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Toxicity of Ammonia to Algae in Sewage Oxidation Ponds

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Ammonia, at concentrations over 2.0 mM and at pH values over 8.0, inhibits photosynthesis and growth of *Scenedesmus obliquus*, a dominant species in high-rate sewage oxidation ponds. Photosynthesis of *Chlorella pyrenoidosa*, *Anacystis nidulans*, and *Plectonema boryanum* is also susceptible to ammonia inhibition. Dark respiration and cell morphology were unaffected by any combination of pH and ammonia concentrations tested, thus limiting the apparent effect to inhibition of the normal function of the chloroplasts. Methylamine had the same effect as ammonia, and its penetration into the cells was found to be pH dependent. Therefore, the dependence of toxicity of amines to algae on pH apparently results from the inability to penetrate the cell membrane in the ionized form. When operated at 120-h detention time of raw wastewater, the high-rate oxidation pond maintained a steady state with respect to algal growth and oxygen concentration, and the concentration of ammonia did not excced 1.0 mM. Shifting the pond to 48-h detention time caused an increase in ammonia concentration in the pond water to 2.5 mM, and the pond gradually turned anaerobic. Photosynthesis, which usually elevates the pH of the pond water to 9.0 to 10.0, could not proceed beyond pH 7.9 because of the high concentration of ammonia, and the algal population was washed out and reduced to a concentration that could maintain a doubling time of 48 h without photosynthesis bringing the pH to inhibitory levels. Under these conditions, the pH of the pond becomes a factor that limits the operational efficiency of the oxidation pond.

The high-rate oxidation pond, also named accelerated photosynthetic pond (20) or high-rate waste stabilization pond (9), has received increased attention in recent years as a means for treatment of wastewater, as well as for production of algal biomass (9, 12).

High growth rates of algae are critical for a successful operation of such a coupled system. However, growth of algae in raw sewage seems to present problems that are specific to this medium, since their growth rate is very slow, with a doubling time of 50 h reported in laboratory batch culture experiments (7). In actual continuous operation of an oxidation pond, steady-state algal growth (*Scenedesmus obliquus* was generally the dominant species) was obtained only at a retention time of about 100 h (10, 13, 15). This is compared to 5- to 8-h doubling time for the same alga under optimal laboratory conditions (8), indicating the presence of either inhibitors or limiting factors in sewage.

Jerusalem domestic raw sewage contains high concentrations of ammonia, 4 to 8 mM (A. Abeliovich and Y. Azov, unpublished data), and as a result ammonia concentration in the oxidation ponds can reach high concentrations when operated at short detention times.

Although low concentrations of ammonia, up to 0.2 mM, were found to be beneficial to various algae in field condition (1), un-ionized ammonia at higher concentrations is known to be toxic to a wide range of organisms, such as *Prymnesium parvum* (21, 22), marine diatoms (16), and plants and fish (23). According to Golueke et al., high-rate oxidation ponds failed to operate when supplemented with overdoses of ammonia (10). It was therefore of interest to study the effects of ammonia on growth and photosynthesis of axenic cultures of algae and its effects on growth rate and photosynthesis of algae in a high-rate oxidation pond, as part of an attempt to increase the efficiency of the operation of oxidation ponds.

MATERIALS AND METHODS

Organisms and culture conditions. Axenic cultures of *S. obliquus* (an isolate from a local sewage oxidation pond and kindly supplied by I. Dor, Human Environmental Sciences Laboratory, Hebrew University, Jerusalem), *Chlorella pyrenoidosa* (from the Department of Botany, Hebrew University, Jerusalem), *Anacystis nidulans* 6311 (from the Department of Bacteriology and Immunology, University of California at Berkeley), and *Plectonema boryanum* 594 *(Gomot)* (University of Indiana culture collection, Bloomington, Ind.) were used. *Plec-*

tonema boryanum was grown in modified Chu No. 10 medium (19). *S. obliquus*, *C. pyrenoidosa*, and *A. nidulans* were grown on Zehnder and Gorham medium No. 11 (24), with the $NaNO_3$ concentration modified to 1.0 g/liter. The algae were grown at 30 C in 250-ml flasks containing 100 ml of medium placed on a rotary shaker. Illumination was provided by 40 W cool while fluorescent lamps, giving an incident light intensity of 2×10^3 ergs/cm^2 per s at the surface of the flasks.

Oxygen measurements. Cells were suspended in medium and placed in a 3.0-ml Perspex cell. Rates of oxygen evolution and uptake were measured at 30 C using a Clark-type oxygen electrode (YSI 4004, Yellow Springs Instruments Co., Yellow Springs, Ohio) connected to a recorder (RE541 Goertz potentiometric recorder, Vienna). A 1,000 W quartz iodine lamp (P1/15, Atlas, England) was used for illumination. The light intensity was 10^6 ergs/s at the surface of the cell.

Photoassimilation of CO_2. Cells at the end of the logarithmic phase of growth were collected by centrifugation and suspended in reaction mixtures to a final concentration of about 1.0 mg of protein per ml. Unless otherwise stated, reaction mixtures at various pH values (final volume, 10 ml in 25-ml flasks) contained growth medium, 0.01 M glycine (brought by NaOH to pH 8.5 to 9.0, according to experimental conditions) or 0.01 M tricine (pH 8.0), and $NaH^{14}CO_3$ (Radiochemical Centre, Amersham, England) at a final concentration of 1.0 mM and a final specific activity of 0.2 mCi/mM. The flasks were incubated with agitation at 30 C under constant illumination of 10^4 ergs of cool white fluorescent light/cm^2 per s at the surface of the flasks. Unless otherwise stated, reaction mixtures were incubated for 60 to 75 min, and aliquots (1.0 ml) of the cell suspension were transferred every 15 min into 5% cold trichloroacetic acid, filtered, washed on a fiber glass filter (Whatman, GF/C), and dried at 50 C.

The samples were counted in a gas flow, end window planchet counter (model D-47, Nuclear-Chicago Corp., Des Plaines, Ill.). Counting efficiency was 16%. All data are presented after subtraction of incorporation of $^{14}CO_2$ by dark controls.

Field work. Field work was carried out in a high-rate sewage oxidation pond during the months of July through September. The pond was 40 cm deep and had a total area of 300 m^2. Its mode of operation has been published elsewhere (20). Oxygen concentration measurements were made by a portable oxygen meter (YSI model 54), and the pH was measured by a Metrohm Herisau portable pH meter (E488).

Analytical methods. Chlorophyll *a* or protein concentration were used as a measure of algal biomass.

(i) Chlorophyll *a*. Chlorophyll *a* was extracted by boiling methanol and determined with a Zeiss PMQ II spectrophotometer, using the absorption coefficients reported by Parsons and Strickland (17).

(ii) Protein. Protein was determined by the method of Lowry et al. (14).

(iii) Ammonia. Ammonia was determined by the method of Nessler (2) after filtering the sample through a membrane filter (type HA, Millipore Corp.). No prewashing of the filters was necessary.

RESULTS

Effect of ammonia on growth and photosynthesis. Growth of *S. obliquus* in mineral medium in light is severely inhibited by ammonia at high pH (Fig. 1). Inhibition starts at about 2.0 to 2.5 mM, and no growth was observed at concentrations higher than 3.0 mM. Likewise, ammonia had an inhibitory effect on photoassimilation of CO_2 at alkaline pH values (Table 1). Ammonia concentrations necessary for inhibition of photosynthesis are inversely proportional to the pH of the reaction mixture (Fig. 2). However, it is important to note that dark respiration was uninhibited at any of the combinations of pH and ammonia tested, and no morphological changes, such as swelling,

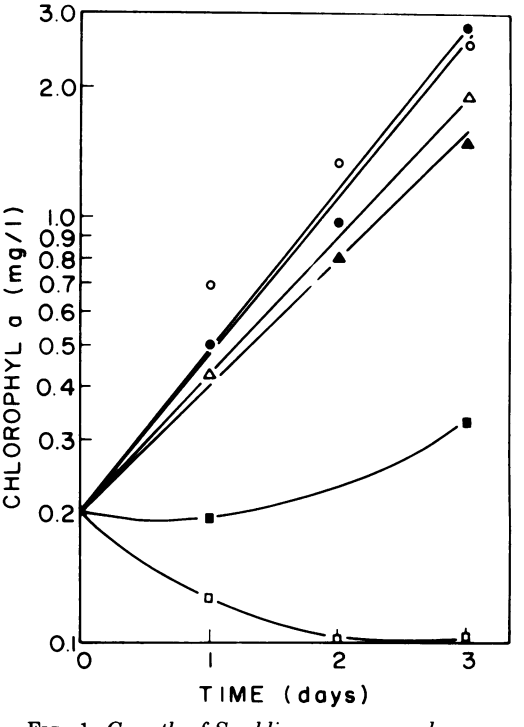

FIG. 1. *Growth of S. obliquus, expressed as concentration of chlorophyll a, in medium containing free ammonia as nitrogen source. Ammonia concentrations were measured and adjusted every day. (Initial and final pH values for each flask are given in parentheses.) Symbols: (○) ammonia-free control, with nitrate as nitrogen source (8.7 → 10.7). Ammonia concentration before and after adjustment: (●) 0.8 to 1.0 mM (9.5 → 9.7); (△) 1.6 to 2.0 mM (9.8 → 9.2); (▲) 2.0 to 2.5 mM (9.8 → 9.2); (■) 2.6 to 3.0 mM (9.8 → 9.0); (□) 4.0 mM (9.9 → 8.9).*

TABLE 1. *Effect of pH on rates of $^{14}CO_2$ photoassimilation by S. obliquus in the presence of nitrate, ammonia, methylamine, and raw sewage*[a]

Treatment	pH[b]					
	7.0	7.9	8.3	9.0	9.5	10.0
Nitrate	100			70	60	50
Ammonia	90	90		12	3	1
Methylamine		135	80	3		
Raw sewage	22			2		
Raw sewage + mineral medium	25			3		
Preincubation[c] with ammonia (5 mM), 1 h at pH 9.0	95					
Preincubation with ammonia (5 mM), 5 h at pH 9.0	66					

[a] Assays were carried for 1 h. The numbers represent percentages of rate of photoassimilation by untreated controls (0.15 μm of CO_2/mg of protein per h). Nitrate, ammonia, and methylamine were added to a final concentration of 5 mM. The concentration of ammonia in the raw sewage was 7.6 mM.

[b] The pH of incubation mixtures was adjusted by tricine·NaOH (pH 7.0 to 8.3, 0.05 N) and glycine·NaOH (pH 9.0 to 10.0, 0.05 N).

[c] Preincubation was carried out in mineral medium supplemented with NH_4Cl (5 mM) and brought to pH by glycine·NaOH (0.05 N, pH 9.0). After preincubation, the cells were washed and resuspended in an ammonia-free incubation mixture. Photoassimilation experiments started within 10 min after the end of preincubation.

could be detected in the cells with a phase-contrast microscope.

As ammonia is mostly ionized below pH 8.0, it was of interest to see if this pH effect is a result of a higher permeability of the cell membrane to un-ionized ammonia as compared to the ion. Methylamine, which uncouples chloroplasts as does ammonia (11) and was shown to affect photosynthesis by intact algae similarly to ammonia (Table 1), was used to test penetration at different pH values. Significant penetration starts only at pH 8.0 and increases with elevation of the pH, similar to the inhibitory effect of ammonia on photosynthesis (Fig. 3). When *S. obliquus* cells were incubated with ammonia at pH 9.0 for 1 h and then washed and transferred to pH 7.0, no inhibitory after effects of ammonia on photosynthesis could be detected (Table 1). However, exposure of the cells to ammonia at pH 9.0 for 5 h led to a 30% inhibition of photosynthesis rate after the cells were washed and transferred to an ammonia-free medium at pH 7.0 (Table 1).

Effect of ammonia on other algae. We tested the effect of ammonia at high pH on the photosynthetic activity of *C. pyrenoidosa* and of two blue-green algae, *A. nidulans* and *P. boryanum* (594). All were subject to severe inhibition of photosynthesis, similar to *S. obliquus*. (Table 2).

Effect of ammonia in sewage on photosynthesis and growth of algae in a high-rate oxidation pond. Photoassimilation of $^{14}CO_2$ by laboratory-grown algae that were transferred to fresh raw sewage was inhibited by ammonia in the absence as well as in the presence of added mineral medium (Table 1). Likewise, algae collected from a sewage oxidation pond (mainly *S. obliquus*, some *Chlorella* sp., *Chlamydomonas* sp., and *Euglena* sp.) could not photosynthesize when incubated in pond water to which ammonia was added at a high pH (Table 3). Table 3 also shows that pond water had an inhibitory effect on the rate of photoassimilation of $^{14}CO_2$ by algae, even without ammonia, at low pH.

To test how this phenomenon affects the actual operation of an oxidation pond, we operated it at two detention time regimes, 48 and 120 h, thus varying the rate of inflow of ammonia and organic matter. During the experi-

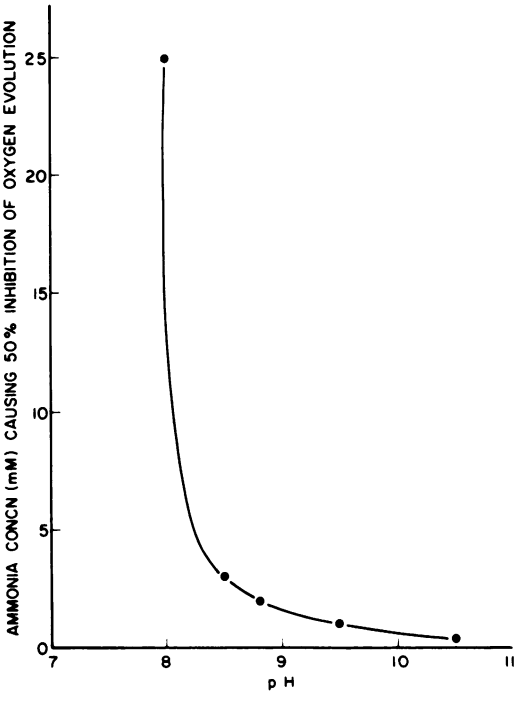

FIG. 2. *Ammonia concentration causing 50% inhibition of oxygen evolution at different pH values by S. obliquus. Initial rate of oxygen evolution was 7.5 mg of O_2/ml per min. Buffers used (0.05 N final concentration) were: sodium phosphate for pH 7.8; tricine for pH 8.0; glycine for pH 8.5 and 8.8; and sodium carbonate for pH 9.5.*

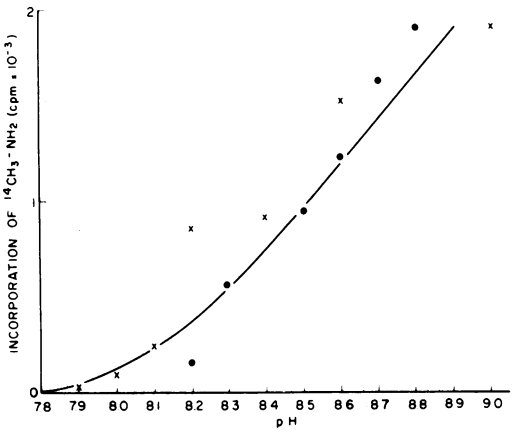

FIG. 3. *Penetration of [^{14}C]methylamine into cells of S. obliquus at different pH values. Each reaction mixture contained algal cell suspension (2 mg of protein), 1.0 μCi of [^{14}C]methylamine, and 1.0 μm of cold methylamine as carrier, in a final volume of 5.0 ml of medium. pH was maintained by tricine (0.1 M) brought to pH before addition of the cells. Reaction mixtures were incubated for 1 h in the light (×) and in the dark (○). After incubation the cells were filtered, washed with phosphate buffer (0.1 M, pH 7.0), dried, and counted for ^{14}C. The data are presented after subtraction of counts incorporated by a control at pH 7.7 (1,200 counts/min).*

TABLE 2. *Rates of $^{14}CO_2$ photoassimilation by C. pyrenoidosa, A. nidulans, and P. boryanum in the presence of nitrate or ammonia*[a]

Organism	pH of incubation mixture[b]	Nitrate (5 mM)	Ammonia (5 mM)
C. pyrenoidosa	7.0	100	89
C. pyrenoidosa	9.0	45	21
A. nidulans	7.0	100	90
A. nidulans	9.0	47	23
P. boryanum	7.0	100	84
P. boryanum	9.0	64	8

[a] See footnote *a*, Table 1.
[b] See footnote *b*, Table 1.

ment, we measured the maximal pH and oxygen and ammonia concentrations for each day, as well as algae concentration, measured as chlorophyll *a*.

The pond was allowed to reach a steady-state condition with a 120-h detention time before experiments were initiated. Days 0 to 4 (Fig. 4) show that under these conditions, chlorophyll *a* concentration reached 11 to 12 mg/liter, ammonia concentration was 0.8 mM, pH was highly alkaline, and oxygen concentration reached 20 mg/liter and over. When the pond was operated at a 48-h detention time, chlorophyll *a* concentration was reduced to 3 to 4 mg/liter, ammonia concentration reached 2.5 to 2.6 mM, and the pH dropped to 7.8 to 7.9. Oxygen concentration dropped rapidly when the pond was operated in

TABLE 3. *Photoassimilation of $^{14}CO_2$ by algae collected from a sewage oxidation pond and incubated in pond water*[a]

pH[b]	Mineral medium	Pond water	
		1.2 mM ammonia[c]	5.0 mM ammonia[c]
7.9	100	53	61
9.0	55	38	7

[a] See footnote *a*, Table 1.
[b] See footnote *b*, Table 1.
[c] Concentration of ammonia in pond water was 1.2 mM when operated at a 5-day detention time. Ammonia (NH_4Cl) was added to pond water to a final concentration of 5 mM.

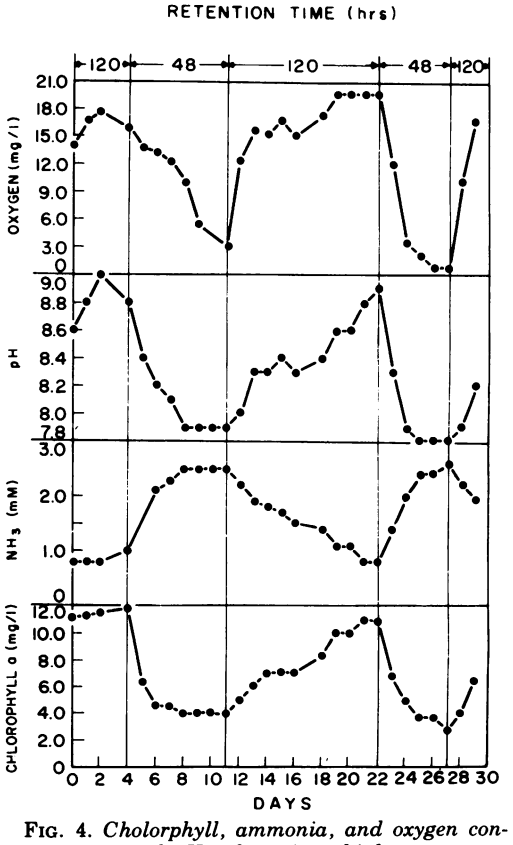

FIG. 4. *Cholorphyll, ammonia, and oxygen concentrations, and pH values, in a high-rate sewage oxidation pond during operation at short and long detention times (48 and 120 h). pH and oxygen concentration were measured at noon, and samples for determination of ammonia and chlorophyll a were taken into the laboratory and analyzed within 30 min.*

this mode, and it tended to turn anaerobic after several days. Bacterica counts performed on water samples during the experiment were between 2×10^8 and 4×10^8/ml regardless of detention time. To determine whether it is the inhibitory effect of ammonia or the effect of other factors, such as excessive organic loading, that inhibits photosynthesis in the pond when it is operated at a detention time of 48 h, we attempted to simulate the effect of varying detention times on the rate of oxygen production by varying the pH and ammonia concentration. Ammonia was added to the pond run at a 120-h detention time at high pH, and, at a detention time of 48 h with ammonia present in the pond, the pH was lowered to 7.0 by HCl to maintain a pH that would allow uninterrupted photosynthesis in spite of the short detention time. The data presented in Fig. 5 indicate that oxygen production by the algae in the pond responds to changes in pH and ammonia concentration similar to the way in which it responds to such changes when tested in the laboratory and to the way it responds to to shifts in detention time (see Fig. 4).

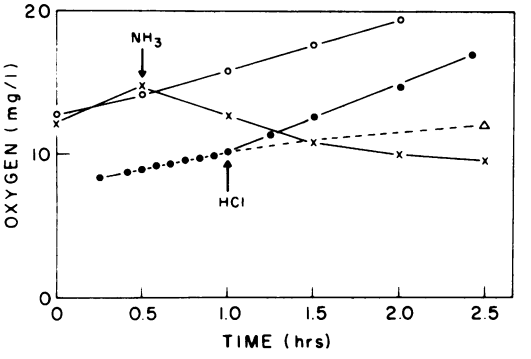

FIG. 5. *Effects of varying pH and ammonia concentration in the pond on oxygen concentration. Zero time was 9:30 a.m. Ammonia concentration was brought to 2.6 mM by addition of NH_4OH. pH was 8.3 prior to the addition of the ammonia and rose to 8.8 after the addition. pH of the pond was 8.3 before addition of HCl and was lowered to 7.0 by the acid. Symbols: (○) Control of pond run at a 120-h detention time, without any additions; ammonia concentration was 0.7 mM and pH at zero time was 8.4 and, at 2.0 h, 8.9. (×) Pond run at at 120-h detention time; at the point indicated ammonia was added. (●) Rate of oxygen evolution 24 h after the pond was shifted from 120- to 48-h detention time regime. At the point indicated pH was lowered by HCl. (△) Expected oxygen concentration at noon in the pond 24 h after shift from 120- to 48-h detention time (from Fig. 4).*

DISCUSSION

Un-ionized ammonia is known to be toxic to various photosynthetic organisms. In the case of *P. parvum*, ammonia, among other weak electrolytes, causes swelling and osmotic lysis of the cells (22), whereas in marine diatoms it was found that photosynthesis is inhibited (16). In the algae we tested in this work, among them species typical to oxidation ponds, ammonia specifically inhibited photosynthesis without affecting dark respiration or cell morphology.

Since photosynthetic activity of algae from several diverse groups, such as green algae, diatoms, and blue-green algae, was found to be susceptible to ammonia poisoning, it seems that the phenomenon is a general one, at least for oxygen evolving photosynthetic microorganisms. In isolated chloroplasts, photoinduced uncoupled electron flow is pH dependent, being inhibited above pH 8.0 (3, 4), probably because of a rise in the internal pH and as a consequence a decrease in the Δ pH (18).

Ammonia acted on whole cells of the algae tested, at high pH, as an apparent inhibitor of photosynthesis rather than as an uncoupler. This is either because of absence of an electron acceptor (5) or because the penetrating ammonia elevates the cell's internal pH to an inhibitory level (3, 5). It is clear from the data presented in Fig. 2 that the upper pH limit for undisturbed photosynthesis in the presence of ammonia is 7.9, which is also close to the lower pH limit for significant penetration of methylamine (Fig. 3). Therefore, it is probably penetration across the cell's membranes that makes the effect of ammonia pH dependent, since ionized ammonia is unable to penetrate freely through algal cell membrane, a phenomenon widely reported earlier (21, 23).

Exposure to ammonia at high pH for several hours has a long-term effect (Table 1). This effect might be of importance in the operation of oxidation ponds, since it can cause low efficiencies of pond operation when it is exposed to sublethal combinations of ammonia and pH, thus turning it anaerobic.

In the high-rate oxidation pond, as long as it was run at a regime of 120-h detention time, ammonia did not reach inhibitory levels nor interrupt photosynthesis, and, therefore, pH values reached 9.0 without leveling off (Fig. 5). At 48-h detention time, ammonia concentration was inhibitory for photosynthesis at pH values over 7.9, the maximal pH measured in the pond after a stabilization period of 48 h. During the first 24 h after the shift to a short detention time, the cells were washed out without any detectable growth. The concentration of algae stabilizes under these conditions only at or below 4 mg of chlorophyll *a* per liter. This is

probably the maximal concentration of algae that survives without being washed out, because it can maintain uninhibited photosynthesis and a doubling time of 48 h in the presence of ammonia without trying to push the pH of the pond water beyond 7.9. Under these conditions, the pH of the pond becomes a factor that limits the operational efficiency of the oxidation pond.

It therefore seems that ammonia and pH are dominant factors in determining the oxygen regime and growth rate in oxidation ponds when they are run at short detention times. It should be noted, however, that other factors that inhibit photosynthesis exist in raw sewage (Table 2), and they probably also contribute to the slow rate of algal growth in the oxidation pond, irrespective of the detention time of wastewater in it. Varying pH values and ammonia concentrations in the pond replace variations in sewage flow rate into the pond. Therefore, the organic matter in it might only play an additional role in inibiting growth and photosynthesis at short detention times.

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