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Effects of Light, Temperature, Nitrate, Orthophosphate, and Bacteria on Growth of and Hepatotoxin Production by *Oscillatoria agardhii* Strains

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The effects of bacteria, temperature, light, nitrate, and orthophosphate on growth of and hepatotoxin (desmethyl-3-microcystin-RR) production by *Oscillatoria agardhii* strains were studied under laboratory conditions. Strains were cultivated in Z8 medium under continuous illumination. Growth was determined by measuring dry weight and chlorophyll *a*, while toxin was analyzed by high-performance liquid chromatography. Two of the three toxic cultures studied produced more toxins in axenic than in nonaxenic cultures. High toxin production correlated with high nitrogen concentrations (test range, 0.42 to 84 mg of N per liter) and low light intensity (test range, 12 to 95 microeinsteins/m^2 per s). Toxin production depended on phosphorus concentration at low levels of phosphorus (0.1 to 0.4 mg of P per liter) and higher concentrations had no additional effect. The optimum temperature for toxin production and growth of green *O. agardhii* was 25°C. Red *O. agardhii* produced almost similar amounts of toxin at temperatures of 15 to 25°C. The lowest toxin production by both strains was at 30°C.

Common mass occurrences of toxic cyanobacteria in eutrophic fresh and brackish waters all over the world cause animal deaths and health hazards for humans (1, 2, 4, 18–20). The most common hepatotoxin-producing genera of cyanobacteria in fresh waters are *Microcystis*, *Anabaena*, and *Oscillatoria* (1, 2, 4, 19). Mass occurrences of hepatotoxic *Oscillatoria agardhii* were found in lakes from three Scandinavian countries, Norway, Sweden, and Finland (1, 19), as well as in The Netherlands (10), while *O. rubescens*, another toxic species, was reported from Italy (12). The toxins isolated and identified from *O. agardhii* to date have been shown to be cyclic arginine-containing heptapeptides from two Norwegian blooms (desmethyl-3-microcystin-RR and desmethyl-3,7-microcystin-RR) (2, 9) and a Norwegian culture (desmethyl-3-microcystin-RR) (14) having 50% lethal doses of 250 to 1,000 μg/kg (intraperitoneally, mouse).

To date, most studies on the influence of environmental factors on toxin production by cyanobacteria have been done with *Microcystis aeruginosa* strains with a mouse bioassay to detect toxicity (3–5, 16, 22, 23, 26, 27), but none have been concerned with *Oscillatoria* spp. Many studies have concluded that toxicity (3, 5, 22, 26) and toxin content of the cells (25) are highest at the late logarithmic growth phase. The effects of temperature (3, 5, 16, 23, 27) and light (4, 5, 23, 27) on toxin production of *M. aeruginosa* were the most commonly studied parameters, and only a few studies consider the influence of other factors such as main nutrients (4, 27) or pH (22). More information is needed regarding the influence of these factors on toxin production and growth of cyanobacteria to understand the dynamics of toxic blooms in nature. Since toxins are also produced for research purposes, optimal conditions for toxin production would give better yields of these compounds.

In this study, the effects of temperature, light, nitrogen, phosphorus, and the presence or absence of bacteria on hepatotoxin production of *O. agardhii* strains were studied.

MATERIALS AND METHODS

Organisms. Four *O. agardhii* strains isolated from Finnish lakes were used in this study: 97 (green; isolated in 1986 from L. Maarianallas, Finland), CYA 128 (red; isolated in 1984 from L. Vesijärvi, Finland), CYA 126 (green; isolated in 1984 from L. Långsjön, Finland), and 18 (green; isolated in 1985 from L. Långsjön, Finland). CYA 128 and 126 cultures were kindly provided by O. M. Skulberg, Norwegian Water Research Institute, Oslo, Norway. Strains 97 and 18 were isolated on Z8 medium, and all cultures were rendered axenic by the method of Vaara et al. (21) at our laboratory. Cultures 97, CYA 128, and CYA 126 all produced the same main hepatotoxin, desmethyl-3-microcystin-RR (molecular weight, 1,023; Sivonen et al., unpublished results). Strains produced minor amounts of other hepatotoxins which were not quantified or characterized because of the lack of isolated material. Strain 18 was used as a nontoxic control culture.

Culturing and analysis. Five growth experiments were done; in the first experiment growth and toxin production curves of the axenic and nonaxenic cultures of all four strains were determined under standard conditions, and in the other four experiments the effects of temperature, nitrate nitrogen, phosphorus, and light on growth and toxin production were studied (Table 1). In all experiments the culture medium was liquid Z8 (7, 8), and culture vessels were 250-ml Erlenmeyer flasks (Schott, Duran, Federal Republic of Germany) which contained 100 ml of medium; the cultivation took place in continuous illumination (cool white fluorescent tubes; Daylight Deluxe; Airam, Helsinki, Finland). Light intensity was measured with a Li-Cor Mc. model LI-185 B Quantum/radiometer/photometer. In the first experiment the axenic and nonaxenic clones of strains 97, 18, CYA 128, and CYA 126 were cultivated at 20 ± 2°C in an incubation room. For the remaining experi-

TABLE 1. *O. agardhii* strains and growth conditions used in the five experiments of this study

Variable[a]	Strains	Culture conditions			
		Temp (°C)	Nitrogen (mg/liter)	Phosphorus (mg/liter)	Light (microeinsteins/ m^2 per s)
Axenity	18, 97, CYA 128, CYA 126	20	84	5.5	24
Temp	18, 97, CYA 128	15, 20, 25, 30	84	5.5	24
Nitrogen	18, 97, CYA 128	25	0.42, 4.2, 21, 84	5.5	24
Phosphorus	18, 97, CYA 128	25	84	0.1, 0.4, 1.5, 5.5	24
Light	18, 97, CYA 128	25	84	5.5	12, 24, 50, 95

[a] Nonaxenic (unialgal but not bacterium-free) and axenic (bacterium-free) strains were used in this experiment. In the temperature, nitrogen, phosphorus, and light experiments only axenic cultures were used.

ments, axenic strains 97, 18, and CYA 128 were cultivated in constant-temperature water baths. In these experiments, one parameter was changed at each time, and the axenic 97, CYA 128, and 18 strains were cultivated under the conditions listed in Table 1. Limited space in water baths led to the exclusion of strain CYA 126 from these experiments. The nutrient concentrations were chosen to represent low and high values for natural waters and laboratory medium. The Z8 medium contains nitrogen in nitrate form. The inocula in the nitrogen and phosphorus experiments were grown in original Z8 medium but filtered, washed, and suspended in nutrient-free medium before inoculation. In addition, inocula for the phosphorus experiments were grown for 5 days in phosphorus-free medium prior to beginning experiments to deplete stored phosphorus. With each strain, 20 to 40 culture flasks inoculated per concentration were randomized and incubation locations were changed on different working days. The contents of three to six Erlenmeyer flasks were pooled for each sampling, and the pH, dry weight (by filtering 30 to 60 ml of sample to tared GF/C glass-fiber filters which were then dried for 24 h at 100°C and weighed), chlorophyll *a* (6), and toxin content (13, 17) in cells and in culture media were determined. Axenity was determined from each flask separately before pooling by cultivating a drop of the culture on tryptone-yeast extract-glucose agar plates.

In the bacterial and temperature experiments, samples were taken every three to five days during a 3- to 4-week period. For the rest of the experiments, samples were taken weekly. Toxin concentrations of cells harvested by filtration (nylon cloth; 10-μm pore size) were analyzed after lyophilization. Toxin within the culture medium was determined after filtering through GF/C glass-fiber filters and passage through octadecyl C_{18} cartridges (Bond Elut; Amersham Corp.). The toxin was eluted from the cartridges with 100% methanol, and each sample was air dried. Air-dried samples to test toxicity in medium were suspended in 0.5 ml of 5% 1-butanol–20% methanol (vol/vol) in water, and lyophilized cell samples to test toxins in the cells were extracted twice with the same solution (100 μl/mg of lyophilized cells) (13) prior to high-performance liquid chromatography analysis. The toxin concentrations were then determined with a high-performance liquid chromatograph, which had a diode array detector (17). Toxins isolated and purified from the respective strains were used as standards. The calibration curve was linear from 2.0 to 300 μg/ml ($n = 6$; $r > 0.999$).

Statistics. Differences in toxin production by axenic and nonaxenic strains were studied by Student's paired test. Correlation coefficients between toxin production and biomass parameters were calculated for each experiment.

RESULTS

O. agardhii 18, the nontoxic reference culture, did not produce toxins under any conditions, whereas strains CYA 126, CYA 128, and 97 produced toxins under all conditions studied (Fig. 1 and 2 to 6).

Most of the toxins were detected within the cell in all experiments. Toxin concentrations in the medium were low during the first week but increased at the end of the experiment (Fig. 2). The average toxin concentrations in the medium (mean of all determinations) were <10% for strain 97 and about 20% for strain CYA 128. Total toxin expressed as micrograms per liter correlated positively with both biomass parameters dry weight ($P < 0.001$) and chlorophyll *a* ($P < 0.001$), which were also correlated to each other ($P < 0.001$) in most of the experiments. With the red *O. agardhii* strain CYA 128, the total toxin had a more signifi-

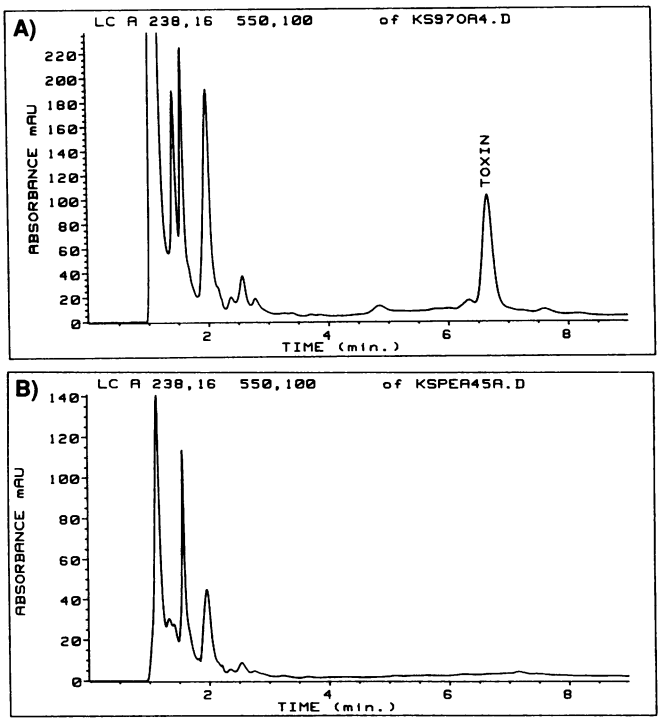

FIG. 1. High-performance liquid chromatograms of *O. agardhii* strains. (A) Toxic strain 97 showing the toxic peak at retention time 6.5; (B) nontoxic reference strain 18.

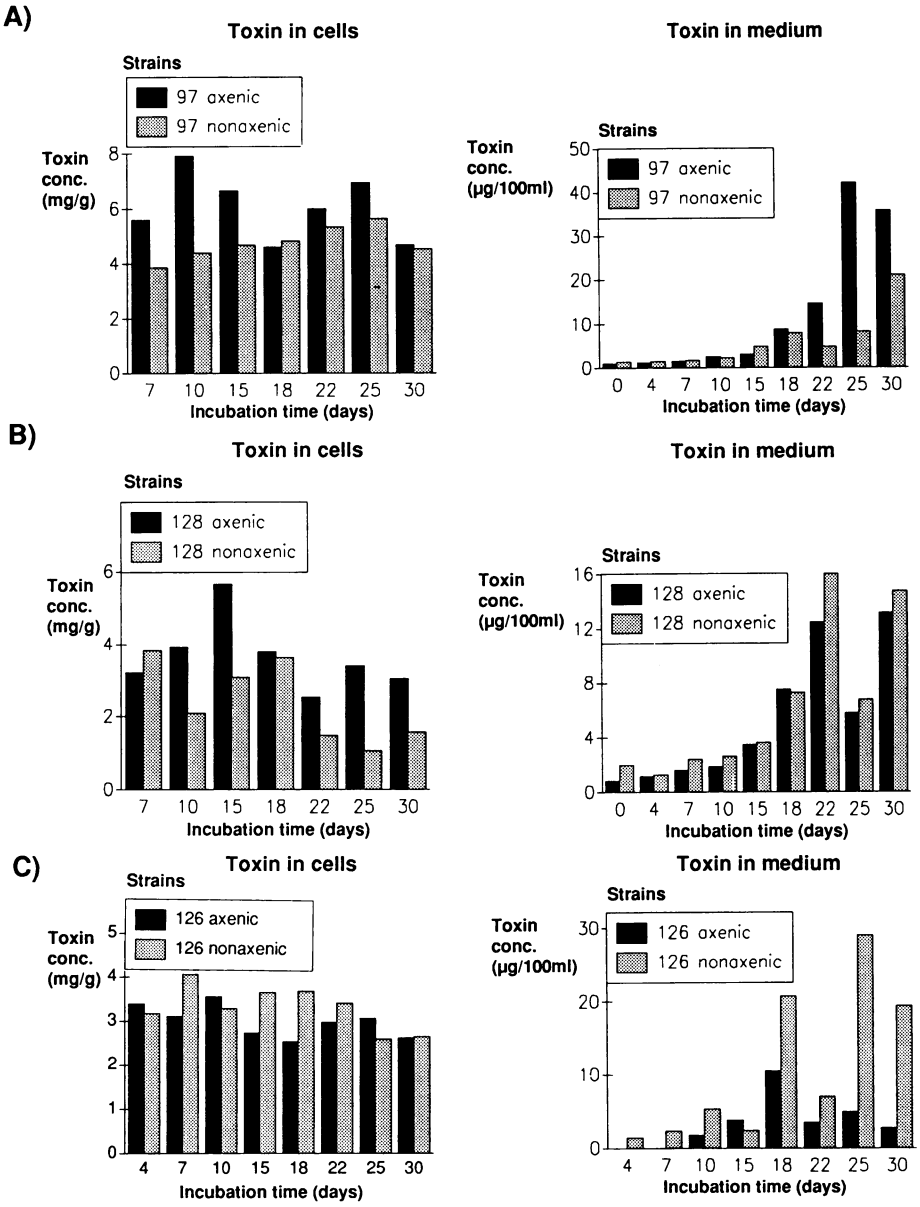

FIG. 2. Toxin production of axenic and nonaxenic *O. agardhii* strains. (A) Left, Toxins in the cells; right, toxin in the medium of strain 97. (B) Left, Toxin in the cells; right, toxin in the medium of strain CYA 128. (C) Left, Toxin in the cells; right, toxin in the medium of strain CYA 126.

cant correlation ($P < 0.001$) with dry weight than with chlorophyll *a* ($P < 0.05$).

Toxin production by axenic strains 97 and CYA 128 was significantly higher ($P < 0.05$) than that by nonaxenic clones. This relationship was not true for strain CYA 126 (Fig. 2).

The effect of temperature on growth and toxin production is shown in Fig. 3. The green *O. agardhii* strain 97 had an optimum temperature 25°C for growth and toxin production. The red strain CYA 128 produced almost equal amounts of toxins at 15, 20, and 25°C. With both strains toxin production was lowest at 30°C.

High nitrogen contents in the culture medium favored both growth of and toxin production by both of the *Oscillatoria* strains studied (Fig. 4). Growth and toxin production did not seem to be affected by phosphorus concentration within the limits of 0.4 to 5.5 mg/liter. At a concentration of 0.1 mg/liter, the growth was poor and inadequate to support high toxin production (Fig. 5).

Light markedly influenced toxin production by both *Oscillatoria* strains. At low light intensities, toxin production was higher than at high light intensities (Fig. 6). The leakage of toxin from the cells was higher at high light intensities (data not shown). The culture of green *Oscillatoria* strain 97 was yellowish at high light intensities. Also, the chlorophyll *a* levels were lower than at low intensities, but the growth on a dry-weight basis was almost equal at all light intensities.

The toxin production levels with different cultures showed similar trends; strain 97 produced more toxins (mean of all

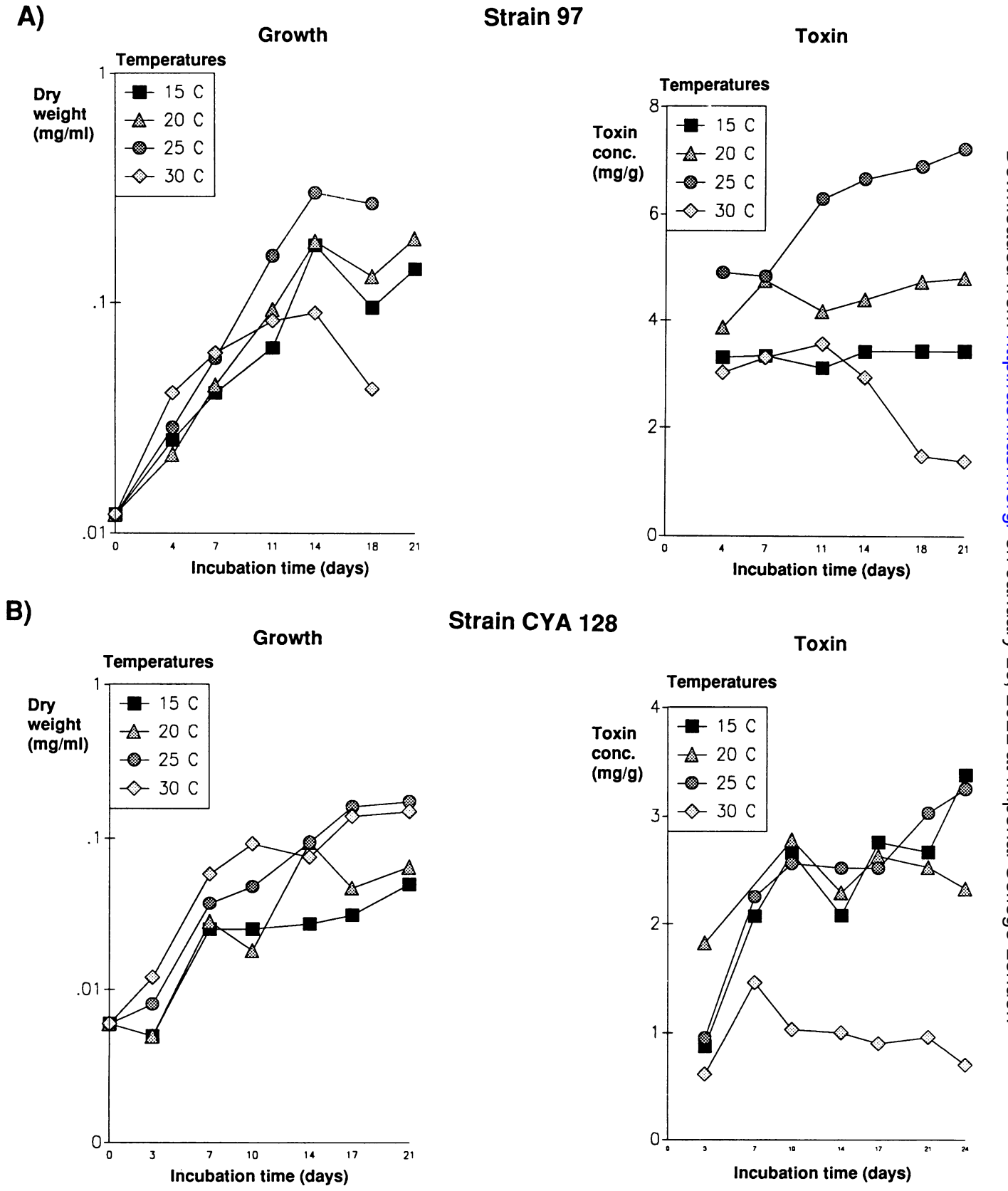

FIG. 3. Effect of temperature on growth of and toxin production by *O. agardhii* strains: (A) 97; (B) CYA 128.

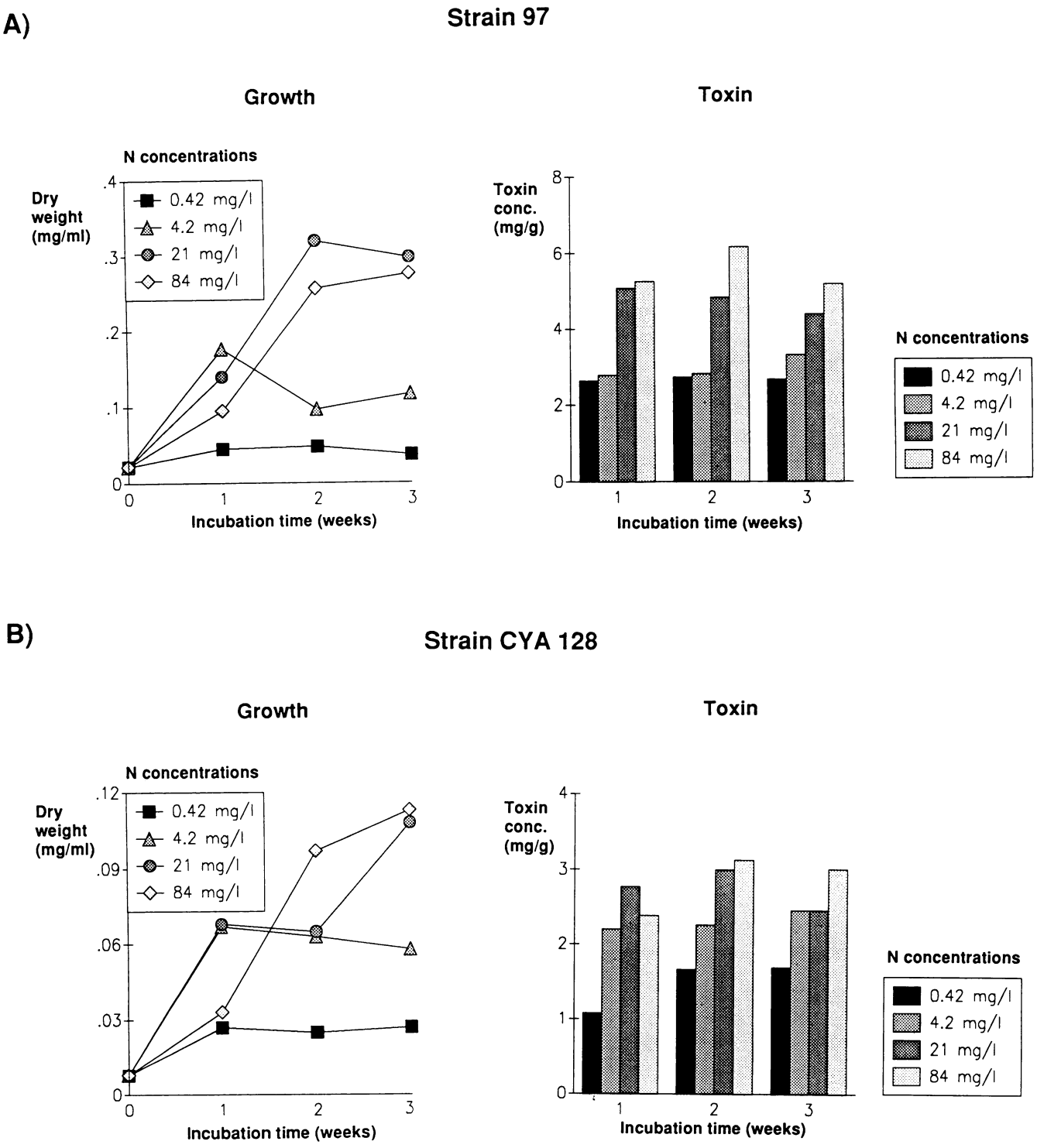

FIG. 4. Effect of nitrogen on growth of and toxin production by *O. agardhii* strains: (A) 97; (B) CYA 128.

experiments, 4.84 mg/g; range, 1.38 to 7.9 mg/g) than CYA 128 (mean, 2.35 mg/g; range, 0.61 to 5.65 mg/g).

Toxin concentration in the cells expressed per unit volume of culture medium also showed that strain 97 (mean of all the determinations, 1,521 μg/liter; range, 6.33 to 2,695 μg/liter) was a better toxin producer than strain CYA 128 (mean, 580 μg/liter; range, 4.35-1,020 μg/liter). The lowest toxin concentrations (<50 μg/liter) were found with strain 97 at the lowest phosphorus concentration and with strain CYA 128 at the lowest concentrations of both phosphorus and nitrogen.

DISCUSSION

Changes in environmental parameters did not induce toxin formation in the nontoxic strain of *O. agardhii* studied, nor

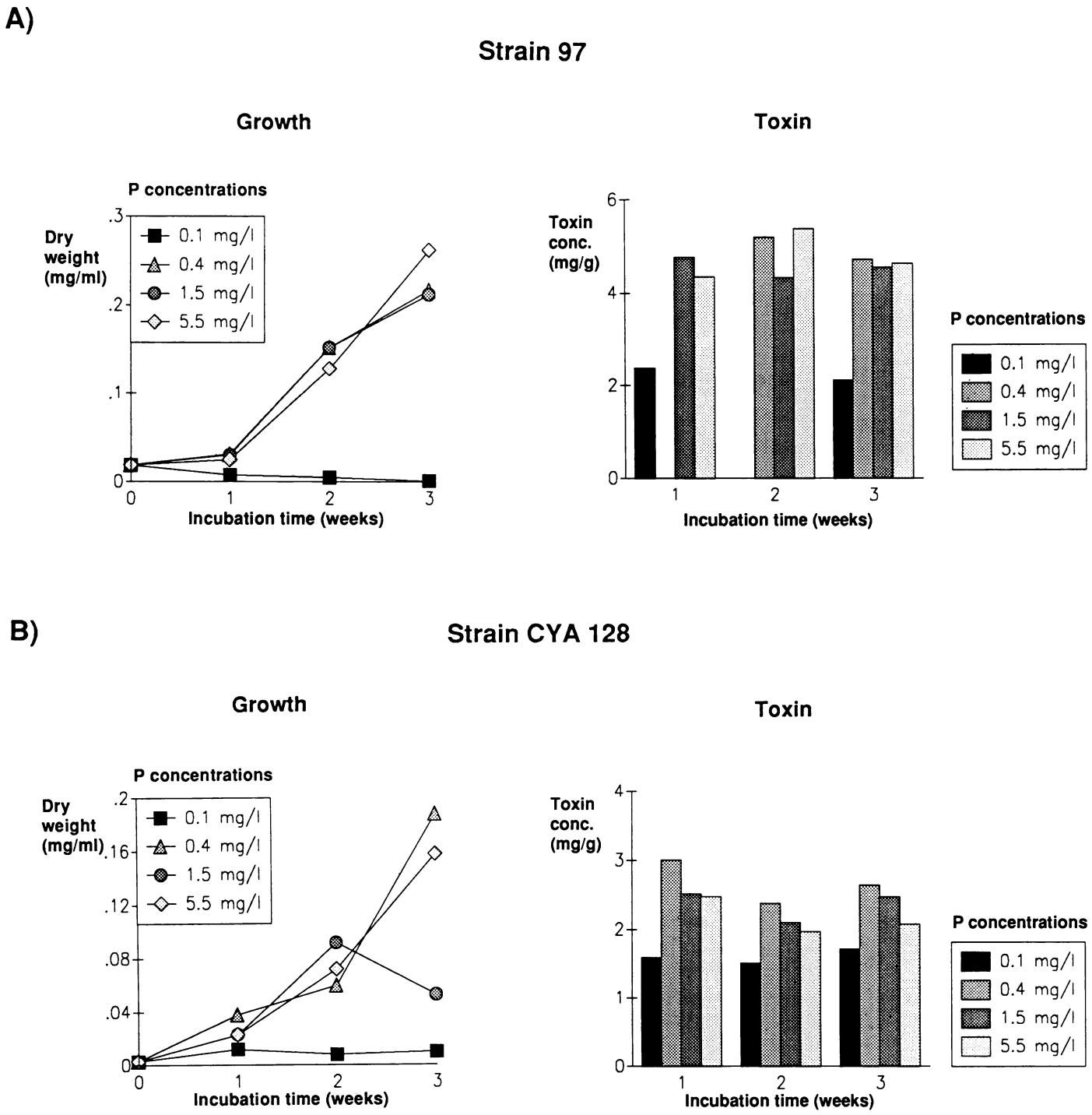

FIG. 5. Effect of phosphorus on growth of and toxin production by *O. agardhii* strains: (A) 97; (B) CYA 128.

did the toxin-producing strains lose their ability to produce toxin under different environmental conditions. This study confirms the fact that hepatotoxins are largely kept within the cells during growth, although leakage of the toxins increased towards the end of the 3- to 4-week growth period. High light intensities and high temperature had adverse effects on growth and promoted toxin leakage from the cells. Hepatotoxins are known to be released into the medium in batch cultures when the cells die (3; J. Kiviranta, K. Sivonen, R. Luukkainen, K. Lahti, and S. I. Niemelä, unpublished data). Coexisting bacteria do not seem to induce hepatotoxin production since with two of three strains the toxin production was higher in axenic cultures.

Toxin production at different temperatures seems to be strain dependent, and for strain 97 the optimum temperature was 25°C, which was the same temperature optimum detected for *M. aeruginosa* by Gorham (5) and Codd and Poon (4). The highest toxin production by *M. aeruginosa* occurred

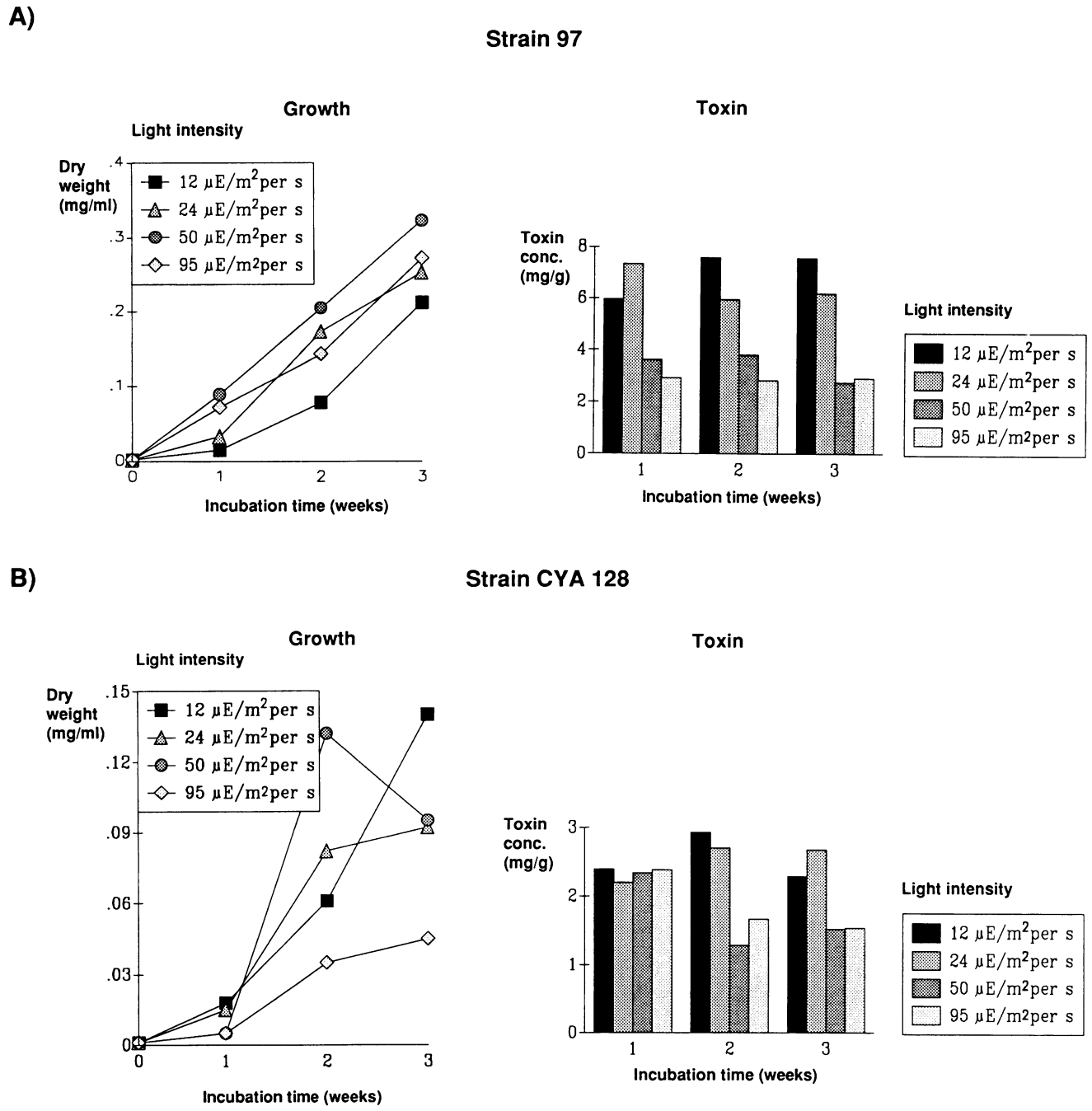

FIG. 6. Effect of light on growth of and toxin production by *O. agardhii* strains: (A) 97; (B) CYA 128.

at 18 to 20°C rather than at higher temperatures (16, 23, 24, 27). Toxin production of *O. agardhii* CYA128 was equally good at 15 through 25°C, while lowest values in both cultures occurred at 30°C, a temperature not found in Finnish lakes.

Van der Westhuizen and co-workers (23, 24) found lower toxin production at very low and high light intensities with an *M. aeruginosa* strain, with an optimum fluence rate for that culture of 145 μmol of photons/m^2 per s. Watanabe and Oishi (27) found 3.7-fold-higher toxicity levels at 75 than at 7.5 microeinsteins/m^2 per s. In contrast, Codd and Poon (4) reported that light intensities of 5 to 50 microeinsteins/m^2 per s and Gorham (5) reported that those of 2,150 to 17,220 lx had no influence on toxin production of *M. aeruginosa* strains they tested. *Oscillatoria* strains in this study showed the highest toxin production at the lowest light intensities. Conflicting results may be due to differences in light sources (spectrum of the light sources), different behavior of strains and species, different culture media used, and/or differences in toxin detection methods. *O. agardhii* is known to prefer low light intensities in nature (11, 15), and it has been shown to occur in mass several meters below the water surface (11). This study indicates that its toxin production under such conditions may be even higher than in higher light intensity.

A direct relationship between the nitrate concentration

and toxin production was observed with both of the *Oscillatoria* strains studied. Similar results were obtained in the studies of Codd and Poon (4), in which they omitted the nitrogen source for *M. aeruginosa* and found 10 times less toxin than in reference cells. A slight reduction in toxin production was also noticed by Watanabe and Oishi (27) with lower nitrogen levels. The effect of nitrogen on toxin production by non-nitrogen-fixing cyanobacterial cultures such as *M. aeruginosa* and *O. agardhii* might be explained logically by the peptide nature of the toxins. Considerably lower levels of phosphorus are needed for toxin production and a saturation level of 0.4 mg of phosphorus per liter was noticed in this study. In lakes, phosphorus is usually the limiting factor, and small changes in the phosphorus levels may influence the growth and toxin production of cyanobacteria.

In all experiments, the green *Oscillatoria* strain (strain 97) produced clearly more toxin than the red strain (CYA 128). Generally, both strains behaved similarly, especially regarding nutrients and light. The most apparent effects were the increases in toxin production increasing nitrate nitrogen concentrations and low light intensities. Toxin concentrations in the cells were usually highest under conditions which also favored growth. Toxin production was lowered by high light intensities and temperatures, as well as by deficiency in nutrients. Thus, toxin production by *O. agardhii* strains is probably not a response to environmental stress conditions, whereas van der Westhuizen and Eloff (22, 23) found that optimum conditions for growth (pH, temperature, and light) did not coincide with those for toxin production by the *M. aeruginosa* culture they studied.

Lindholm et al. (11) measured toxin concentrations by using a high-performance liquid chromatography method similar to that used in this study, in a lake where *O. agardhii* formed in mass. The highest toxin concentration recorded in their study was 37 μg/liter, which was exceeded manyfold by this laboratory study. Only phosphorus and nitrogen deficiency in the cultures kept the toxin concentrations below 50 μg/liter. This indicates that the increase of both nutrients in lakes may markedly promote the growth of and toxin production by toxin-producing *Oscillatoria* strains.

Further studies are needed to evaluate whether other species and strains produce toxins under similar circumstances. Also, the role of other factors such as pH, different N sources, and micronutrients on toxin production should be determined. The insights gained from the laboratory experiments should be tested under field conditions before further conclusions are made. Future studies are also needed to determine the genetic or biochemical factors in regulation of cyanobacterial toxin production.

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