APPLIED MICROBIOLOGY, Aug. 1968, p. 1191-1199

Vol. 16, No. 8
Printed in U.S.A.

Attached Growth of *Sphaerotilus* and Mixed Populations in a Continuous-flow Apparatus[1]

F. F. DIAS,[2] NORMAN C. DONDERO[3], AND M. S. FINSTEIN

Department of Environmental Sciences, Rutgers, The State University, New Brunswick, New Jersey 08903

Received for publication 13 May 1968

The effects of NH_4Cl concentration, organic nitrogen compounds, glucose concentration, dissolved oxygen concentration, and flow rate on the attached growth of pure cultures of *Sphaerotilus natans* and of a mixed population in a continuous-flow apparatus are described. Low concentrations of NH_4Cl and oxygen, and high flow rates resulted in attached populations that were dominated by *Sphaerotilus*. The conditions that allowed maximal attached growth in pure culture did not correspond to the conditions that promoted attached growth of *Sphaerotilus* in a mixed population.

The term "sewage fungus" describes a group of organisms comprised of bacteria and protozoa, as well as true fungi, that grow attached to surfaces in polluted water courses. Butcher (3) defined the term as "the community of polysaprobic organisms with a fungus-like growth." The attached growth has also been referred to as a "slime infestation" (8). Probably the most important form of slime infestation, if not the most common as well, is that caused by *Sphaerotilus natans*, a bacterium in which the individual rod-shaped cells are encased in filamentous sheaths. The nature and economic significance of *Sphaerotilus* have been reviewed (6, 8).

Interest in *Sphaerotilus* stems also from its frequent association with failures in the treatment of waste waters by the activated-sludge method. Profuse growth of the filamentous *Sphaerotilus* is found in some types of bulking activated sludges that do not settle.

The environmental factors that allow *Sphaerotilus* to grow massively in successful competition with other members of the biota are not clear, although a number of suggestions have been made (6, 8). The goal of our investigations was to elucidate these factors, and this paper gives the results we have obtained. These investigations are presented at this time, also, because they show the usefulness of the continous-flow technique in studying the ecology of attached aquatic organisms, both in pure culture and in mixed populations. We have previously used the continuous-flow apparatus to study the nutrition of *Sphaerotilus* (5; Dias and Dondero, Bacteriol. Proc., p. 48, 1967).

[1] Paper of the Journal Series, New Jersey Agricultural Experiment Station, Rutgers, The State University, New Brunswick, N.J.

[2] Present address: Anil Starch Products, Ahmedabad, India 2.

[3] Present address: Department of Food Science, Cornell University, Ithaca, N.Y. 14850.

MATERIALS AND METHODS

The continuous-flow apparatus, comprised of 12 culture vessels, has been described (5). It must be emphasized that we are not dealing with continuous cultures in the usual sense. In our experiments, we are interested in the growth that occurs attached to the culture vessels (a situation that must be avoided in conventional continuous cultures).

The culture vessels, each containing 150 ml of medium, were inoculated with the desired amounts and types of inocula (*S. natans*, sewage, or both), and growth was allowed to proceed batchwise for 24 hr. The flow of medium at the desired rate was then started and allowed to continue until the requisite amount had passed through. The attached growth was harvested and estimated as described previously (5, 14). The values recorded in the Results represent the total attached growth adhering to all surfaces of the culture vessel, including the sparger and the magnetic stirring bar. For convenience and clarity, details are given with each experiment.

All experiments were conducted at 20 C. Gases, when used, were sparged through the medium in each culture vessel at the rate of 400 ml/min.

Strain 47 of *S. natans* was obtained from lyophilized stocks (7). After revival, the culture was maintained at 20 C in CGY broth (14). When used as inoculum, the culture was grown in 5% CGY broth (5 ml of CGY broth diluted to 100 ml with water), at 20 C, on a rotary shaker for 24 to 36 hr. A 10-ml amount of this culture was added to each culture vessel. When

TABLE 1. *Effect of NH_4Cl concentration on the amount of attached growth of Sphaerotilus natans in the continuous-flow apparatus*

Expt	NH_4Cl concn in medium[a]	Attached growth		
		Dry wt	Nitrogen	
			Amt	Per cent (w/w)
	mg/liter	*mg*	*mg*	
1	0.05	4.4	0.18	4.1
	0.1	5.5	0.2	3.6
	0.2	10.6	0.37	3.6
	0.4	17.6	0.64	3.6
2	0.5	26.5	0.86	3.3
	2.5	45.0	2.23	4.9
	5.0	44.9	3.2	7.1

[a] Added to the basal medium containing 50 mg of glucose/liter. The flow rate was 180 ml/hr, and 9 liters was passed through each culture vessel.

TABLE 2. *Effect of NH_4Cl concentration and organic nitrogen compounds on the amount of attached growth of mixed populations in the continuous-flow apparatus*

NH_4Cl concn in medium[a]	Additional nitrogen source[b]	Attached growth		
		Dry wt	Nitrogen	
			Amt	Per cent (w/w)
mg/liter		*mg*	*mg*	
0.2	None	13.6	0.46	3.4
1.0	None	40.3	1.36	3.4
5.0	None	45.1	3.24	7.2
10.0	None	49.5	5.62	11.4
10.0	Casitone	63.1	7.9	12.5
10.0	Sodium glutamate	56.0	6.64	11.5
10.0	L-Methionine	52.8	6.23	11.9

[a] Added to the basal medium containing 50 mg of glucose/liter. The flow rate was 180 ml/hr, and 9 liters was passed through each culture vessel.

[b] Added at a concentration of 40 mg/liter of medium.

mixed populations were studied, the culture vessels received 10 ml of settled sewage in addition. The sewage was allowed to become stale for 24 hr at 20 C prior to use.

Basal medium refers to the following: $MgSO_4$ (0.1 mM), $CaCl_2$ (0.1 mM), $FeCl_3$ (0.01 mM), phosphate buffer (K^+, *p*H 7.2, 1 mM), vitamin B_{12} (5 μg/liter), micronutrient solution [(4) 0.001 ml/liter], and $Na_2MoO_4 \cdot 2H_2O$ (0.005 mg/liter), in deionized, distilled water. The buffer was autoclaved separately and added to the cool, sterile medium prior to use. Various additions to this basal medium are described below. Glucose, when used, was autoclaved separately as a 20% (w/v) solution.

Dissolved oxygen was measured by the azide modification of the iodometric method (2). The dissolved-oxygen values were obtained for media collected at the waste-medium (effluent) end of the system.

It should be noted that the term slime, as used in this paper, refers to attached microbial growth without the connotation of production of extracellular mucoid polymeric substances or reference to any anatomical structure.

RESULTS

Effect of nitrogen nutrition. Pure-culture *Sphaerotilus* slimes contained different amounts of nitrogen, depending on the amount of nitrogen in the medium (Table 1). The amount of nitrogen in the slime ranged from 3.2 to 7.1%. All cultures, irrespective of their nitrogen content, contained normally sheathed cells.

The nitrogen content of mixed-population slimes also varied with the amount of NH_4Cl in the medium (Table 2). The types of organisms in the slime were greatly influenced by the amount of nitrogen in the medium. At NH_4Cl concentrations of 1 and 0.2 mg/liter, the slimes contained large numbers of *Sphaerotilus* filaments and could be described as *Sphaerotilus* infestations (Fig. 1 and 2). However, when the concentration of NH_4Cl was increased to 5 mg/liter, the dominance of *Sphaerotilus* was no longer evident (Fig. 3).

The addition of organic nitrogen compounds resulted, in some instances, in an increased amount of attached growth (Table 2) due, no doubt, to the increased amount of available carbon. However, the three compounds tested did not have any selective influence on the growth of *Sphaerotilus* (Fig. 4 and 5).

Effect of glucose concentration. The weight of the attached growth of *Sphaerotilus*, as was expected, increased with mounting glucose concentrations within the range of concentrations tested (Table 3).

The amount of mixed-population slime also increased with glucose concentrations, the increase being linear up to a glucose concentration of 50 mg/liter (Table 4). This weight was higher than that recorded for pure-culture slimes (25 mg/liter). Interestingly, the mixed-population slimes developed from media containing less glucose appeared to have greater numbers of filamentous *Sphaerotilus* (Fig. 6–8). In this case, unlike the low-nitrogen slimes, the dominance of *Sphaerotilus* was not overwhelming.

Effect of oxygen concentration. A decrease in the amount of dissolved oxygen from 7.8 mg/liter to 6.9 mg/liter had no influence on the amount of attached growth of *Sphaerotilus* (Table 5). The amount of growth produced when the amount of dissolved oxygen was decreased to 1

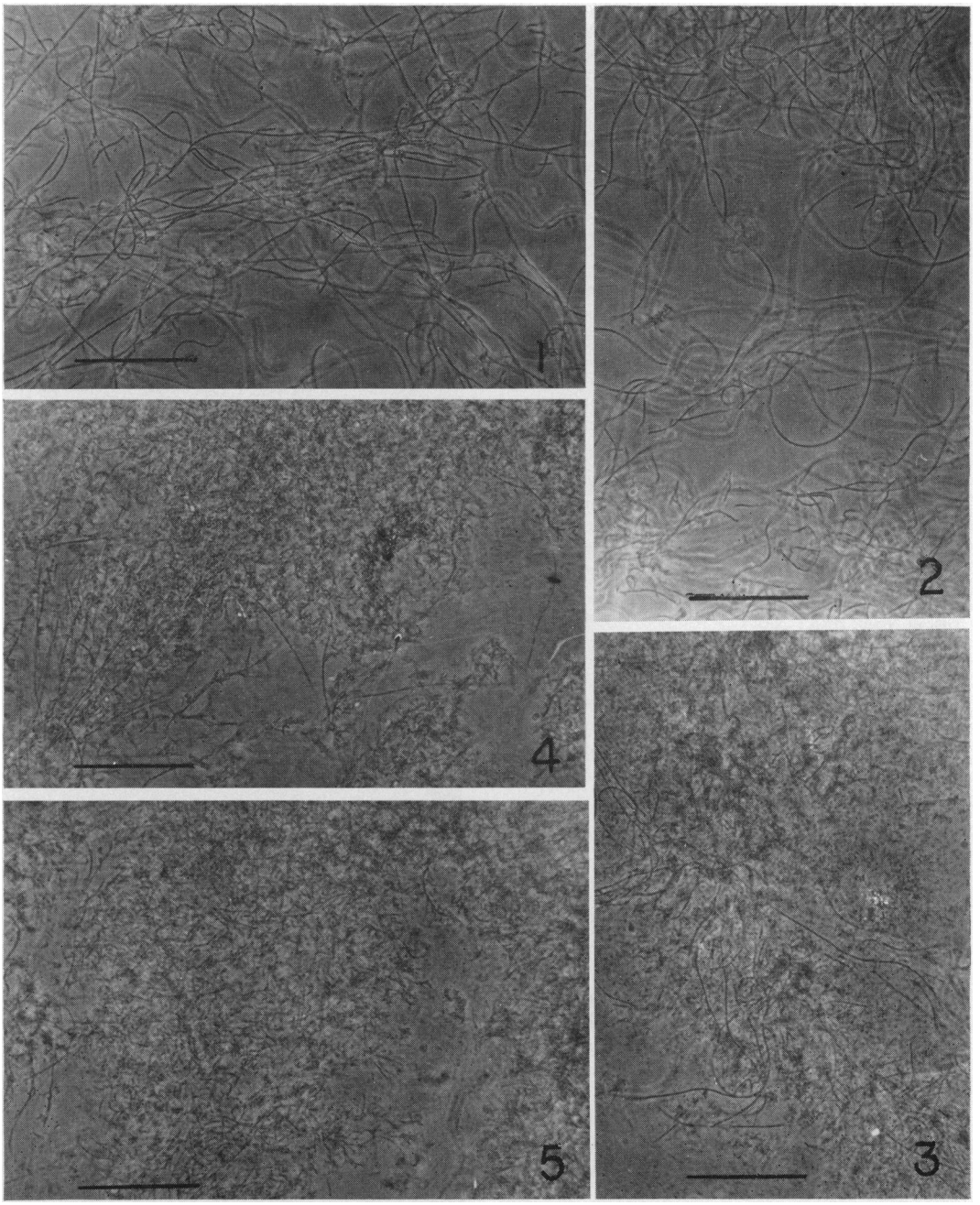

FIG. 1–5. *Effect of NH_4Cl concentration and organic nitrogen compounds on the nature of the slime developing in the continuous-flow apparatus. The inoculum was a mixture of Sphaerotilus natans and sewage. The line represents 100 μ. Figure 1: 0.2 mg of NH_4Cl/liter of medium; Fig. 2: 1 mg of NH_4Cl/liter of medium; Fig. 3: 5 mg of NH_4Cl/liter of medium; Fig. 4: 10 mg of NH_4Cl/liter and 40 mg of casitone/liter of medium; Fig. 5: 10 mg of NH_4Cl/liter and 40 mg of sodium glutamate/liter of medium.*

mg/liter was greatly reduced. The growth at the reduced oxygen tension was comprised of filamentous sheathed cells that were indistinguishable from cells grown at the higher oxygen tension. The attached growth of mixed populations was reduced when the amount of dissolved oxygen was decreased from 7.8 to 6.9 mg/liter (Table 5). However, the amount of attached growth pro-

TABLE 3. *Effect of glucose concentration on the attached growth of Sphaerotilus natans in the continuous-flow apparaus*

Glucose concn[a]	Attached growth		
	Dry wt	Nitrogen	
		Amt	Per cent (w/w)
mg/liter	*mg*	*mg*	
10	9.8	1.14	11.6
25	27.8	2.41	8.7
50	38.1	3.11	8.2
100	54.5	4.02	7.4

[a] Added to the basal medium containing 10 mg of NH_4Cl/liter. The flow rate was 180 ml/hr, and 9 liters was passed through each culture vessel.

TABLE 4. *Effect of glucose concentration on the attached growth of mixed populations in the continuous-flow apparatus*

Glucose concn	NH_4Cl concn[a]	Attached growth		
		Dry wt	Nitrogen	
			Amt	Per cent (w/w)
mg/liter	*mg/liter*	*mg*	*mg*	
5	1	11.5	1.18	10.3
10	2	14.8	1.56	10.5
10	10	16.8	1.97	11.8
25	5	31.1	3.48	11.2
50	10	55.0	6.2	11.3
100	20	69.6	7.53	10.9

[a] Added to basal medium. The flow rate was 180 ml/hr, and 9 liters of medium was fed through each culture vessel.

duced by the mixed population when the amount of dissolved oxygen was decreased to 1 mg/liter was greater than that produced by *Sphaerotilus* alone. Microscopically, the mixed-population slime developed at 1 mg/liter contained a larger proportion of filamentous *Sphaerotilus* (Fig. 9) than did the slimes developed at the higher oxygen concentrations (Fig. 10). The slimes developed at an oxygen concentration of 1 mg/liter did not contain either protozoa or fungi. On the other hand, the slimes growing at the higher oxygen concentrations contained bacteria, protozoa, and a fungus. This is the only experiment in the series using synthetic media in which a fungus was selected. The proportion of fungi was not as overwhelming, however, as is indicated by Fig. 10 or as was encountered in the experiment described below in which sewage was used. The reason for the enriched growth of the fungus is not clear; the *p*H of the effluent did not differ from that in other experiments. When this experiment was repeated, fungus was not encountered; however, the results with respect to the selection of *Sphaerotilus* were identical.

Effect of flow rate. Pure cultures of *Sphaerotilus* accumulated more attached growth from 9 liters of medium at lower flow rates (Table 6). However, the rate of growth was slower at the lower flow rates. This was proven by the quantity of attached

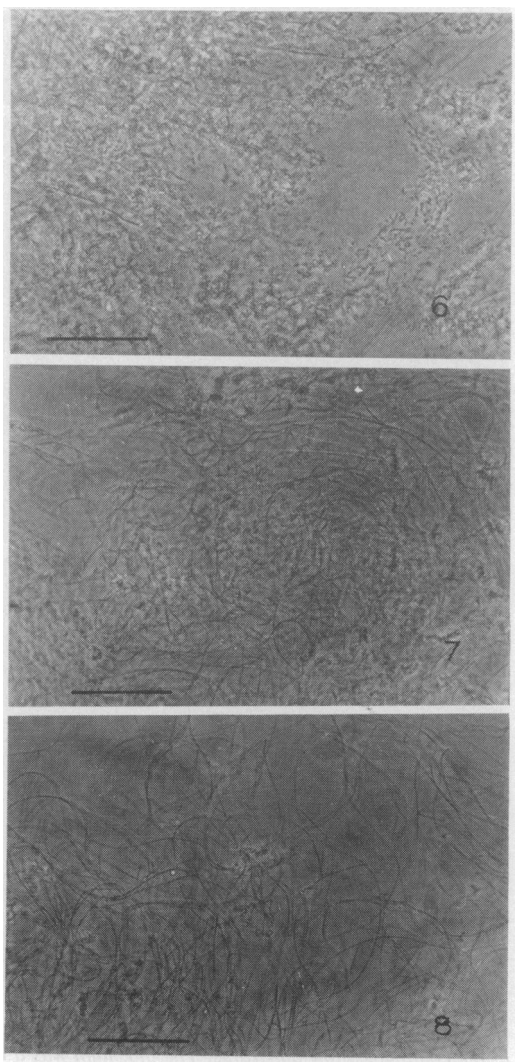

FIG. 6–8. *Effect of glucose concentration on the type of slime developing in the continuous-flow apparatus. The inoculum was a mixture of Sphaerotilus natans and sewage. The line represents 100 μ. Figure 6: 100 mg of glucose/liter; Fig. 7: 25 mg of glucose/liter; Fig. 8: 5 mg of glucose/liter.*

TABLE 5. *Effect of oxygen concentration on the attached growth of Sphaerotilus and mixed populations*

Gas passed through medium[a]	Dissolved oxygen[b]	Attached growth			
		Sphaerotilus		Mixed population	
		Dry wt	Nitrogen	Dry wt	Nitrogen
	mg/liter	*mg*	*mg*	*mg*	*mg*
None.....	6.9	80	5.5	36	4.1
Air.....	7.8	83	5.5	60	6.2
Nitrogen....	1.0	11	0.7	25	2.7

[a] Basal medium containing 10 mg of NH_4Cl/liter and 100 mg glucose/liter. The flow rate was 180 ml/hr, and each culture vessel received 9 liters of medium. During the initial 24-hr batch-culture growth of the culture before flow of medium was started, no gases were sparged through the medium.

[b] The dissolved oxygen in the effluent emerging from the continuous-culture vessels which had received no inoculum. The dissolved oxygen concentration in the effluent of inoculated culture vessels, collected a few hours before growth was harvested, was essentially the same in the case of the aerated units. It was depleted by about 1 mg/liter for the mixed cultures which were not aerated or which were gassed with nitrogen. No observations were made in the case of the *Sphaerotilus* cultures which were not aerated or which were sparged with nitrogen.

growth harvested after a 20-hr period. At the lower flow rate, less medium had passed through the culture vessels and the amount of attached growth was less than that achieved at a higher flow rate (Table 7).

By contrast, the flow rate did not have as great influence on the attached growth of a mixed population (Table 6). Slightly more growth occurred at a flow rate of 180 ml/hr than at either 90 or 250 ml/hr. The lower flow rate, as was expected, allowed a greater quantity of unattached growth; this amount was not estimated. Qualitatively, the slimes produced at the higher flow rates (180 and 250 ml/hr) had *Sphaerotilus* filaments. The higher flow (250 ml/hr) produced the larger number of filamentous *Sphaerotilus* (Fig. 11). The slime developed at a flow rate of 90 ml/hr was free of *Sphaerotilus* filaments (Fig. 12).

Experiments with sewage. These experiments were done during the earlier phases of this study and, although no quantitative data were recorded, the observations are presented here because they demonstrate the usefulness of the continous-flow apparatus in the study of microbial ecology. The medium used in this study was settled domestic sewage diluted with water (1:20, v/v; 5-day biochemical oxygen demand, about 20 mg/liter) and fortified with glucose (20 mg/liter). The inoculum was activated sludge (10 ml) that contained visible *Sphaerotilus* filaments.

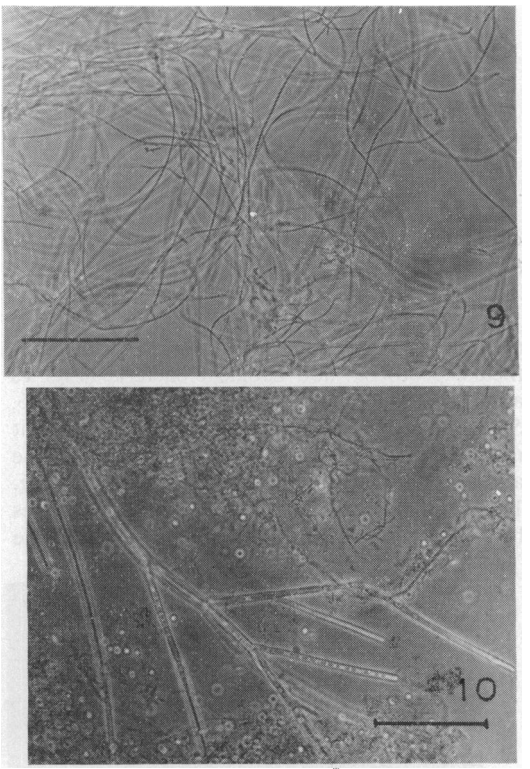

FIG. 9–10. *Effect of dissolved oxygen concentration on the nature of the slime developing in the continuous-flow apparatus. The inoculum was a mixture of Sphaerotilus natans and sewage. The line represents 100 μ. Figure 9: 1 mg of dissolved oxygen/liter; Fig. 10: 7.8 mg of dissolved oxygen/liter.*

TABLE 6. *Effect of flow rate on the attached growth of Sphaerotilus natans and mixed populations in the continuous-flow apparatus*

Flow rate of medium[a]	Attached growth			
	Sphaerotilus		Mixed population	
	Dry wt	Nitrogen	Dry wt	Nitrogen
ml/hr	*mg*	*mg*	*mg*	*mg*
250	60.4	4.46	41.6	4.33
180	71.5	4.14	61.7	6.18
90	138.9	10.34	42.6	4.82

[a] The medium was composed of the basal medium containing 10 mg of NH_4Cl/liter and 100 mg of glucose/liter; 9 liters of medium was passed through each culture vessel.

TABLE 7. *Effect of flow rate on the amount of attached growth of Sphaerotilus natans produced in the continuous-flow apparatus fed varying amounts of medium*

Flow rate of medium[a]	Amt of medium fed through each culture vessel in 20 hr	Attached growth	
		Dry wt	Nitrogen
ml/hr	*liters*	*mg*	*mg*
180	9.0	38.1	3.11
145	7.0	36.4	3.12
90	4.5	12.5	1.2

[a] The medium was made up of the basal medium containing 10 mg of NH_4Cl/liter and 50 mg of glucose/liter.

The slime that developed in the above medium (*p*H 7.4) had large numbers of *Sphaerotilus* filaments, protozoa, and zoogleal bacteria (Fig. 13 and 14). When the *p*H was adjusted to 5.6, *Sphaerotilus* filaments no longer were evident (Fig. 15). When the *p*H was decreased to 4.5, the attached slime was dominated by a fungus (Fig. 16), tentatively identified as a *Trichosporon* species by William B. Cooke. Attempts to study the growth of this fungus in pure culture were not successful because unequal sloughing of the attached growth resulted in poor quantitative replicability.

DISCUSSION

An unexpected observation, in the light of earlier reports on the bulking of activated sludges (9–12, 15, 16), was that increased levels of glucose did not result in the selection of *Sphaerotilus*. In fact, the opposite seemed to be the case, although the results were not entirely clear. It is a well-documented fact (15, 16) that the addition of carbohydrates to normal activated sludge results in *Sphaerotilus* bulking. Our results indicate that this is probably due to nitrogen-deficient conditions rather than the presence of carbohydrates per se. This has been indicated also by the work of Ingols and Heukelekian (12) and by that of Hattingh (9). However, it should be noted that *Sphaerotilus* infestations have also been described under conditions of nitrogen abundance [e.g., streams receiving ammonia-base spent sulfite liquor (1)]. In addition to its enriched growth in low nitrogen concentration, reported herein,

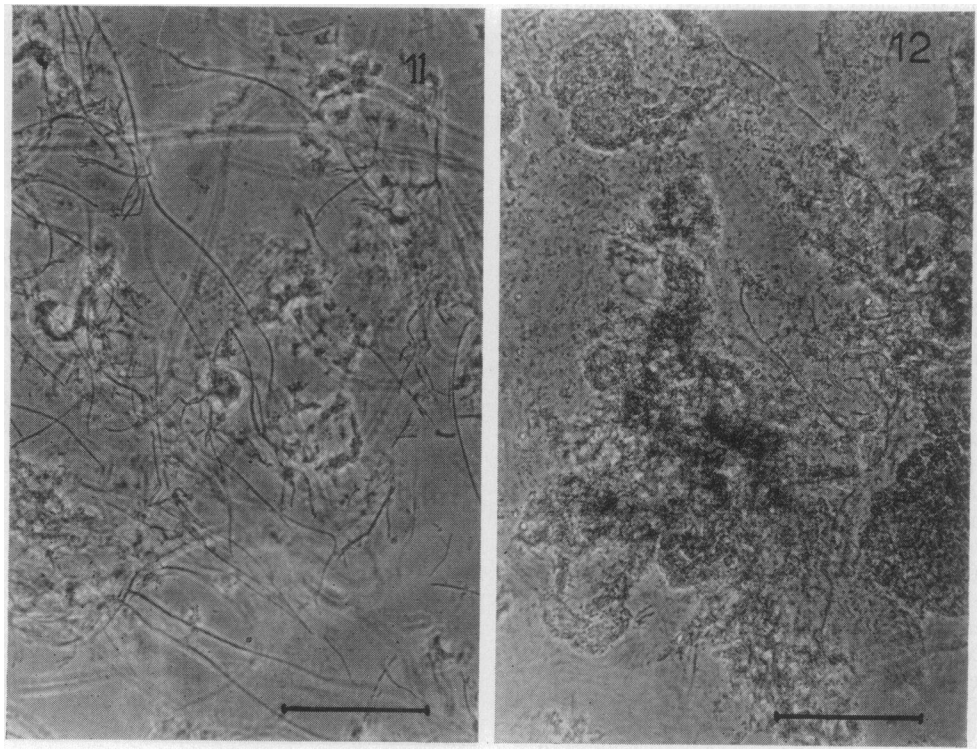

FIG. 11–12. *Effect of flow rate on the nature of the slime developing in the continuous-flow apparatus. The inoculum was a mixture of sewage and Sphaerotilus natans. The line represents 100 μ. Figure 11: 250 ml/hr; Fig. 12: 90 ml/hr.*

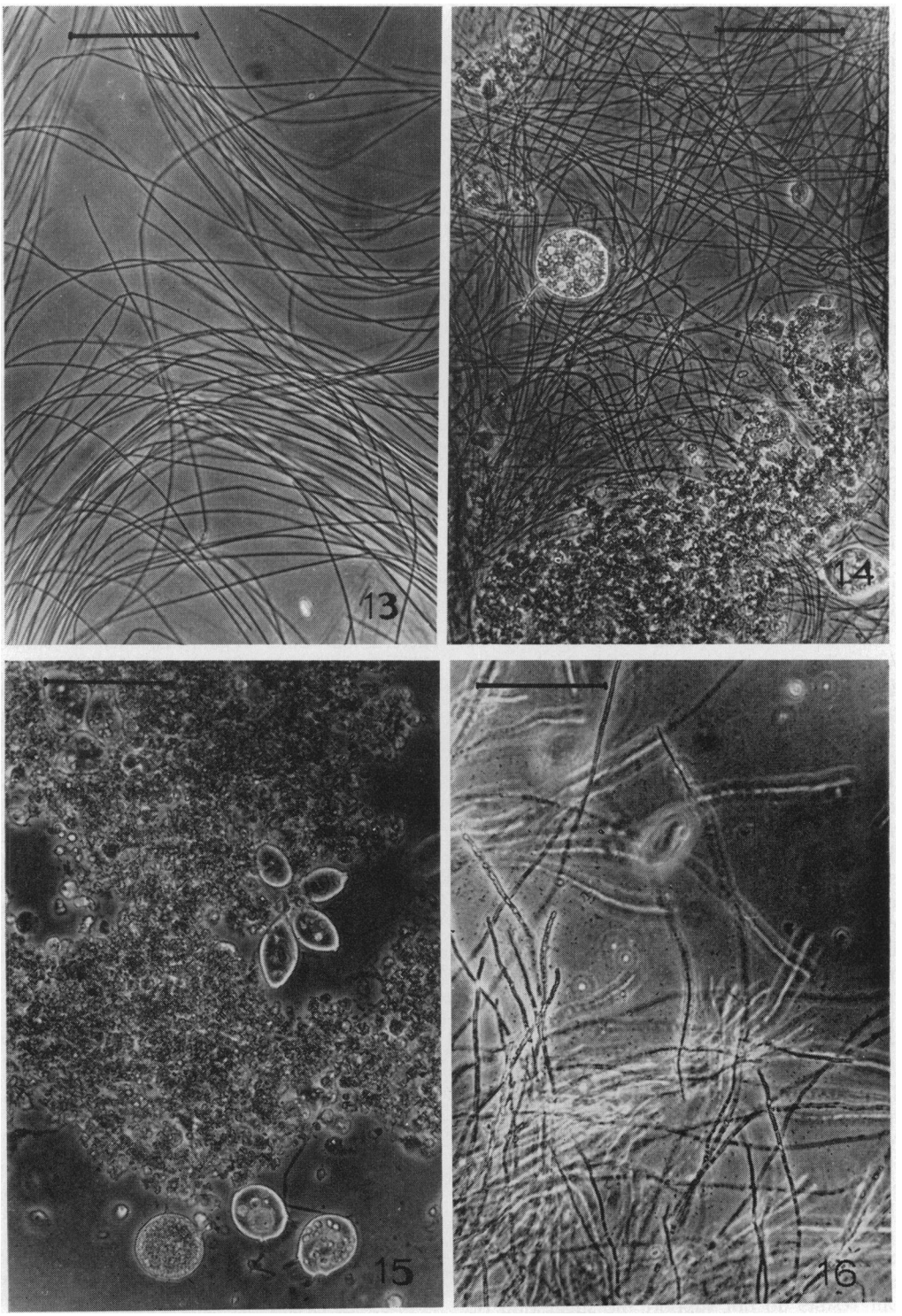

FIG. 13–16. *Nature of the attached growth developing in the continuous-flow apparatus fed a dilute sewage fortified with glucose and adjusted to different pH values. The inoculum was activated sludge. Figures 13–14: pH 7.4; Fig. 15: pH 5.6; Fig. 16: pH 4.5.*

Sphaerotilus has been reported to thrive when the milieu is deficient in phosphorus (9).

The observation that initial low oxygen tension favors growth of *Sphaerotilus* is probably due to its ability to grow at reduced oxygen tension. The observation that a small decrease in dissolved oxygen concentrations has no effect on pure *Sphaerotilus* slimes while greatly affecting the amount of attached growth of a mixed population points in this direction. The results also reveal that very low oxygen concentrations are not conducive to the growth of pure cultures of *Sphaerotilus*. The observation that very low oxygen concentrations seem to promote growth of *Sphaerotilus* in a mixed population is possibly an indication that the very low oxygen concentrations are even less favorable for the other bacteria that grow attached. The results do not permit one to determine the minimal oxygen concentration that will allow maximal growth of *Sphaerotilus*. Mulder (13) reported that *Sphaerotilus* is not affected by changes in the rate of aeration (shake versus stationary-batch cultures) as are certain *Arthrobacter* species isolated from waste waters.

The experiments with flow rate showed that results obtained with pure *Sphaerotilus* cultures do not necessarily represent the behavior of *Sphaerotilus* growing in association with other organisms. Changes in flow rate have a much greater influence on the amount of attached growth of pure *Sphaerotilus* slimes than with mixed-population slimes because, at low flow rates and with mixed populations, a large number of organisms grow in an unattached state and are able to compete for food with the attached slime. At higher flow rates, the unattached organisms are washed out of the system. However, if the flow rate is increased beyond a certain limit, the detention time of nutrients is too small to allow efficient utilization, and the amount of attached growth produced is less per unit of medium fed. At low detention periods, the ability of *Sphaerotilus* to be retained in the growth chamber as tassels puts it in a more advantageous position, and *Sphaerotilus* dominates.

The above results on the effect of flow rate on attached growth are at variance with those recorded by Amberg and Cormack (1). This difference is easily explained by the very low nutrient concentrations used in their experiments. Under such conditions, one would obtain an increase in attached growth with increased flow rates. Amberg and Cormack's results have greater relevance to conditions in streams and rivers.

Our results do not support the suggestion of A. D. Adamse (Ph.D. Thesis, Agricultural University, Wageningen, The Netherlands, 1966) that organic nitrogen sources favor the growth of *Sphaerotilus*. Dias and Heukelekian (5) showed that *S. natans* utilizes inorganic nitrogen compounds as readily as it does organic nitrogen compounds.

We have not been able to secure identical growth yields in the various experiments even though identical cultural conditions were employed. However, preliminary experiments showed that the coefficient of variation among the 12 culture vessels treated identically in the same experiment was about 10%; therefore, all conclusions drawn were based on results obtained in the same experiment.

The successful utilization of the continuous-flow system is propably the most significant observation of this investigation. Continous-flow systems have been used to study mixed populations, notably, those described by Wuhrmann (17). However, the open channels used by most investigators probably are more difficult to control and are difficult, if not impossible, to use for pure-culture studies. Our continuous-flow system is fairly simple, and could be used in any microbiology laboratory. It is amenable to the study of pure cultures as well as mixed populations. It should be possible to study not only the sessile bacteria but other organisms, such as stalked ciliate protozoa, as well. One disadvantage of our apparatus is that the medium has an appreciable detention time in the culture vessels, a departure from conditions obtaining in a stream. On the other hand, by altering the stirring rate, the effects of velocity of flow can be studied apart from the rate of feeding.

In conclusion, various environmental conditions were found to enhance the growth of *Sphaerotilus* in a mixed population. The common occurrence of this organism is probably a result of the many cultural and nutritional attributes that give the organism a competitive advantage over other aquatic organisms.

ACKNOWLEDGMENTS

This investigation was supported by research grant WP 212-07 from the U.S. Department of the Interior, Federal Water Pollution Control Administration.

We thank William B. Cooke for identifying the fungus.

LITERATURE CITED

1. Amberg, H. A., and J. F. Cormack. 1960. Factors affecting slime growth in the lower Columbia river and evaluation of some possible control measures. Pulp Paper Mag. Can. **61**:70–80.
2. American Public Health Association. 1965. Standard methods for the examination of water and waste water, 12th ed. American Public Health Association, Inc., New York.
3. Butcher, R. W. 1932. Contributions to our knowledge of the ecology of sewage fungus. Trans. Brit. Mycol. Soc. **17**:112–123.

4. Dias, F., and J. V. Bhat. 1962. A new levan producing bacterium, *Corynebacterium laevaniformans*, nov. spec. Antonie van Leeuwenhoek J. Microbiol. Serol. **28**:63–72.
5. Dias, F. F., and H. Heukelekian. 1967. Utilization of inorganic nitrogen compounds by *Sphaerotilus natans* growing in a continuous-flow apparatus. Appl. Microbiol. **15**:1083–1086.
6. Dondero, N. C. 1961. *Sphaerotilus*, its nature and economic significance. Advan. Appl. Microbiol. **3**:77–107.
7. Dondero, N. C., R. A. Phillips, and H. Heukelekian. 1961. Isolation and preservation of cultures of *Sphaerotilus*. Appl. Microbiol. **9**:219–227.
8. Harrison, M. E., and H. Heukelekian. 1958. Slime infestation. Literature review. Sewage Ind. Wastes **30**:1278–1302.
9. Hattingh, W. H. J. 1963. Activated sludge studies. 3. Influence of nutrition on bulking. Water Waste Treat. J. **9**:474–480.
10. Heukelekian, H. 1941. Activated sludge bulking. Sewage Works J. **13**:39–42.
11. Ingols, R. S., and H. Heukelekian. 1939. Studies on activated sludge bulking. I. Bulking of sludge by means of carbohydrates. Sewage Works J. **11**:927–945.
12. Ingols, R. S., and H. Heukelekian. 1940. Studies on activated sludge bulking. II. Bulking induced by domestic sewage. Sewage Works J. **12**:694–714.
13. Mulder, E. G. 1964. Iron bacteria, particularly those of the *Sphaerotilus-Leptothrix* group, and industrial problems. J. Appl. Bacteriol. **27**:151–173.
14. Okrend, H., and N. C. Dondero. 1964. Requirement of *Sphaerotilus* for cyanocobalamin. J. Bacteriol. **87**:286–292.
15. Ruchhoft, C. C., and J. F. Kachmar. 1941. Studies of sewage purification. XIV. The role of *Sphaerotilus natans* in activated sludge bulking. Sewage Works J. **13**:3–32.
16. Smit, J. A study of conditions favouring "bulking" of activated sludge. Sewage Works J. **4**:960–972.
17. Wuhrmann, K. 1964. River bacteriology and the role of bacteria in self purification of rivers. *In* H. Heukelekian and N. C. Dondero (ed.). Principles and applications in aquatic microbiology. John Wiley & Sons, Inc., New York.