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Phototrophic Purple and Green Bacteria in a Sewage Treatment Plant

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In all purification stages of a biological sewage treatment plant, phototrophic bacteria were detected by the method of viable cell counts. The predominant species identified belonged to the genus *Rhodopseudomonas* of purple nonsulfur bacteria. The number of phototrophic bacteria was highest in wastewater containing sludge. In activated sludge, an average of 10^5 viable cells/ml was found; the number depended upon concentration of sludge rather than on seasonal changes in light conditions in the course of a year. Bacteriochlorophyll a was extracted from activated sludge. Relative to the viable counts of phototrophic bacteria, the content of bacteriochlorophyll a was 5- to 10-fold higher than that of three representative pure cultures. By incubation of activated and digester sludge under different environmental conditions, it was shown that phototrophic bacteria can compete with other bacteria only under anaerobic conditions in the light.

Phototrophic purple and green bacteria are found in nearly all aquatic environments. Purple or green sulfur bacteria can be easily recognized when they form water blooms. Purple nonsulfur bacteria, however, rarely appear in visible concentrations (19). Their distribution in nature, therefore, can only be evaluated from results obtained by enrichment techniques (8) or the membrane filter method (1, 17).

The presence of purple bacteria is particularly dependent upon the degree to which water is polluted by organic matter. Their growth contributes to the purification of heavily polluted water exposed to sunlight, as, for example, in sewage lagoons (4, 7). In Japan, phototrophic bacteria are used in the main purification stage of organic wastewater treatment (9-11).

The occurrence of phototrophic bacteria in conventional organic wastewater purification plants has not been previously studied. This paper presents viable cell counts and identification of phototrophic purple and green bacteria in all aerobic and anaerobic purification stages at the sewage treatment plant in Göttingen (West Germany). Additionally, experiments were carried out to determine which type of metabolism (phototrophic, respiratory, or fermentative) is used by the phototrophic bacteria to compete with other bacteria in the different parts of the sewage plant.

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MATERIALS AND METHODS

Agar media. The medium for purple nonsulfur bacteria contained, per liter: 2.0 g of disodium succinate (anhydrous); 0.5 g of KH_2PO_4; 0.4 g of $MgSO_4 \cdot 7H_2O$; 0.4 g of NH_4Cl; 0.5 g of $CaCl_2 \cdot 2H_2O$; 2.0 g of yeast extract (Difco); 0.5 ml of ethanol; 5.0 ml of iron(III)-citrate solution (100 mg/100 ml); 10 ml of trace element solution (14); 12 g of agar (Difco). The pH was adjusted before autoclaving to 6.9.

The medium for purple and green sulfur bacteria was prepared as described by Pfennig and Lippert (14).

Method for viable cell counts. (i) Purple nonsulfur bacteria. Sludge samples were first agitated for 10 min by a Vibromixer (Fa. Sartorius) to obtain homogeneous suspensions. Serial dilutions (1:10) were made in sterile 0.2% NaCl. From each of three suitable dilution steps, 1 ml was pipetted into 100 ml of liquefied agar medium at 40°C. After the agar was mixed it was distributed into four petri dishes. The plates were incubated in tall glass jars under an atmosphere of 95% H_2 and 5% CO_2 (28 to 30°C, 2,000 to 3,000 lx). Colonies were counted after 10 to 14 days of incubation.

(ii) Green and purple sulfur bacteria and purple nonsulfur bacteria. Serial dilutions (1:10) of homogeneous sludge suspension were prepared in agar shake tubes according to the method of Pfennig (13) and were sealed with paraffin-paraffin oil (1:3). The agar tubes were incubated for 14 days at 28°C and 2,000 lx of incandescent light. Phototrophic sulfur and nonsulfur bacteria in digester sludge were counted using agar shake tubes with the medium of Pfennig (13), supplemented by 0.05% acetate, and the vitamin solution of Pfennig and Lippert (14).

Samples. Samples were obtained at the Göttingen sewage treatment plant between 8:00 and 9:00 a.m. in sterile 250-ml bottles. The analysis was started within 1 h after collection. In the case of activated sludge samples, the bottles were not completely filled to avoid anaerobic conditions.

Test for decomposition of gelatin. Gelatin was added to the agar medium for purple nonsulfur bacteria to a final concentration of 0.4%. When colonies of the test strains had developed, the plates were covered with a solution containing 1.5 g of $HgCl_2$ and 2 ml of 12 M HCl in 10 ml of distilled water. The appearance of a clear zone around the colonies indicated hydrolysis of gelatin.

Experiments with sludge samples under different culture conditions. (i) Activated sludge. Wastewater from the primary sedimentation tank was added to an equal volume of activated sludge. Three hundred-milliliter amounts of this mixture were incubated in 1,000-ml Erlenmeyer flasks on a rotary shaker at 150 rpm for 2 weeks at 28°C. Anaerobic flasks were flushed with N_2 and closed with rubber stoppers. For aerobic cultivation, fluted Erlenmeyer flasks capped with felt cloth were used. Illuminated cultures were incubated at 2,000 lx of incandescent light.

(ii) Digester sludge. Digester sludge or digester sludge-raw sludge (1:1) was diluted with distilled water (1:1). Four 200-ml portions of diluted sludge were incubated in 1,000-ml Erlenmeyer flasks. The flasks were flushed with N_2 and closed with rubber stoppers, which contained an air seal filled with paraffin oil to compensate for gas pressure. The flasks were incubated at 28°C for 4 weeks on a rotary shaker at 150 rpm. For illuminated flasks, a light intensity of 2,000 lx was used.

Extraction of bacteriochlorophyll a from activated sludge. Two liters of activated sluge was centrifuged, and the pellet was resuspended in 100 ml of distilled water. To extract bacteriochlorophyll a, 900 ml of acetone-methanol (7:2) was added and stirred for 1 h in the dark at +4°C. Before centrifugation, 25 ml of 12 M HCl was added to convert bacteriochlorophyll into stable magnesium-free bacteriopheophytin.

After centrifugation, 100 ml of carbon tetrachloride was added to the decanted supernatant. By addition of 1,000 ml of water, an acetone-methanol-water phase and a carbon tetrachloride phase were obtained. The latter phase, containing bacteriopheophytin and other lipid-soluble pigments, was separated and washed several times with distilled water. Remaining water was bound by the addition of anhydrous Na_2SO_4.

Chromatography. Bacteriopheophytin was separated from other pigments by column chromatography on silica gel (Mallinckrodt, 100 mesh) at 4°C. As solvent, carbon tetrachloride-acetone (85:15) was used.

The bacteriopheophytin fraction was further purified by repeated chromatography on silica gel plates (Merck, 5553). Mixtures of carbon tetrachloride and acetone in ratios between 85:11 and 97:3 were used as solvents. Bacteriopheophytin a was recovered from the plates by extraction of the silica gel fraction with carbon tetrachloride. Purified bacteriopheophytin a, isolated from *Rhodospirillum rubrum*, served as a reference.

Spectrophotometric determinations. Absorption spectra of pigments dissolved in carbon tetrachloride were measured with a Zeiss spectrophotometer (DMR 21).

The concentration of bacteriopheophytin a was estimated from the height of the peak at 760 nm. The extinction coefficient of bacteriopheophytin a in carbon tetrachloride was calculated from a calibration curve according to Thiele (thesis, University of Göttingen, Göttingen, West Germany, 1966): $\epsilon_{760} = 37$ liters/mmol · cm. The value for bacteriopheophytin a was multiplied by the factor 1.025 to obtain the corresponding bacteriochlorophyll a concentration.

Determination of bacteriochlorophyll a. Bacteriochlorophyll a was extracted from pure cultures of purple nonsulfur bacteria with acetone-methanol (7:2). Ten milliliters of cell suspension was centrifuged, the pellet was resuspended in 1 ml of distilled water, and 9 ml of solvent mixture was added. After 30 min at 4°C, the mixture was centrifuged. The absorbance of the supernatant was measured at 775 nm. The bacteriochlorophyll a concentration was calculated using the extinction coefficient $\epsilon_{775} = 75$ liters/mmol · cm given by Clayton (2).

RESULTS

Viable cell counts of phototrophic bacteria in different parts of the sewage treatment plant in Göttingen. Phototrophic bacteria were found in all purification stages of the biological sewage treatment plant in Göttingen, which was constructed by Loos and Preuss (12). Most of the species identified belonged to the purple nonsulfur bacteria (Table 1). A small percentage of purple and green sulfur bacteria was found in samples containing either activated sludge or sludge from the primary settling tank. The total number of phototrophic bacteria in these samples was high.

Phototrophic bacteria were found to be already present in the raw sewage of the inlet. During the mean residence time of about 4 h in the primary settling tank, the viable cell count increased, especially in the sedimented raw sludge. This sludge is subsequently digested in the methane digester. The mean residence time of the sludge in this digester is about 20 days. Table 1 shows that the number of phototrophic bacteria in the digester sludge was less than that in the raw sludge, indicating that the death rate of phototrophic bacteria under digester conditions cannot be compensated for by an assumed growth rate of any of the species.

The wastewater leaving the primary settling tank is purified in an aerobic activated sludge process. In this purification step the number of phototrophic bacteria increased about 10-fold (Table 1).

Phototrophic bacteria in activated sludge. To establish by which type of metabolism the phototrophic bacteria grow in activated sludge, the following experiments were performed.

TABLE 1. *Numbers of purple and green bacteria in the different tanks of the sewage treatment plant in Göttingen*

Sample	No. of samples	Colony counts/ml			
		Purple nonsulfur bacteria		Purple sulfur bacteria	Green sulfur bacteria
		Mean value	Range		
Raw sewage	1	1,700		0 (in 10 ml)	0 (in 10 ml)
Supernatant of the primary settling tank	2	8,100	7,350–8,910	50	0 (in 0.1 ml)
Settled raw sludge of the primary settling tank	5	116,000	4,100–550,000	0 (in 0.1 ml)	800
Digester sludge	5	16,000	100–80,000		0 (in 0.1 ml)
Activated sludge	15	100,000	2,900–650,000	1,000	1,300

(i) Determination of illumination conditions in the activated sludge tank. Anaerobic tube cultures, freshly inoculated with *Rhodopseudomonas capsulata*, were incubated for 3 weeks in different depths of the tank. During this period the weather was sunny. Light-dependent growth occurred only to a depth of about 10 cm below the surface of the activated sludge tank. It is apparent that anaerobic light-dependent growth of phototrophic bacteria must be strongly light-limited in the activated sludge tank, which has a depth of 3.8 m. We expected, therefore, that the number of phototrophic bacteria would vary with the different light conditions of the seasons.

(ii) Effect of seasonally changing light conditions. Phototrophic bacteria were counted monthly over 1 year. The numbers of phototrophic bacteria fluctuated during the year from 10^3 to more than 10^5 per ml, but the fluctuations were not in accordance with the changing light conditions throughout the year. Rather, it was found that the cell number increased with the sludge concentration: a linear relationship was obtained when the square root of the cell number was plotted against the dry-weight concentration of the activated sludge (Fig. 1b). The same correlation was observed for those chemoorganotrophic bacteria that grew under anaerobic conditions on the agar plates simultaneously with the phototrophic bacteria (Fig. 1a). The numbers of these chemoorganotrophic bacteria correlated strictly to the numbers of the phototrophic bacteria. It is reasonable to conclude, therefore, that growth of both photo- and chemoorganotrophic bacteria is favored by the microaerobic conditions at high sludge concentrations.

(iii) Detection of bacteriochlorophyll a in activated sludge and its concentration related to the viable counts of phototrophic bacteria. When pure cultures of purple nonsulfur bacteria grow aerobically as chemoorganotrophs, their bacteriochlorophyll synthesis becomes repressed by oxygen (3). To find out whether the purple nonsulfur bacteria in activated sludge contain bacteriochlorophyll or whether their bacteriochlorophyll formation is repressed by oxygen, the content of bacteriochlorophyll a of activated sludge was determined and related to the viable cell counts. The absorption spectra of the acid extract (Fig. 2a) show that activated sludge contains detectable amounts of bacteriochlorophyll a. Successive pu-

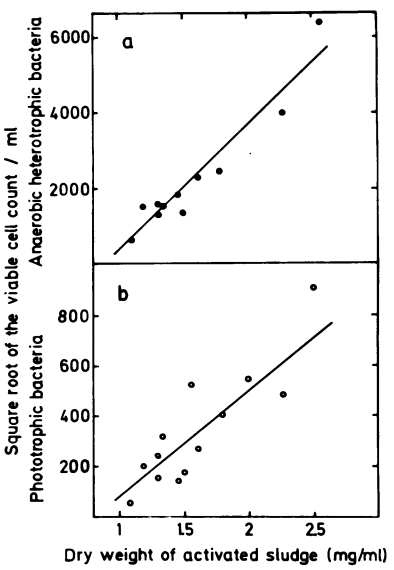

FIG. 1. *Dependence of colony counts of anaerobic heterotrophic (a) and phototrophic (b) bacteria on the varying concentrations of activated sludge within 1 year.*

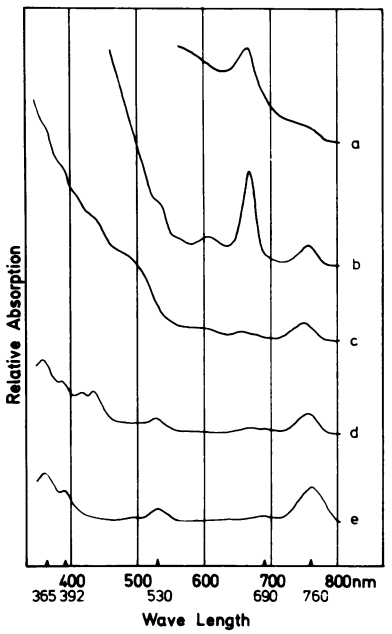

FIG. 2. *Spectrophotometric detection of bacteriopheophytin a in activated sludge extract and its chromatographic separation from other pigments. (a) Activated sludge extract; (b) after separation on a silica gel column; (c) after thin-layer chromatography; (d) after a second thin-layer chromatography; (e) absorption spectrum of pure bacteriopheophytin a (Thiele, thesis).*

rification of bacteriopheophytin a from other pigments, e.g., pheophytin of algae and carotenoids, provided evidence that the shoulder at 760 nm of spectrum a (Fig. 2) was caused by the absorption of bacteriopheophytin a.

When the bacteriopheophytin content of the activated sludge was related to the number of viable cells of phototrophic bacteria in the sludge, a theoretical bacteriochlorophyll content of these cells was obtained that was 5- to 10-fold higher than that of viable cells from pure cultures of *Rhodopseudomonas sphaeroides, R. capsulata,* and *R. gelatinosa,* which were grown anaerobically under light limitation (Table 2).

(iv) Determination of environmental conditions under which phototrophic bacteria multiply in activated sludge. Activated sludge was incubated aerobically or anaerobically, both in the light or in the dark. Phototrophic bacteria were found to grow only under anaerobic light conditions (Table 3). Both the viable cell count and the dry weight increased under such conditions, indicating photoassimilation of soluble organic compounds present in the wastewater by phototrophic bacteria. The viable cell counts decreased in anaerobically dark- and aerobically light- and dark-incubated activated sludge. The decrease was largest under aerobic dark conditions and least under anaerobic conditions. Also, a loss of activated sludge dry weight took place (Table 3). The decrease in the viable cell count was proportionally larger than could be expected from the mineralization of the sludge alone.

TABLE 2. *Bacteriochlorophyll a content of activated sludge and of pure cultures (isolated from activated sludge)*

Source of bacteriochlorophyll a	Bacteriochlorophyll a ($\mu g/10^5$ viable cells)
Activated sludge (29 Aug. 1972)	2.5×10^{-3}
Activated sludge (22 Sept. 1972)	1.9×10^{-3}
Activated sludge (4 Oct. 1972)	1.8×10^{-3}
R. capsulata[a]	0.62×10^{-3}
R. sphaeroides	0.48×10^{-3}
R. gelatinosa	0.15×10^{-3}

[a] Pure cultures were grown anaerobically under light limitation at 500 lx from a tungsten lamp.

TABLE 3. *Survival and growth of phototrophic bacteria in activated sludge incubated under different conditions*

Incubation	At the beginning		After 7 days	
	Phototrophic bacteria (colony counts/ml)	Dry wt of activated sludge (mg/ml)	Phototrophic bacteria (colony counts/ml)	Dry wt of activated sludge (mg/ml)
Light				
Aerobic	8.6×10^4	0.89	4.2×10^3	0.60
Anaerobic	8.6×10^4	0.89	1.8×10^7	0.99
Dark				
Aerobic	8.6×10^4	0.89	2.3×10^3	0.59
Anaerobic	8.6×10^4	0.89	4.4×10^3	0.77

Genera and species of phototrophic bacteria in activated sludge. Several genera and species of phototrophic bacteria can be recognized by the morphology of their cells (15) and the color and shape of their colonies on agar. The percentage of the different species was estimated using the same agar plates for the viable counts (Table 4). The identification of *Rhodospirillum tenue, Rhodopseudomonas viridis* and *R. palustris, Rhodomicrobium vanielii, Chromatium vinosum, Thiocapsa roseopersicina,* and *Chlorobium limicola* was quite easy. In contrast it was sometimes difficult to distinguish *R. sphaeroides, R. capsulata,* and *R. gelatinosa.* The three latter species formed the largest number of colonies on the agar plates. Three representative strains were isolated and identified unequivocally by growth tests on 37 different substrates. For one strain, however,

TABLE 4. *Distribution of different species of phototrophic bacteria in activated sludge*

Species	Distribution (%)
Rhodopseudomonas sphaeroides *Rhodopseudomonas gelatinosa*	51–75
Rhodopseudomonas palustris	6–25
Rhodopseudomonas capsulata	6–25
Rhodospirillum tenue	1–5
Rhodopseudomonas viridis	1–5
Chromatium vinosum	<1
Thiocapsa roseopersicina	<1
Chlorobium limicola	<1

these growth tests did not give sufficient information to identify it as *R. gelatinosa* or *R. sphaeroides*.

Gelatin hydrolysis was tested to identify *R. gelatinosa*, because this character has been described as typical for this species. Half of the strains that were identified as *R. capsulata*, however, also hydrolyzed gelatin. Other strains having the cell morphology and colony color of *R. gelatinosa* did not hydrolyze gelatin. Gelatin hydrolysis, therefore, did not prove to be conclusive for the identification of *R. gelatinosa*.

The strictly anaerobic brown-colored species of the genus *Rhodospirillum, R. fulvum, R. molischianum*, and *R. photometricum*, were not detected on plates used to determine viable cell counts. It was possible, however, to enrich *R. photometricum* repeatedly in activated sludge by incubating samples in test tubes at 1,000 lx and 30°C. Growth spots of accumulated *R. photometricum* appeared after a few days on the illuminated side of the tube. From such enrichments, one strain was isolated in pure culture.

Some phototrophic bacteria, picked from a single colony, failed to grow on second plates of the same medium. Among these, *R. tenue* strains proved to have a requirement for vitamin B_{12}, which was not added to the original agar medium.

Culture experiments with digester sludge. The numbers of phototrophic bacteria decreased in the methane digester (Table 1). To determine whether this was due to the conditions in the digester, e.g., any inhibitory effects of its microbial population on the phototrophic bacteria or the complete absence of light, the following experiments were done. Diluted digester sludge with and without the addition of raw sludge was incubated anaerobically in the dark and in the light. The illuminated flasks showed a large increase in the numbers of phototrophic bacteria after 2 and 4 weeks. In flasks kept in the dark, neither growth of the whole population of phototrophic bacteria (Table 5) nor growth of individual species could be observed, judging from the appearance of the different types of colonies. Thus, phototrophic bacteria in digester sludge have a selective advantage only in the presence of light.

TABLE 5. *Survival and growth of phototrophic bacteria in digester sludge incubated anaerobically in the dark or in the light*

Time	Phototrophic bacteria (colony counts/ml)			
	Diluted digester sludge mixed with raw sludge		Diluted digester sludge	
	Light	Dark	Light	Dark
At the beginning	1×10^5	1×10^5	2.8×10^3	2.8×10^3
After 2 weeks	8.3×10^8	2×10^4	2.9×10^8	1.6×10^3
After 4 weeks	3.0×10^9	8×10^3	8.0×10^8	9.0×10^3

DISCUSSION

Phototrophic bacteria are present in all purification stages of the municipal sewage treatment plant in Göttingen; their contribution to the purification process, however, is negligible, as shown by the viable cell counts presented in this paper. Even the unexpectedly high colony counts of phototrophic bacteria in the activated sludge tank, 5×10^4/mg (dry weight) of activated sludge, is small in comparison to total viable counts of aerobic bacteria amounting to 10^7/ml (dry weight) (16). In heavily polluted waters or highly eutrophic ponds, the number of phototrophic bacteria is in the same range as that found in activated sludge: 10^4 to 10^5 cells/ml as reported by Biebl and Drews (1) and Kaiser (8). The presence of phototrophic bacteria in activated sludge was demonstrable both by the plate count method and by detection of bacteriochlorophyll a in activated sludge. Quantitative determinations of bacteriochlorophyll a showed that activated sludge contained much more bacteriochlorophyll a than would be expected from the colony counts (Table 2). There are several possible explanations for this finding. First, the plate count medium used did not support growth of all phototrophic cells present in the sludge; second, due to cell aggregates, one phototrophic colony from an activated sludge sample represented more than one single cell; and third, the activated sludge contained bacteriochlorophyll a or bacteriopheophytin a in dead cells of phototrophic bacteria which could not be recorded by the viable count method.

In spite of these uncertainties, one can conclude from the relatively high content of bacteriochlorophyll a in activated sludge that phototrophic bacteria grow under the reduced oxygen tension in the sludge flocs, because bacteriochlorophyll synthesis would be repressed if the

bacteria were exposed to the oxygen concentration measured in the free water phase of the aeration tank (2 to 4 mg of O_2 per liter).

Full pigmentation usually indicates that the bacteria were grown anaerobically by photosynthetic energy conversion, but they might also have grown by respiratory energy conversion at low oxygen partial pressure (5). It is likely that phototrophic bacteria take advantage of both types of metabolism. In activated sludge, the photosynthetic metabolism appears to be less significant, since only the uppermost 10 cm of the total depth of 3.8 m receives sufficient light to allow phototrophic growth, as we have found. Due to the mixing procedure, phototrophic bacteria stay, intermittently at best, for 2.6% of the day in that layer, where they can grow photosynthetically.

Although this quantity of light in our experience is rather low for phototrophic growth, light does not appear to be the growth-limiting factor, since no correlation was observed between colony counts and seasonal light conditions. This apparent contradiction is solved if one assumes that phototrophic bacteria obtain energy by respiration during their stay in the dark. This assumption would also explain why the facultative aerobic species *R. gelatinosa, R. capsulata*, and *R. sphaeroides* predominated in activated sludge.

The assumption that phototrophic bacteria grow using mainly respiratory energy applies only to microaerobic conditions. Two facts indicate that too much oxygen is unfavorable. First, with decreasing sludge concentration in the aeration tank aerobic conditions become improved, but the colony counts of both phototrophic and anaerobic heterotrophic bacteria are diminished quadratically (Fig. 1a and b). Second, phototrophic bacteria in activated sludge decreased in number when cultivated both in the light and in the dark under strict aerobic conditions in fluted Erlenmeyer flasks on a rotary shaker (Table 3).

Low oxygen concentrations at which phototrophic bacteria can grow fully pigmented in activated sludge are probably present in the activated sludge flocs. About 95% of the total number of phototrophic bacteria are attached to the sludge flocs (Siefert, thesis, University of Göttingen, Göttingen, West Germany, 1972).

As reported by Uffen and Wolfe (18), phototrophic bacteria might also grow under strongly reducing conditions anaerobically in the dark by a fermentative metabolism. If this metabolism is of ecological significance, phototrophic bacteria should increase in viable cell counts in the strictly anaerobic environment of the methane digester. Colony counts of digester sludge, however, were much lower than those of the raw sludge entering the digester. Also, it was impossible to obtain an increase in viable cell counts of phototrophic bacteria when digester sludge, enriched with raw sludge, was incubated anaerobically in the dark (Table 5). In illuminated control vessels, colony counts greatly increased. It is, therefore, conclusive that under anaerobic conditions in the absence of light phototrophic bacteria cannot compete with chemotrophic anaerobes, because their fermentative energy metabolism is not efficient enough (6).

Our results show that in conventional sewage treatment plants under normal operating conditions, phototrophic bacteria are not capable of competing with chemotrophic bacteria because of either the limited availability of light or the presence of too much oxygen, or both. Nevertheless, these bacteria are permanently present in all stages of the plants and can be easily enriched, if samples are incubated under appropriate conditions. If light and oxygen supply are adjusted to the requirements of the phototrophic bacteria, they should become the predominant bacteria involved in the purification process. Sewage treatment plants utilizing phototrophic bacteria for the main purification step already are in operation in Japan (11). The use of phototrophic bacteria has some advantages over the conventional biological sewage treatment. First, the major organic and inorganic constituents of sewage are assimilated rather than dissimilated, resulting in an augmented uptake of minerals. Thus, a third purification step for removal of inorganic nutrients could possibly be saved. Second, the cell protein of the phototrophic bacteria is a useful by-product, which can be utilized as a supplement in animal nutrition.

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