of compounds which promoted growth of

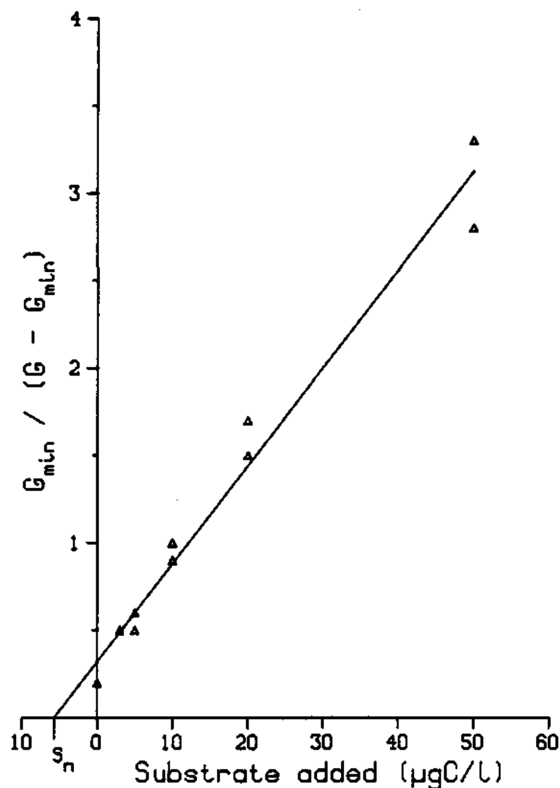

Fig. 7 Linearization of the relationship between the generation time (G, h) of strain NOX and the concentration (ΔS) of oxalate added to tap water. S_n is the natural substrate concentration.

strains NOX and P17, the so-called easily assimilable organic carbon compounds (AOC), were calculated from the obtained N_{max} values and the Y values for acetate. The AOC concentrations for both strains were expressed in amount of acetate-carbon equivalents to compare these concentrations with each other. In ozonated water AOC concentrations available for strain NOX (AOC-NOX) were 1.4 to 1.8 times larger than AOC-P17 (Table 5).

Growth of strain NOX in ozonated water, in which *P.fluor-*

Table 4. Growth constants of strain NOX for oxalate and acetate at 15 °C

Substrate	Y (CFU/μg of C)	G_{min} (h)	K_s (μg of C/liter)
Oxalate	2.9×10^6	4.1	15.2[a] (17.8[b], 11.5[c])
Acetate	1.2×10^7	17.6[d]	-[e]

[a] Calculated from equation 2 (see the text).
[b] Calculated from equation 3 (see the text).
[c] Calculated from equation 2 in which $S = \Delta S + Sn$ (see the text).
[d] G at 1 mg of acetate C/liter.
[e] -, Not determined.

escens P17 had already reached N_{max} values, revealed that strain P17 did not utilize a significant amount of substrate that can be consumed by strain NOX. From the N_{max} values of strain NOX in ozonated water in which strain P17 had already reached N_{max} values and the yield of strain NOX with oxalate, it may be concluded that concentrations of about 130 (pilot plant) to 220 (Kralingen) μg of oxalate C per liter may have been present in the water after ozonation (Table 5). Assuming that the value of AOC-P17 expressed in acetate-C equivalants is close to the real concentration of carbon available for strain P17, then the total concentration of organic carbon (C_T) available for strains NOX and P17 in ozonated water can be estimated from:

$$C_T = \text{AOC-P17} + \text{oxalate C conc.} \quad (4)$$

Table 5 reveals that C_T ranged from 190 μg of C/l (pilot plant) to 315 μg of C/l (Kralingen) and varied between 4.2 % (Weesperkarspel) and 12.6 % (Kralingen) of the total organic carbon concentration.

DISCUSSION

Properties of strain NOX

Tests for the detection of extracellular enzymes (Table 1) and

Table 5. Growth of strain NOX and *P. fluorescens* P17 at 15 °C in water before and after ozonation

Origin of water	Total organic carbon (mg/l)	NOX			P17			NOX with P17[a]		
		N_{max} (CFU/ml)[b]	G (h)[b]	AOC (µg of C/l)[c]	N_{max} (CFU/ml)[b]	G (h)[b]	AOC (µg C/l)[c]	N_{max} (CFU/ml)[b]	Oxalate (µg of C/liter)[d]	C_T (µg of C/liter)[e]
Weesperkarspel (Amsterdam Waterworks)										
before ozonation	6.9	1.0×10^5	7.5	8.3	1.2×10^5	4.7	28	–[f]	–	–
after ozonation (2.2 mg O_3/l)	6.4	1.6×10^6	3.5	133	4.1×10^5	4.6	97	5.1×10^5	175	272
Kralingen (Rotterdam Waterworks)										
before ozonation	2.9	1.5×10^5	6.3	13	6.4×10^4	13	15	–	–	–
after ozonation (3.2 mg O_3/l)	2.5	2.1×10^6	3.8	175	4.0×10^5	5.9	95	6.4×10^5	220	315
Pilot plant										
before ozonation	1.8	7.5×10^4	14	6.2	2.5×10^4	30	5.9	–	–	–
after ozonation (2.0 mg O_3/l)	1.6	1.4×10^6	–	117	2.7×10^5	–	64	3.7×10^5	127	191

[a] Strain NOX was inoculated when strain P17 had reached N_{max}; colonies of strain NOX grown on the counting plates differ from those of strain P17, thus enabling simultaneous determination of the colony counts of both types of bacteria.
[b] Average values of duplicate experiments.
[c] Assimilable organic carbon in micrograms of acetate-C equivalents per liter calculated from N_{max} and Y for acetate; the Y value of P17 on acetate is 4.2×10^6 CFU/µg of acetate C (23).
[d] Calculated from N_{max} and Y of strain NOX for oxalate.
[e] Total concentration easily assimilable organic carbon compounds; C_T = AOC-P17 + conc. of oxalate C.
[f] –, Not determined.

the determination of the nutritional versatility of the organism at high and very low concentrations of substrates revealed that strain NOX is specialized in the utilization of a number of carboxylic acids with a low molecular weight. The results presented in this paper and those presented in a previous communication (19) demonstrated that experiments with mixtures of substrates are very useful for a rapid screening of the nutritional versatility of a bacterium at very low substrate concentrations. For more detailed information, separate growth experiments with low concentrations of selected substrates are required. The selection of substrates as used in the experiments described in this paper was based on the results of the colony growth test (Table 2).

The colony growth test may be regarded as a variation of the replica plating technique. Typical aquatic bacteria, like *Flavobacterium* sp. strain S12 (19) and strain NOX, which have both been isolated from tap water after enrichment with a very low concentration of substrate, grew very slowly and gave relatively poor responses in the replica test. For this reason the colony growth test, which gives semiquantitative information about the growth response, was conducted with strain NOX. Moreover, the colony growth test allows detection of toxic effects, which may be revealed either by a retarded growth as compared with growth of the blanks or by failure of the organism to grow on the plates.

Investigations at very low concentrations of individual substrates give essential information as is shown by the inability of strain NOX to grow with 10 µg of C of glycine and alanine, both of which promoted colony growth at a concentration of 1 g per liter. Growth at a high concentration but no growth at very low concentrations was also observed with a number of substrates for *Aeromonas hydrophila* (21), *P.fluorescens* (23), *P.aeruginosa* (20) and *Flavobacterium* sp. strain S12 (19). Such observations may be explained by high substrate saturation constants of the organism for these compounds. The reverse situation, namely, the promotion of growth of strain NOX at 10 µg of C per liter, but no enhanced growth of the colonies at a concentration of 1 g per liter, as was observed with formate, glycolate and aspartate, needs further investigation.

Strain NOX closely resembles the polarly flagellated vibrio-shaped *Vibrio oxaliticus*, isolated from garden soil and described by Bhat and Barker (2), sharing the following

physiological properties: growth with oxalate as sole source of carbon and energy, relatively slow growth with acetate, poor growth with amino acids and no reduction of nitrate. At present, the genus *Vibrio* includes only vibrio-shaped bacteria with a fermentative character. Therefore it is suggested to reclassify strain NOX and *V. oxaliticus* into a new species of *Spirillum*. The polarly flagellated vibrio-shaped organism, *Spirillum delicatum*, isolated from distilled water and described by Leifson (12), also resembles strain NOX. From the origin of *S. delicatum* and from the results obtained with strain NOX it may be concluded that the genus *Spirillum* includes species which are adapted to very low substrate concentrations.

Kinetics of growth on oxalate and acetate

The nonlinear relationship between growth rate of strain NOX and reciprocal values of the concentrations of oxalate below 50 μg of C/l (Fig. 6) shows that oxalate and compounds contributing to S_n were utilized concurrently at very low concentrations. The linear relationship observed between G of this strain and the reciprocal values of ΔS when $\Delta S \geq 50$ μg of C/l (Fig. 6) may have been due either to preferential uptake of oxalate at these relatively high concentrations or to an insignificant contribution of S_n to these substrate concentrations. The K_s value (15.2 μg of oxalate C/l) calculated from this linear plot is much lower than the ΔS values used in this calculation. The K_s value calculated with equation 3 when $\Delta S \leq 50$ μg of C per liter (Table 4) is very close to the one obtained with the Lineweaver-Burk plot. This result suggests that both models are adequate for the ΔS values mentioned. Calculation of the best fitting curve seems unsatisfactory because the value of G_{min} obtained in this way deviates from the observed values. Moreover, the S_n value obtained in this way differs more from the value calculated from N_{max} and Y on oxalate than the one obtained by using equation 3. This unsatisfactory result indicates that the assumption of concurrent utilization of oxalate and S_n at all applied oxalate concentrations is not correct.

The similarity between the initial growth rates of strain NOX in tap water supplied with acetate and the growth rate of the blanks suggests that, despite the low S_n value (6 μg of acetate C equivalents/l), natural substrates were preferently utilized. A generation time of 17.6 h in the presence of 1 mg of acetate C per

liter suggests that this compound is not rapidly utilized at very low concentrations.

Effect of ozonation on the concentration of substrates available for strain NOX and P.fluorescens strain P17

The data presented in Table 5 clearly demonstrate that ozonation of water results in a pronounced increase of the concentration of easily biodegradable substrates, particularly carboxylic acids. The concentration of AOC expressed in acetate-C equivalents per liter in water prior to ozonation as calculated from the N_{max} values of the applied organisms and their Y values with acetate was only in one case clearly higher for strain P17 than for strain NOX. This difference may be explained by the large nutritional versatility of strain P17 as has been shown previously (23). The similarity between values of AOC-NOX and AOC-P17 in the two other samples of non-ozonated water suggests that a large part of AOC-P17 consisted of carboxylic acids which were also available to strain NOX. The values of AOC-NOX (in acetate C equivalants) in ozonated water were much larger than the AOC-P17 values (Table 5). Hence, the specialized strain NOX seems better suited for the determination of the AOC concentration of ozonated water than strain P17. The use of strain NOX in combination with strain P17 may give information about the concentration of oxalate because this compound reacts much slower with ozone than glyoxylate and formate (7) which also do not serve as a source of carbon for strain P17 (23). This procedure thus enables a more precise assessment of the concentration of organic carbon available for strains NOX and P17 (C_T) in ozonated water than the use of strain NOX alone, particularly because of the large difference between yields on acetate and oxalate. It is clear that the data on oxalate obtained by the technique described above should be verified by the application of an appropriate chemical analysis.

Bacteria growing in large numbers in ozonated water, may cause considerable deterioration of drinking water quality in distribution systems, when this water is distributed without further treatment. Rapid sand filtration reduces the AOC concentration (22). Further investigations using the techniques described in this paper may give practical information about the ability of filtration procedures to minimize the concentration of biodegradable compounds formed by ozonation.

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10. General discussion

METHODOLOGY

Knowledge about the activities and growth of microorganisms at very low substrate concentrations is important for drinking water supply, waste water treatment and for ecological studies of marine and fresh water environments. Specific techniques, which may be used either with pure cultures or natural populations, are required to obtain relevant data about the activities and growth of micro-organisms at very low substrate concentrations. Techniques usually applied for this purpose are (a) measurement of uptake kinetics of pure cultures or natural populations by using compounds with a radioactive label in batch experiments and (b) determination of growth kinetics and bacterial behaviour in continuous-culture experiments.

Many data about transport constants (K_t, the substrate concentration at which the uptake rate is half of the maximum uptake rate) have been reported for a great diversity of bacteria and detailed studies have been conducted by using the first-mentioned technique. A selection of reported data is presented in Table 1. Also the heterotrophic activity of natural populations at very low substrate concentrations has frequently been studied by using labelled compounds (3, 14, 33, 34, 38). Finally, this technique enables to study the uptake and biodegradation of anthropogenic compounds at extremely low concentrations (2).

Even a copiotrophic bacterium as *Escherichia coli* has K_t values below 1 µM for a number of substrates, but extremely low K_t values (< 0.1 µM) have in particular been observed for aquatic bacteria (Table 1). Unfortunately, it is not clear to which extent these K_t values are similar to the substrate concentration (K_s) at which these bacteria have half of their maximum growth rate.

Several chemoheterotrophic bacteria have been selected and studied in continuous-culture experiments. Only in a few cases K_s values were determined (Table 1). These K_s values are relatively high in comparison with the reported K_t values. Table 1 contains also data obtained in batch-culture experiments with *E.coli*. The K_s values estimated in these studies are close to the K_t values observed in that organism. Nevertheless, batch-culture experiments are believed to be less suited than continuous culture experiments for determination of growth kinetics, or for selecting bacteria which are able to grow at very low concentrations of substrates (11, 12, 36).

Table 1. A selection of transport constants (K_t) and substrate saturation constants (K_s), as reported in literature.

Organism	Substrate	K_t (μM) ; K_s (μM)	Technique	Temperature (°C)	Ref.
E.coli	glucose	- ;0.38	Batch cult.	30	(30)
		- ;70[b]	"	30	(30)
E.coli	glucose	- ;1.0	Batch cult.	37	(37)
E.coli	glucose	- ;44	Cont.cult.	20	(16)
E.coli	glucose	- ;13	Batch cult.	-	(20)
E.coli	glycerol	1.3 ;0.9	Batch exp.	37	(13)
E.coli	galactose	0.49;-	Batch exp.	25	(27)
E.coli	galactose	4.0 ;-	Batch exp.	25	(27)
E.coli	maltose	1 ;-	Batch exp.	25	(31)
E.coli	maltotriose	2 ;-	Batch exp.	25	(31)
E.coli	succinate	14 ;-	Batch exp.	25	(19)
E.coli	succinate	30 ;-	Batch exp.	37	(21)
E.coli	amino acids	1-10;-	Batch exp.	37	(25)
P.aeruginosa	amino acids	0.1-1;-	Batch exp.		(15, 18)
P.aeruginosa	glycerol	7.8;-	Batch exp.	37	(32)
		480[b]			(32)
P.aeruginosa	glucose	8.0;-	Batch exp.	37	(24)
P.aeruginosa	glucose	11.0;-	Batch exp.	30	(6)
P.fluorescens	glucose	1.0;-	Batch exp.	30 and 5	(22) (22)
Pseudomonas sp.	lactate	20;-	Batch exp.		(23)
Pseudomonas sp.	lactate	-;91	Cont. cult.		(23)
Spirillum sp.	lactate	5.8;-	Batch exp.		(23)
Spirillum sp.	lactate	-;23	Cont. cult.		(23)
Heterotrophic bact.	glucose	13;-	Batch exp.	20	(1)
Asticcacaulis biprosthecum	glucose	1.8;-	Batch exp.		(26)
		34[b];-			(26)
Unknown rod	glucose	0.03;-	Batch exp.		(14)
Achromobacter aquamarinus	glucose	0.04;-	Batch exp.	6	(33)

Table 1. Continued.

Organism	Substrate	K_t (μM)	K_s (μM)	Technique	Temperature (°C)	Ref.
Marine bacteria	glucose	0.03-1.7;	-	Batch exp.	5	(10)
Vibrio sp.	arginine	0.017;	-	Batch exp.	5	(9)
Vibrio sp.	arginine	4.5 ;	-	Batch exp.	5	(9)
Natural pop.	glutamate	0.025;	0.04;	Batch exp.		(3)
Natural marine population	toluene	0.03;	-	Batch exp.		(5)

[a] -, Not determined
[b] Low affinity transport system.

Information about the growth constants (K_s and minimum generation time, G_{min}) of bacteria with hygienic, technical and ecological importance is very scarce. In the present study the batch-culture technique was applied to determine the growth (generation time and maximum colony counts) of such bacteria at very low substrate concentrations. This technique was also selected for the determination of the growth constants, because (a) it requires very simple equipment, (b) is theoretically very simple, (c) only small amounts of sometimes expensive or scarce compounds are needed, (d) it seems less laborious than continuous-culture experiments and (e) extremely low substrate concentrations are easier to control in batches than in the continuous-culture vessel.

The results presented in the preceding chapters clearly show that with batch-culture experiments it is very simple (a) to select bacteria which are able to grow on very low concentrations of a of a specific substrate and (b) to determine growth kinetics of pure cultures at extremely low substrate concentrations. The application of the technique is mainly limited to those bacteria which are able to form colonies on solid media. Most bacteria important in water supply and many other aquatic bacteria are able to do so. Also in continuous-culture experiments colony counts are used at very low substrate concentrations (16, 17).

The amount of substrate naturally present in the water should be very low when it is applied for selection of bacteria growing at

very low concentrations of substrates or for growth experiments conducted to determine growth kinetics. Drinking water prepared by water treatment processes with biological activity, and in particular by slow sand filtration, is suited for this purpose. Table 2 summarizes the growth kinetics of bacteria determined in batch-culture experiments as described in the preceding chapters.

OLIGOTROPHY AND COPIOTROPHY

Low substrate-saturation constants for growth (K_s) seem an important property of so-called oligotrophic bacteria. This in contrast to copiotrophic bacteria which require high substrate concentrations for rapid growth (26). The K_s values shown in Table 2 reveal large differences between K_s values of a specific organism for various substrates and between K_s values of various organisms for a specific substrate. These observations reveal, that designation of an organism as an oligotrophic bacterium cannot solely be based on a few estimations of K_s values or, more correctly, a few substrate affinities $\left(\left[G_{min}.K_s\right]^{-1}\right)$. Unfortunately, comparison of the K_s values shown in Table 2, with K_s values of oligotrophic bacteria is difficult because of a notable lack of such data in the literature.

The K_t values as reported for *Asticcacaulis biprosthecum,* which is considered to be an oligotrophic bacterium (26), and for an oligotrophic marine bacterium (1) are not really low when compared to the K_s values presented in Table 2 for the *Flavobacterium* strains. The lowest K_t values (0.02-0.04 μM) available in literature, seem those reported for natural populations and a few, mostly unidentified organisms (Table 1). The K_s values obtained for the *Flavobacterium* strains and of *Pseudomonas fluorescens* P17 growing on arginine are similar to these values. These observations suggest that oligotrophic bacteria have K_t and K_s values below 0.1 μM for compounds which are utilized as a sole source of carbon and energy.

A limitation of using K_s values (or substrate affinities, for individual compounds to explain bacterial behaviour at low concentrations of substrate is demonstrated by the small difference in growth rate between *P.fluorescens* P17, and P.aeruginosa P1525 in drinking water (Chapter 4). This difference, which is much smaller than the observed differences between K_s values, may nearly completely be explained by the difference in G_{min} values as caused

Table 2. Substrate saturation constants (K_s) and minimum generation times (G_{min}) as obtained for a number of organisms in batch-culture experiments at 15 °C

	A.hydrophila strain 315		*P.fluorescens* strain P17		*P.fluorescens* strain P500		*P.aeruginosa* strain P1525		*Flavobacterium* spp. strain 166		*Flavobacterium* spp. strain S12	
	K_s(μM)	G_{min}(h)	K_s(μM)	G_{min}(h)	K_s(μM)	G_{min}(h)	K_s(μM)	G_{min}(h)	K_s(μM)	G_{min}(h)	K_s(μM)	G_{min}(h)
Acetate	1.7	15.0	0.17	5.5	0.95	5.2	1.2	11.7	-	-	-	-
Succinate	-[a]	-	0.48	3.0	2.6	2.8	-		-	-	-	-
Aspartate	-	-	0.34	3.1	1.9	2.9	2.3	6.0	-	-	-	-
Arginine	-	-	0.04	3.9	-	-	0.4	6.0	-	-	-	-
Glucose	0.17	7.2	0.79	4.6	5.2	3.6	-	-	0.045	4.7	1.7	6.2
Maltose	-	-	-	-	-	-	-	-	-	-	0.17	2.8
Maltotriose	-	-	-	-	-	-	-	-	-	-	0.025	2.5
Maltotetraose	-	-	-	-	-	-	-	-	-	-	0.027	2.1
Maltopentaose	-	-	-	-	-	-	-	-	-	-	0.017	2.2
Maltohexaose	-	-	-	-	-	-	-	-	-	-	0.014	2.1
Starch	(73)[b]	3.3	-	-	-	-	-	-	(3.9)	3.7	(13.7)	2.2

[a]-, Not determined
[b]( ), In μg of C per liter.

by the effect of temperature on the growth of these organisms. Most likely, the presence of a mixture of substrates in water is responsible for this phenomenon. This suggestion is supported by the observation, that *P.fluorescens* P17 and P500, had similar growth rates when growing on mixtures of compounds despite a five times difference in K_s values and similar G_{min} values for a series of individual compounds (Chapter 2). Therefore, growth constants obtained for individual substrates should be used with caution in explaining bacterial behaviour in natural situations.

Apart from K_s and G_{min} values, a number of other characteristics such as maintenance requirements, attachment properties, storage of substrates and a high surface-volume ratio determine the oligotrophic or copiotrophic nature of a bacterium (23, 26).

THRESHOLD SUBSTRATE CONCENTRATIONS

The kinetic data as summarized in Table 2, and also in Table 1, demonstrate that the concentration of substrate in drinking water should be very low indeed for a complete prevention of multiplication of bacteria. The substrate concentration, below which a bacterium is unable to multiply, the threshold concentration (S_t), is determined by the growth kinetics (G_{min}, K_s) and the maintenance requirements of the organism, which in the absence of exogenous substrate results in a certain death rate. The mean half-life (t_e), the period in which 50 % of the organisms died from starvation, can be used to quantify this death rate. No increase in numbers of bacteria occurs when the generation time calculated from G_{min}, K_s and the substrate concentration S equals t_e, thus:

$$t_e = G = G_{min} + (G_{min} \cdot K_s/S_t) \quad (1)$$

$$\text{as} \quad G_{min} << t_e \quad (2)$$

$$S_t = \frac{G_{min} \cdot K_s}{t_e} \quad (3)$$

Equation (3) shows the dependency of S_t on G_{min}, K_s and t_e. For *Aeromonas hydrophila* strain 315 a t_e value of 94 h was calculated (Chapter 5); starvation experiments with *Flavobacterium* sp. strain S12 (unpublished data) gave a t_e value of 90 h. Minimum values of G_{min} x K_s observed for strain S12, *Flavobacterium* sp. strain 166 and *P.fluorescens* P17 are about 13 to 15 μg C/l.h

(Chapters 2, 6, 8). Assuming that all these bacteria have a t_e of about 90 h, than S_t should be about 0.14 μg of C per liter. Larger S_t values can be calculated for bacteria with lower substrate affinities (larger values for $G_{min}.K_s$). Depending on the number of suitable substrates present in water, the concentration of individual substrates should be less than the S_t value.

The described theoretical approach shows that only extremely low concentrations of easily assimilable organic compounds can be allowed in drinking water to prevent completely the regrowth of oligotrophic bacteria. Allowing a doubling of colony counts in drinking water during distribution (G = 24-72 hours) would require concentrations of about 0.18-0.54 μg of C per liter for bacteria with $G_{min}.K_s$ = 13 to 15 μg C/l.h. The maximum colony counts at these concentrations can reach 10^3-10^4 CFU/ml.

The very low G_{min}. K_s values have been obtained for specific substrates. The inability of *Flavobacterium* sp. strain S12 to multiply in slow sand filtered water (Chapters 7,8) reveals that biological treatment reduces the concentrations of substrates in which this strain is specialized to values below S_t. This reduction is most likely the result of the activities of bacteria resembling strain S12 and of other bacteria which are able to utilize also other substrates. Otherwise these bacteria would be unable to maintain themselves in the filter bed. In general compounds for which bacteria have the highest substrate affinities, possibly amino acids and carbohydrates, are removed to extremely low levels whereas other compounds for which bacteria have lower affinities remain present at higher concentrations. Further data on maximum substrate affinities (minimum values of $G_{min}.K_s$) and on t_e values for representative compounds and bacteria are needed for improved calculations of S_t values.

EASILY ASSIMILABLE ORGANIC CARBON (AOC)

The investigations reported in previous chapters, show that organic compounds which may be utilized as a source of carbon and energy for growth determine both the growth rate and the maximum colony count of bacteria in water. Ozonation of water favours bacterial growth whilst added specific compounds raise the maximum colony count of either the natural population (Chapter 6, Fig. 1), or that of isolated bacteria. This clearly demonstrates that the presence of so-called easily assimilable organic carbon (AOC) is

more important for the growth of bacteria than the total concentration of organic carbon (TOC). The AOC concentrations usually are very small fractions of the TOC concentrations (Chapters 2, 3, 4, 9). Many compounds can be arranged into a category between easily assimilable and non-assimilable or recalcitrant compounds like humic and fulvic acids. AOC measurements are based on the growth of selected bacteria, which represent the natural flora of drinking water. It is likely that also a number of compounds which are less readily assimilable than simple amino acids, carbohydrates, and carboxylic acids are included in these determinations. In any case, easily assimilable compounds are included and, for simplication, growth (N_{max} values) of the test strains is regarded as a measure for the AOC concentration.

Three types of bacteria that may be used to determine the following AOC categories in drinking water have been described in the previous chapters.

(1) A very versatile fluorescent pseudomonad, strain P17 for determining the AOC concentration for general use in water types where oxalic acid, and maltose- and starch-like (MSL) compounds are not expected to be present in relatively large amounts;

(2) A *Flavobacterium* sp. strain S12 to determine the concentration of MSL compounds when such compounds are expected to be present, in particular when starch-based coagulant aids have been applied in water treatment;

(3) A *Spirillum* sp. strain NOX, to determine the concentration of carboxylic acids, including oxalic acid, in particular when ozonation is applied in water treatment.

Next to the categories of compounds included in these determinations, other compounds which can be utilized rapidly by various chemoheterotrophic bacteria but not by the selected organisms, may be present in water. An example is methane, that is particularly present in anaerobic ground water, and may occur in drinking water. Determination of methane is achieved by a simple gas-chromatographic technique with a detection level of 0.01 mg/1.

Drinking water prepared by water treatment, including stages with biological activity, in particular filtration, usually contains a low concentration of AOC utilizable for strain P17 (Chapter 3). In this case it is reasonable to expect that the natural bacterial population, either present in the water, in the ground or in the filterbeds, also removed those easily assimilable organic compounds

that could not be utilized by strain P17. Therefore, in this situation the AOC concentration determined with strain P17 may serve as a more general indicator for the presence of easily assimilable organic compounds than only for those compounds utilized by the test organism. The situation is different if the AOC is affected by specific treatments (ozonation, addition of a starch-based coagulant aid) or by materials, which release biodegradable compounds into the water.

The AOC concentrations (P17) reported in the preceding chapters, were very low in the effluents of filters with biological activity (Chapter 3) and in ground water (35). *Aeromonas hydrophila* did not multiply in the water prepared by slow sand filtration (Chapter 5). The growth rate of *P.aeruginosa* in this water was low (Chapter 4) and *Flavobacterium* sp. strain S12, specialized in MSL compounds, was unable to multiply in such a filtrate (Chapters 7, 8). Moreover, growth experiments with various coliform bacteria (species of *Citrobacter, Klebsiella* and *Enterobacter*) learned that these organisms were always unable to multiply in the effluent of slow sand filters (unpublished data). These observations indicate that in sand filtered water with AOC concentrations of about 10 g of acetate-C equivalents per liter, no significant growth of copiotrophic bacteria may occur. However further research is needed to define the effects of low AOC concentrations on the growth of oligotrophic bacteria, and on the development of animals in the distribution system.

Already in the beginning of this century it was observed that non-metallic construction and plumbing materials, used in distribution systems, promoted the growth of bacteria in drinking water (8). A number of bacteriological techniques for determining the growth-promoting effect of non-metallic materials has recently been developed in various countries (4, 7, 28). Release of growth promoting compounds may also be assessed by AOC determinations (35). The presence and growth of *Legionella* species in hot water systems (see Chapter 1) which appears to be related to the release of growth-promoting compounds by non-metallic packings (40) show that such materials may have a significant effect on the water quality. The effects of non-metallic materials on the growth of bacteria in drinking water fall beyond the scope of this study and will therefore not be further discussed here. The subject has recently been reviewed by Schoenen and Schöler (29).

In conclusion, it can be stated that the techniques reported in the foregoing chapters have shown to be very valuable for the estimation of low concentrations of easily assimilable organic carbon in water. When using selected cultures and selected compounds detailed information can be obtained on either the growth kinetics of a specific organism at very low substrate concentrations or about the effects of low concentrations of a specific compound on the growth of bacteria in water. Further application of the developed technique will therefore not only contribute to a further improvement of drinking water quality, but will also be useful in ecological studies, in particular on oligotrophic bacteria, and in studies on the growth of bacteria on very low concentrations of anthropogenic compounds.

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Summary

Water, in particular surface water, used for the preparation of drinking water usually contains a large variety of bacteria, amongst others organisms directly originating from pollution, e.g. bacteria of faecal origin, and bacteria which have multiplied on organic compounds present in the water. The removal of faecal pollution by various treatments and protection of distributed water against pollution are main objectives in drinking water supply.

Multiplication of chemoheterotrophic bacteria results in a reduction of the concentration of many natural and anthropogenic organic compounds in the raw water. Growth of bacteria also occurs during water treatment processes, and may even continue in drinking water during distribution when the water contains sufficient suitable substrates.

Growth ("regrowth") of bacteria in drinking water distribution systems results in a deterioration of the water quality. The hygienic condition of the water is affected by the multiplication of bacteria with pathogenic properties. Bacteria which produce taste and odour compounds, and animals developing on bacteria grown in the system influence the aesthetic quality of drinking water. The activities of specific bacteria may enhance corrosion of piping materials, resulting in turbid or coloured water.

Regrowth depends on the presence of compounds that serve as a nutrient source for bacteria. Such compounds present in drinking water either originate from (a) the raw water, (b) water treatment processes, or (c) construction and plumbing materials.

The objectives of the present study were: (a) the development of a bacteriological method for the estimation of organic compounds that occur in low concentrations in water and are used as growth substrates for bacteria; (b) the determination of the effect of water treatments on the concentration of these compounds and (c) the determination of the ability of oligotrophic aquatic bacteria and bacteria of hygienic, aesthetic or technical significance, to multiply in drinking water at very low substrate concentrations.

A batch-culture technique was developed to study the growth of bacteria in drinking water. The maximum colony counts of pure cultures of bacteria grown in water were used as a measure for the concentration of easily assimilable organic carbon (AOC). Three strains of bacteria were selected for the AOC determinations:

(1) *Pseudomonas fluorescens* strain P17, an organism with a great nutritional versatility, for general use.

(2) A *Flavobacterium* sp. strain S12, to determine the concentration of maltose-and starch-like compounds.

(3) A *Spirillum* sp. strain NOX to determine the concentration of carboxylic acids, including oxalic acid.

The properties of these bacteria were extensively studied in batch-culture experiments with drinking water supplemented with very low amounts of selected organic compounds. The obtained results prove that growth and growth kinetics of bacteria at very low concentrations can be determined in simple experiments using simple equipment.

Chapter 2 contains a report of experiments with two fluorescent pseudomonads, strains P17 and P500. These isolates, belonging to different biotypes of *P.fluorescens*, were tested for growth in tap water supplied with different concentrations of acetate and glutamate, and low concentrations (10 and 20 μg of C per l) of various other substrates and mixtures of related substrates, the latter being present in amounts corresponding to 1 μg of C per l each. Amino acids appeared to be excellent substrates for both isolates, but many other substrates were utilized at very low concentrations as well. Saturation constants (K_s) of strain P17 with acetate, arginine, aspartate, glutamate, lactate, succinate, malonate, *p*-hydroxybenzoate and glucose were all below 1 μM. The K_s values of strain P500 were about 5 times higher than those of P17. Since especially P17 is able to utilize a large number of different substrates at low concentrations, assessment of maximal colony counts of this organism by growth measurements in various types of tap water gives information on the concentrations of easily assimilable organic carbon (AOC). These concentrations may be calculated from the maximum colony count (colony-forming units per ml) of the organism grown in the water to be tested and its yield on acetate (4.2×10^9 CFU/mg of acetate C).

Chapter 3 describes investigations conducted to determine the AOC concentration in water samples collected from the various treatment stages of 8 treatment plants. In 7 of these plants surface water was used as the raw water source. AOC concentrations usually lay between 10 and 100 μg of acetate-C equivalents per l and were below 1 % of the concentration of dissolved organic carbon in the waters examined. Higher concentrations were observed in open storage basins and in water treated by ozonation. Rapid sand filtration caused maximum AOC reductions of about 80 % at influent AOC concen-

trations above 50 μg of C per l. At AOC concentrations below 25 μg of C per l, small reductions (< 50 %) were achieved by sand filtration.

Experiments with *P.aeruginosa*, an opportunistic pathogen, known for its ability to multiply at very low substrate concentrations, are described in Chapter 4. Five *P.aeruginosa* strains were tested for the utilization of 47 low-molecular-weight compounds as sole sources of carbon and energy for growth at a concentration of 2.5 g/l. Of these compounds, 31 to 35 were consumed. Growth experiments in tap water at 15 °C were carried out with one particular strain (P1525) isolated from drinking water. This strain was tested for the utilization of 30 compounds supplied at a concentration of 25 μg of C per l. The growth rate (expressed as number of generations per hour) of strain P1525 in this tap water was approximately 0.005 h^{-1}, and only with 10 individual compounds it was above 0.03 h^{-1}. An average yield of 6.2×10^9 colony-forming units per mg of C was obtained from the maximum colony counts. The average yield and maximum colony count of strain P1525 grown in tap water supplied with a mixture of 45 compounds, each at a concentration of 1 μg of C per l, enabled to calculate that 28 compounds were utilized. Growth rates of two *P.aeruginosa* strains (including P1525) in various types of water at 15 °C were half of those of a fluorescent pseudomonad. The AOC concentrations calculated from maximum colony counts and average yield values amounted to 0.1 to 0.7 % of the total organic carbon concentrations in five types of tap water. In river water and in ozonated water the percentages were about 10 times larger than in tap water.

Aeromonas hydrophila is frequently observed in drinking water. The organism may affect the determination of coliform bacteria by causing false positive reactions. Moreover, it is considered as an opportunistic pathogen. An *A.hydrophila* isolate obtained from sand-filtered river water was studied in batch-culture experiments with drinking water supplied with low concentrations of substrate. The isolate multiplied in the used drinking water, which contained 2-3 mg of dissolved organic carbon per liter, only after the addition of an adequate amount of a suitable substrate. Of five compounds tested at a concentration of 1 mg of C per l (glucose, acetate, glutamate, succinate and DL-lactate), glucose promoted growth most strongly. Growth was not promoted by lactate and extremely slow growth was observed with

succinate. Glucose gave growth of the organism even at initial concentrations below 10 μg of C per l. At initial glucose concentrations below the K_s value (12 μg of C per l), generation times showed nonlinear relationships with 1/initial glucose concentrations. From these observations, the maintenance coefficient m was calculated (m = 0.015 mg of glucose per mg dry wt per h at 12 °C). At initial concentrations below the K_s value of starch (73 μg of C per l), no growth was observed, but complete use of starch occurred in these situations after the addition of 10 μg of glucose C per liter.

The experiments in well-cleaned glass flasks as described in Chapter 6 revealed that addition of starch in concentrations of 10 and 25 μg of substrate C per l to the filtrate of slow sand filters stimulated the development of a yellow-pigmented bacterium which was identified as a *Flavobacterium* sp.. The isolate, strain 166, was able to multiply in tap water without substrates added, but addition of starch and glucose in amounts as low as 1 μg of substrate C per l clearly enhanced growth. The substrate saturation constants (K_s) of the *Flavobacterium* sp. for these compounds were 3.9 μg of starch C and 3.3 μg of glucose C per l. The results of this study demonstrate that microorganisms rapidly utilizing starch at a level of a few micrograms per l commonly occur in water. Moreover, they show that isolation of a bacterium that utilizes a compound at a very low concentration is very simple by using batch-culture experiments.

Chapter 7 reports another investigation on a starch-utilizing yellow-pigmented bacterium. This organism, strain S12, isolated from tap water, was tested for the utilization of 64 natural compounds at a concentration of 1 g/l by measuring colony growth on agar media. Only 12 carbohydrates and glycerol promoted growth. Experiments were carried out with pasteurized tap water supplied with mixtures of substrates at concentrations of 1 or 10 μg of C of each substrate per l and experiments with a number of separate carbohydrates at 10 μg of C per liter. It was shown that of the 64 natural compounds tested only sucrose, maltose, raffinose, starch, and glycerol promoted growth at very low concentrations. Also maltotriose, -tetraose, -pentaose, -hexaose, and stachyose, which were not included in the mixtures, supported growth with generation times of 3 to 5 h at 10 μg of C per l. Strain S12, tentatively identified as a *Flavobacterium* sp., thus appeared to be highly specialized in the utilization of glycerol and a number of oligo- and polysaccharides

at very low concentrations.

The growth kinetics of *Flavobacterium* sp. strain S12 were determined for a number of compounds at 15 °C in pasteurized tap water supplied with very low amounts of substrates (Chapter 8). Substrate saturation constants (K_s) for the growth on maltotriose, maltotetraose, maltopentaose and maltohexaose were 0.1 μM or less, i.e. below those for glucose (1.7 μM) and maltose (0.17 μM). K_s values for starch, amylose and amylopectine were 13.7, 31 and 10.8 μg of C per l, respectively. A yield of 2.3×10^7 colony-forming units per μg of C of the oligo- and polysaccharides was calculated for strain S12 from the linear relationships between maximum colony counts in pasteurized tap water and the concentrations (all below 25 μg of C/l) of supplied compounds. The maximum colony counts of strain S12 grown in various types of raw water and tap water revealed that the former contained only a few μg of maltose- and starch-like compounds per l; in tap water the concentrations were below 1 μg, and usually below 0.1 μg of C per l. The application of starch-based coagulant aids gave increased concentrations of maltose- and starch-like compounds in the water during treatment, but these concentrations were greatly reduced by coagulation and sedimentation, rapid sand filtration and slow sand filtration.

Chapter 9 deals with the determination of the AOC concentration in ozonated water using an oxalate-utilizing organism (strain NOX). This vibrio-shaped organism was identified as a *Spirillum* sp.. Its nutritional versatility was determined by growth experiments with low-molecular-weight carbon compounds at high (g/l) and very low concentrations (μg/l). The organism appeared to be specialized in the utilization of a number of carboxylic acids. Yields of 2.9×10^6 colony-forming units (CFU)/μg of oxalate C and 1.2×10^7 CFU/μg of acetate C were obtained from growth experiments with tap water supplied with various low amounts of either oxalate or acetate. A substrate saturation constant of 0.64 μM oxalate was calculated from the relationship between growth rate and concentration of added oxalate. Maximum colony counts of strain NOX grown in ozonated water (dosages of 2.0-3.2 mg of O_3 per l) were 15 to 20 times larger than the maximum colony counts of strain NOX grown in water prior to ozonation. Based on the nutritional requirements of strain NOX it was concluded that carboxylic acids were produced by ozonation. Oxalate concentrations were calculated from the maximum colony counts of strain NOX grown in samples of ozonated water in

which a non-oxalate-utilizing strain of *P.fluorescens* had already reached maximum growth. The oxalate concentrations obtained by this procedure ranged from 130 to 220 μg of C/l.

The above-described experiments reveal that the growth of bacteria at very low substrate concentrations may be studied by simple batch-culture experiments. The data obtained demonstrate that typical aquatic bacteria, as they may occur in drinking water, are able to multiply at very low concentrations of substrates. To prevent multiplication of such bacteria, the AOC concentrations of drinking water should be very low. AOC determinations performed with selected bacteria show that biological filtration processes in particular are very effective in reducing the concentrations of compounds which promote bacterial growth in drinking water.

Samenvatting

Water, in het bijzonder oppervlaktewater waaruit drinkwater wordt bereid, bevat gewoonlijk een grote verscheidenheid van bacteriën. De aanwezigheid van veel van deze micro-organismen, met name bacteriën van faecale oorsprong, is een rechtstreeks gevolg van verontreinigingen met deze bacteriën. Tevens vermenigvuldigen veel bacteriën zich door voeding met verbindingen die in het water aanwezig zijn. De verwijdering van verontreinigingen van faecale herkomst door middel van waterbehandelingsprocessen alsmede het voorkomen van verontreining van het water tijdens distributie, nemen een zeer belangrijke plaats in bij de drinkwatervoorziening.

Een vermeerdering van bacteriën heeft een daling tot gevolg van de concentratie van vele in het water aanwezige natuurlijke, en door menselijke activiteit geproduceerde stoffen. Het vermogen van bacteriën om deze verbindingen uit het water te verwijderen speelt dan ook een belangrijke rol bij diverse zuiveringsprocessen. Indien in het drinkwater voldoende geschikte voedingsstoffen aanwezig zijn, kan vermeerdering van bacteriën tijdens het verblijf in het leidingnet optreden ("nagroei"). Verbindingen die nagroei veroorzaken kunnen afkomstig zijn van het ruwe water, kunnen worden geïntroduceerd bij waterbehandelingsprocessen of door constructiematerialen worden afgegeven aan het water. Nagroei van bacteriën in drinkwaterdistributiesystemen leidt tot kwaliteitsverslechtering van het water. De hygiënische gesteldheid van het water wordt beïnvloed door de vermeerdering van bacteriën met ziekteverwekkende eigenschappen. Bacteriën die reuk- en smaakstoffen produceren en hogere organismen die zich voeden met bacteriën zijn van invloed op de esthetische kwaliteit van het water. De activiteiten van bepaalde groepen van bacteriën kunnen de aantasting van leidingen versnellen, waardoor troebel of bruingekleurd water ontstaat.

De doelstellingen van het beschreven onderzoek waren: (a) de ontwikkeling van een bacteriologische methode voor de bepaling in water van organische verbindingen die als voeding voor bacteriën kunnen dienen; (b) de bepaling van de invloed van waterbehandelingsprocessen op de concentratie van deze verbindingen en (c) onderzoek naar het vermogen van oligotrofe bacteriën en van bacteriën van hygiënische, esthetische of technische betekenis, om zich in drinkwater met lage substraatconcentraties te vermeerderen.

Voor het onderzoek naar het voorkomen van verschillende organische verbindingen in drinkwater werd gebruik gemaakt van geselecteerde stammen van bepaalde bacteriesoorten die zich kenmerkten door

hun vermogen deze verbindingen in zeer lage concentraties te kunnen opnemen en gebruiken voor groei. Voor deze groeimetingen werd de toename bepaald van het aantal geënte specifieke bacteriën in een bepaalde hoeveelheid (600 ml) van het te onderzoeken gepasteuriseerde water (zgn. "batch-cultures"). Deze batch-cultures werden bij 15 °C geplaatst omdat deze temperatuur dicht bij de temperatuur van het water in het distributiesysteem ligt. Het verband tussen substraatconcentratie en de groei van de specifieke bacteriën werd vastgesteld in bovengenoemde cultures voorzien van verschillende zeer kleine hoeveelheden van de te testen substraten. Op deze wijze werden ijklijnen en opbrengstcoëfficiënten verkregen. De bacteriegroei in de kolven met drinkwater werd gemeten door bepalingen van het aantal koloniëvormende bacteriën (colony-forming units, aangegeven als CFU-waarden in de tabellen en de figuren). Voor deze tellingen werden de juiste verdunningen van het drinkwater uitgestreken op geschikte voedingsbodems. Daar bij een vermeerdering van zeer lage aantallen bacteriën de substraatconcentratie in de culture aanvankelijk nauwelijks afneemt kan in batch-cultures ook de invloed van de substraatconcentratie op de groeisnelheid worden bepaald.

Door gebruik te maken van de hier ontwikkelde werkwijze is men in staat op eenvoudige wijze gegevens te verzamelen over groeisnelheid en groeiopbrengst bij zeer lage substraatconcentraties. Deze grootheden worden bij bacteriën die grotere hoeveelheden substraat consumeren vaak bepaald met zgn. continue cultures waar kwantitatieve gegevens worden verkregen door weging van de gevormde biomassa. Deze bepalingen zijn onuitvoerbaar wanneer het gaat om substraatconcentraties zoals die in drinkwater voorkomen. Door in de voor bacteriegroei ontwikkelde wetmatigheden, celopbrengsten te vervangen door koloniegetallen, kan men de voor continue cultures geldende formules ook voor batch-cultures met zeer lage substraathoeveelheden gebruiken.

Ook voor het selecteren en isoleren van bovenbedoelde specifieke bacteriestammen, die zijn aangepast aan de zeer lage concentraties van substraten in drinkwater, en die werden gebruikt voor het beschreven onderzoek, werd gebruik gemaakt van batch-cultures.

Voor de bepaling van de concentratie van gemakkelijk assimileerbare organische koolstofverbindingen (AOC) werden 3 bacteriestammen gebruikt, afkomstig uit drinkwater, namelijk:

1. *Pseudomonas fluorescens* stam P17; dit organisme kan een grote verscheidenheid van organische verbindingen benutten als

voedselbron.

2. Een *Flavobacterium* sp. stam S12; dit organisme kan worden gebruikt voor de bepaling van maltose- en zetmeelachtige verbindingen.
3. Een *Spirillum* sp., stam NOX; voor de bepaling van de concentratie van carbonzuren, waaronder oxaalzuur.

In hoofdstuk 2 worden een aantal proeven beschreven met uit drinkwater geïsoleerde biotypen van *P. fluorescens* (de bovengenoemde stam P17 en stam P500). Beide stammen bezaten de voor deze soort karakteristieke eigenschap om een groot aantal verbindingen te benutten voor de groei. Om na te gaan of deze eigenschap ook bij lage substraatconcentraties nog tot uiting kwam werden groeiproeven met deze stammen uitgevoerd in drinkwater waaraan zeer geringe hoeveelheden acetaat waren toegevoegd. Tevens werd het effect nagegaan van de toevoeging van kleine hoeveelheden (10 en 20 μg C per 1) van een aantal andere substraten en van mengsels hiervan (iedere verbinding in een concentratie van 1 μg C per 1). Aminozuren bleken uitstekende voedingsstoffen voor beide stammen. Maar ook vele andere verbindingen werden bij zeer lage concentraties benut. De K_s waarden (substraatverzadigingsconstanten) van stam P17 voor acetaat, arginine, aspartaat, glutamaat, lactaat, succinaat, malonaat, p-hydroxybenzoaat en glucose waren alle kleiner dan 1 μM. De K_s waarden van stam P500 waren ongeveer 5x hoger dan die van stam P17. Duidelijk komt hieruit naar voren dat stam P17 in staat is een groot aantal verschillende verbindingen nog bij zeer lage concentraties te benutten voor de groei. De bepaling van het maximum koloniegetal van dit organisme in water kan daarom als een maat voor de concentratie van gemakkelijk assimileerbare organische koolstof (AOC) worden gebruikt. Met behulp van de opbrengstcoëfficiënt van stam P17 voor acetaat (4.2×10^9 CFU/mg acetaat-C) kan dit maximum koloniegetal worden omgerekend in μg acetaat-C equivalenten per 1.

In hoofdstuk 3 wordt melding gemaakt van een onderzoek naar de AOC-gehalten in water in diverse behandelingsstadia van 8 drinkwaterbereidingssystemen. Bij 7 ervan werd oppervlaktewater gebruikt voor de bereiding van drinkwater. De AOC-concentraties van de onderzochte watertypen lagen gewoonlijk tussen 10 en 100 μg acetaat-C equivalenten per 1. Deze concentraties vormden minder dan 1 % van de concentraties aan opgeloste organische koolstof. Relatief hoge AOC-concentraties werden waargenomen in water afkomstig uit spaarbekkens

en water na behandeling met ozon. Snelfiltratie van water zonder vrij chloor leidde tot duidelijke reducties van het AOC-gehalte. Deze reductie bedroeg maximaal 80 %, indien het AOC-gehalte van het aangevoerde water hoger dan 50 µg C per l was. Bij AOC-gehalten beneden 25 µg C per l werden slechts geringe reducties (< 50 %) bij filtratie waargenomen. De AOC-concentraties in het afgeleverde drinkwater lagen tussen 10 en 85 µg acetaat-C equivalenten per l.

Pseudomonas aeruginosa is een facultatief-pathogeen organisme, dat zich bij lage substraat concentraties kan vermeerderen in water. Laatstgenoemde eigenschap van deze bacteriën is nader onderzocht (hoofdstuk 4). Vijf stammen van *P.aeruginosa* werden allereerst onderzocht op hun vermogen een groot aantal organische verbindingen te benutten voor groei. Deze eigenschap werd onderzocht bij een hoge substraatconcentratie (2.5 g/l). Alle stammen bleken 31 à 35 van de geteste verbindingen te kunnen benutten. Van één van de stammen, P1525, geïsoleerd uit drinkwater, werd met behulp van groeiproeven in drinkwater onderzocht, welke van een dertigtal verbindingen dit organisme kon benutten bij een concentratie van 25 µg C/l. Deze stam groeide bij 15 °C zeer traag in drinkwater; slechts in aanwezigheid van een tiental van de onderzochte verbindingen was de generatietijd korter dan circa 30 uur. Vervolgens werd de groei van P1525 gemeten in drinkwater waaraan mengsels van verbindingen waren toegevoegd. Per verbinding was 1 µg C per l aanwezig. Berekend kon worden dat van de in totaal 45 in het mengsel aanwezige verbindingen er 28 werden benut. Het gehalte aan organische verbindingen waarop deze *P. aeruginosa* zich kon vermeerderen in drinkwater was slechts 0.1 à 0.7 % van het totale gehalte aan opgeloste organische koolstof.

Aeromonas hydrophila wordt herhaaldelijk aangetroffen in drinkwater. Dit organisme kan storend werken bij de bepaling van bacteriën van de coligroep en bezit ziekteverwekkende eigenschappen. Om deze redenen werd *A. hydrophila* onderzocht om vast te stellen in hoeverre dit organisme zich kan vermeerderen bij lage substraatconcentraties (hoofdstuk 5). In drinkwater bereid door middel van langzame zandfiltratie trad geen toename van het koloniegetal op. Van de 5 verschillende verbindingen (glucose, acetaat, glutamaat, succinaat en lactaat) die werden toegevoegd in een concentratie van 1 mg C per l was glucose het sterkst groeibevorderend. Lactaat en succinaat werden bij deze concentratie niet of nauwelijks benut. Bij een concentratie van 10 µg glucose-C per l bedroeg de

generatietijd circa 15 uur. Bij glucose concentraties die duidelijk beneden de K_s waarde (12 µg C per l) lagen, waren de generatietijden van het organisme langer dan op grond van de generatietijden bij hogere concentraties kon worden verwacht. Deze waarneming leidde tot de bepaling van de onderhoudscoëfficiënt van het organisme, die 0.015 mg glucose per mg droge stof per uur bedroeg (bij 12 °C). Hierdoor was bij lage concentraties een relatief kleiner deel van het substraat beschikbaar voor groei dan bij hogere substraatconcentraties.

Om na te gaan in hoeverre ook hoog-moleculaire verbindingen in zeer lage concentraties door bacteriën kunnen worden benut, werden geringe hoeveelheden zetmeel (10 en 25 µg C/l) toegevoegd aan kolven met 600 ml van het filtraat van langzame zandfilters (hoofdstuk 6). De toevoeging van zetmeel gaf na incubatie van de watermonsters bij 15 °C een sterke groei van een bacterie met geel-gepigmenteerde kolonies. Het organisme, stam 166, werd geïsoleerd en bleek te behoren tot het geslacht *Flavobacterium*. Nader onderzoek wees uit dat zelfs een toevoeging van 1 µg C per l in de vorm van zetmeel aan drinkwater nog een duidelijk sterkere groei van het organisme tot gevolg had. De substraat-verzadigingsconstanten van stam 166 voor zetmeel en glucose waren respectievelijk 3.9 en 3.3 µg C per l. Uit deze experimenten komt naar voren dat bacteriën die zetmeel in zeer lage concentraties kunnen benutten algemeen voorkomen in water. Duidelijk is gebleken dat bacteriën die zich vermeerderen bij zeer lage concentraties van bepaalde substraten op zeer eenvoudige wijze kunnen worden geïsoleerd met behulp van een groeiproef met een batchculture.

In hoofdstuk 7 wordt een onderzoek beschreven met een andere zetmeelbenuttende geel-gepigmenteerde *Flavobacterium* sp. (stam S12), die op bovenbeschreven wijze was geïsoleerd uit drinkwater. Door middel van de koloniegroei op vaste voedingsbodems werd aangetoond dat stam S12 van de 64 verschillende verbindingen die werden getest slechts 12 suikers en glycerol kon benutten. Uit experimenten met dit organisme in drinkwater waaraan verschillende mengsels van verbindingen waren toegevoegd (iedere verbinding in hoeveelheden die overeen kwamen met 10 en 1 µg C per l) kwam naar voren dat alleen in aanwezigheid van het mengsel van de koolhydraten groei optrad. Door middel van afzonderlijke groeiproeven met de in dit mengsel aanwezige verbindingen bij een concentratie van 10 µg C per l werd vastgesteld dat van de 64 bij het onderzoek betrokken verbindingen er

slechts 5 bij zeer lage concentraties konden worden benut, namelijk sucrose, maltose, raffinose, zetmeel en glycerol. Ook maltotriose, maltotetraose, maltopentaose, maltohexaose en stachyose, die niet inbegrepen waren in de mengsels en de koloniegroeiproeven, werden benut bij een concentratie van 10 μg C per l. *Flavobacterium* stam S12 is dus gespecialiseerd in het benutten van oligosacchariden en polysacchariden waardoor dit organisme in aanmerking kwam voor de bepaling van zetmeelachtige verbindingen in water.

Voor een aantal van de bovengenoemde verbindingen werden de volgende K_s waarden vastgesteld: glucose, 1.7 μM; maltose, 0.17 μM; maltotriose, maltotetraose, maltopentaose en maltohexaose lager dan 0.03 μM (hoofdstuk 8). Voor zetmeel, amylose en amylopectine werden K_s waarden van 13.7; 31 en 10.8 μg C per l vastgesteld. De groeiopbrengst van stam S12 op de bovengenoemde verbindingen bedroeg $2.3x10^7$ kolonievormende eenheden per μg C. Op grond van de maximale koloniegetallen van het organisme in verschillende typen ongezuiverd water werd geconcludeerd dat maltose- en zetmeelachtige verbindingen hierin slechts in geringe hoeveelheden aanwezig waren (enkele μg C per l). Stam S12 was niet of nauwelijks in staat zich te vermeerderen in een aantal drinkwatertypen; de concentratie van maltose- en zetmeelachtige verbindingen berekend uit de maximale koloniegetallen in deze watersoorten was lager dan 1 μg C per l. Toepassing van zetmeelderivaten bij het coagulatieproces had verhoogde concentraties van de genoemde substraten in diverse stadia van de zuivering tot gevolg. Snelfiltratie gevolgd door langzame zandfiltratie reduceerden de concentraties van deze verbindingen zodanig dat stam S12 zich niet kon vermeerderen in het filtraat van de langzame zandfilters.

Onder invloed van ozon wordt onder meer oxaalzuur gevormd uit bepaalde moeilijk afbreekbare verbindingen. Door middel van een batch-culture met drinkwater waaraan een zeer geringe hoeveelheid oxaalzuur was toegevoegd, werd een oxaalzuurbenuttende bacteriestam, (NOX), van het geslacht *Spirillum* geïsoleerd (hoofdstuk 9). Door middel van koloniegroeiproeven op vaste voedingsmedia en experimenten in drinkwater waaraan een viertal verschillende mengsels van verbindingen (1 μg C per verbinding per l) waren toegevoegd werd vastgesteld dat stam NOX was gespecialiseerd in het benutten van een aantal carbonzuren. Groeimetingen in drinkwater waaraan verschillende hoeveelheden acetaat en oxalaat waren toegevoegd wezen uit dat de opbrengst met acetaat ($1.2x10^7$CFU per μg C) circa 4 x hoger was dan de opbrengst met oxalaat ($2.9x10^6$ CFU per μg C). De $K_s$waarde van

stam NOX voor oxalaat was 0.64 µM. De maximum koloniegetallen van stam NOX gegroeid in een drietal watertypen na ozonisatie waren 15 à 20 maal hoger dan de maximum koloniegetallen in deze watertypen vóór ozonisatie. Op grond van de voedingsbehoefte van stam NOX werd hieruit geconcludeerd dat door ozonisatie een hoeveelheid carbonzuren waren gevormd. Stam NOX vermeerderde zich ook sterk in geozoniseerd water waarin *P.fluorescens*, stam P17, die geen oxaalzuur kan assimileren, reeds het groeimaximum had bereikt. Op basis van de groeimaxima van stam NOX gegroeid in aanwezigheid van stam P17 werd berekend dat de oxaalzuurconcentratie in het geozoniseerde water tussen 130 en 220 µg C per 1 lag. Het AOC-gehalte berekend als acetaat-C equivalenten per 1 uit de maximum koloniegetallen in geozoniseerd water was voor stam NOX 1.4 à 1.8 x hoger dan voor stam P17. Stam NOX is, door het vermogen om ook het gevormde oxaalzuur te benutten, dan ook beter geschikt voor AOC-metingen in geozoniseerd water dan stam P17.

Uit het bovenbeschreven onderzoek kan worden geconcludeerd dat de groeimogelijkheden van bacteriën bij zeer lage substraatconcentraties kunnen worden onderzocht met behulp van eenvoudige groeiproeven in batch-cultures. Naar voren komt dat bacteriën, zoals aanwezig in drinkwater, zich bij zeer lage substraatconcentraties nog kunnen vermeerderen. Het AOC-gehalte van drinkwater dient dan ook zeer laag te zijn voor het verhinderen van nagroei. De AOC-bepalingen, uitgevoerd met geselecteerde bacteriën, hebben aangetoond dat met name filtratieprocessen waarbij micro-organismen een rol spelen in staat zijn AOC-gehaltes te verlagen tot een zeer laag niveau.

Curriculum Vitae

De auteur, geboren op 27 juli 1946 te Nieuwe-Tonge (Z-H), behaalde in 1964 het diploma HBS-B aan het Christelijk Lyceum te Alphen aan de Rijn. In 1964 begon hij vervolgens met zijn studie aan de Landbouwhogeschool te Wageningen en in 1972 studeerde hij af in de studierichting Cultuurtechniek (specialisatie Waterzuivering). Het vakkenpakket in de ingenieursfase van deze studie omvatte de cultuurtechniek (specialisatie waterzuivering), de technologie, de microbiologie en de algemene en sociale gezondheidszorg. Sinds 1972 is de auteur werkzaam bij de in Nieuwegein gevestigde Hoofdafdeling Speurwerk van het Keuringsinstituut voor Waterleidingartikelen, KIWA N.V..