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Leucine incorporation by *Microcystis aeruginosa*

Abstract—In experiments with axenic cultures of *Microcystis aeruginosa,* we tested whether this cyanobacterium incorporates leucine, a compound that is often used for the measurement of heterotrophic bacterioplankton production. *Microcystis* showed significant leucine incorporation, and the uptake of exponentially growing cells was higher than the uptake of cells in stationary growth phase. Therefore, the leucine method may not be suitable for measuring bacterial production in highly eutrophic waters with a dominance of cyanobacteria.

For the measurement of bacterioplankton secondary production, two methods are widespread and commonly accepted today: the incorporation of ^{3}H-thymidine into DNA (Fuhrman and Azam 1980, 1982) and the incorporation of ^{3}H-leucine or ^{14}C-leucine into protein (Kirchman et al. 1985; Simon and Azam 1989). It has been shown that both substrates are specific for heterotrophic bacteria and that eukaryotic algae take up neither thymidine (Fuhrman and Azam 1980) nor leucine (Kirchman et al. 1985). However, the phytoplankton of many highly eutrophic lakes is dominated by cyanobacteria. These prokaryotic organisms may potentially take up a considerable amount of the radioactively labeled substrates used for the determination of bacterial production. In case of the colony-forming, non–nitrogen-fixing *Microcystis* spp. often dominating cyanobacterial blooms, it was shown by microautoradiography (Bern 1985), as well as with axenic cultures (Robarts and Wicks 1989), that thymidine is not used by *Microcystis* at nanomolar concentrations. In contrast, similar experiments have not yet been performed with leucine for this important aquatic organism. The aim of this study was to test the incorporation of leucine by *Microcystis* and the suitability of the leucine method for bacterial production measurement in highly eutrophic waters.

An axenic culture of single cells of *Microcystis aeruginosa* PCC 7806 was used for the uptake experiments with leucine. *Microcystis* was cultured in sterilized Z/4 medium (Zehnder and Gorham 1960) in an incubator at 18.5°C and a constant light flux of 85 μE m^{-2} s^{-1} for 24 h per day. Possible bacterial contamination was checked microscopically by DAPI (4′6-diamidino-2-phenylindole) staining (Porter and Feig 1980), the results were negative. Most of the experiments were done with exponentially growing cells at a chlorophyll *a* concentration of 41 μg Chl *a* L^{-1}. One experiment was carried out using cells of stationary growth phase at a concentration of 890 μg Chl *a* L^{-1}, a similar value to that measured in surface scums of cyanobacteria (e.g., 840 μg Chl *a* L^{-1}; Robarts et al. 1994). The concentration of chlorophyll *a* was determined fluorimetrically according to Wetzel and Likens (1991). Extraction of the homogenized cells was performed using 90% ethanol over a 24 h period. The fluorescence of the extract was measured using a luminescence spectrometer (LS 50 B, Perkin Elmer; excitation 434 nm, emission 665 nm), and values were corrected for phaeophytin.

Leucine incorporation was determined following the bacterial production protocol of Simon and Azam (1989). Triplicates of 5 ml of the cultures and triplicates of formalin-killed controls (3.7%, final concentration) were pipetted into sterile glass bottles and spiked with 0.45 μl ^{14}C-leucine (11.1 MBq μmol^{-1}, Sigma, 30 nM final concentration) under sterile conditions in a clean bench. After incubation for 1 h in the dark at 20°C, incorporation was stopped with formalin, and 0.6 ml 50% TCA (trichloracetic acid) was added to each bottle. Samples were filtered onto 0.2-μm Nuclepore filters, filters were rinsed twice with 1 ml 5% TCA and once with distilled water. After adding 10 ml Ultima Gold (Packard) to each scintillation vial, radioactivity was measured using a liquid scintillation analyzer (1600 TR, Packard).

The experiment was repeated under modified incubation conditions that were designed to alter the incorporation rate. Higher leucine concentrations of 50 and 100 nM were used to test the linearity of uptake. In another experiment, sample processing was altered to include boiling for 30 min and cooling after the addition of 50% TCA and before filtering. This protein precipitation step is commonly included when measuring bacterial production and could also influence the incorporation by *Microcystis.* Furthermore, we tried to make leucine unattractive as a carbon and nitrogen source for *Microcystis.* To accomplish this, samples were treated either by (1) incubation in the light (166 μE m^{-2} s^{-1}) to promote autotrophic carbon fixation, (2) nitrogen addition as ammonium molybdate ($H_{24}Mo_7N_6O_{24} \times 4H_2O$, to give 20 mg N L^{-1} in addition to the nitrate-N in the medium), or (3) both light incubation and nitrogen addition. To test the influence of the growth phase, one experiment was carried out with cells from stationary growth phase at 50 nM leucine concentration.

Leucine uptake by exponentially growing *Microcystis aeruginosa* at 30 nM leucine concentration with cold TCA extraction was significantly higher in live samples (27.09 ± 2.70 μmol (g Chl *a*)$^{-1}$ h^{-1}, mean ± SE, $n = 3$) than in formalin-killed controls (0.076 ± 0.008 μmol (g Chl *a*)$^{-1}$ h^{-1}) (*t*-test, $p = 0.001$). In all experiments, the blank values were always below 0.6% of the leucine uptake by the live samples. Although the leucine incorporation at 50 nM was only slightly higher than at 30 nM, the high values at 100 nM indicates that the leucine incorporation by *Microcystis* was not saturated below 100 nM (Fig. 1). Exposure of samples to light resulted in an increase of leucine incorporation (Fig. 2). In contrast, nitrogen addition decreased the incorporation. The combination of light and nitrogen addition caused a slightly lower incorporation than in the control, and the stimulating influence of light almost compensated for the inhibiting influence of nitrogen. A one-way ANOVA showed significant differences between groups ($p = 0.0002$). Using Tukey's post hoc test, differences were significant at $p =$ 0.05 level between control and light and between control and

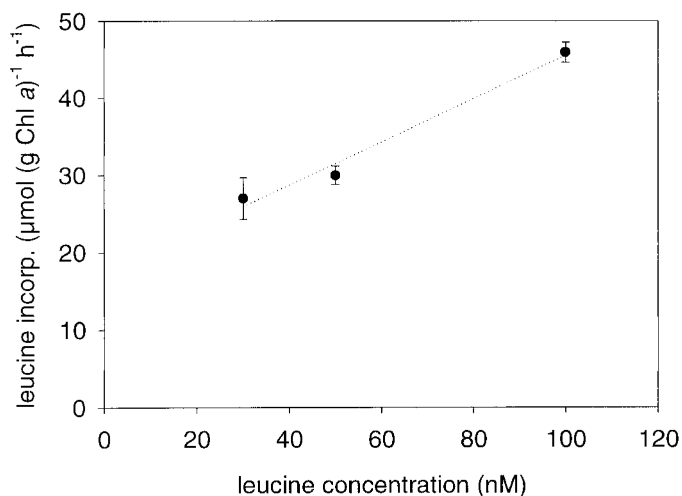

Fig. 1. Leucine incorporation by *Microcystis aeruginosa* (corrected for killed controls) as a function of leucine concentration (bars, standard error).

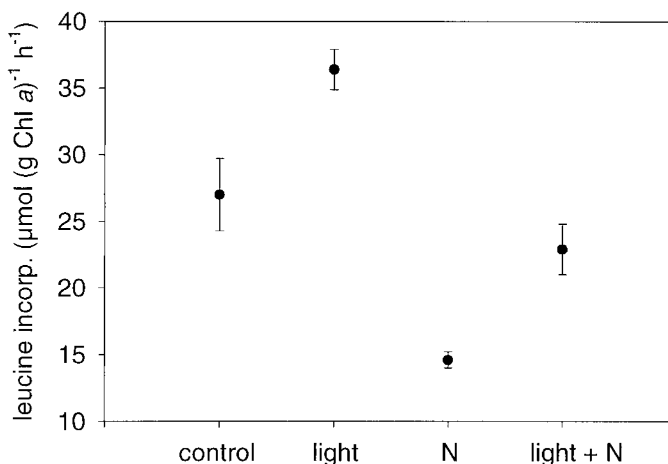

Fig. 2. Leucine incorporation by *Microcystis aeruginosa* (corrected for killed controls) under varying incubation conditions (N, nitrogen; bars, standard error).

nitrogen addition but not between control and the combination of light and nitrogen addition. Hot extraction of samples with TCA decreased leucine incorporation significantly to 14.0 $\pm$ 1.6 μmol (g Chl a)$^{-1}$ h^{-1} or by almost 50% compared to cold TCA extraction (t-test, $p = 0.014$). Leucine uptake of *Microcystis* cells in stationary growth phase was only 2.01 $\pm$ 0.03 μmol (g Chl a)$^{-1}$ h^{-1}, approximately 15 times lower than the uptake of exponentially growing cells.

The significant leucine uptake by *Microcystis* is contradictory to previous suggestions that leucine is a specific substrate for heterotrophic bacteria (Hollibaugh 1994). Other microorganisms besides heterotrophic bacteria were assumed to be of little importance with regard to the uptake of amino acids up to a concentration of 100 nM (Riemann and Azam 1992). Regarding cyanobacteria, Kirchman et al. (1985) did not find uptake by coccoid forms (although at only 0.5 nM final concentration). Torreton and Dufour (1996) tested *Synechococcus* (at 24 nM final concentration) with the same result. To our knowledge, no experiments with *Microcystis* have been carried out thus far, and the results of the present study are the first record of leucine uptake by *Microcystis.*

In contrast to the supposition that heterotrophic leucine uptake should be suppressed when autotrophic carbon fixation is promoted by irradiation, the rate of leucine incorporation by *Microcystis* was higher in the light than in the dark. Hence, the use of leucine increased when cells were actively growing. This is in accordance with our findings of much higher incorporation rates by cells in the exponential growth phase than in the stationary growth phase. This phenomenon might be explained by a higher substrate demand of actively growing cells and the increased toxin production by cyanobacteria during growth. The production of microcystin LR was observed to be highest during the exponential growth of *Microcystis* both in laboratory experiments (Watanabe and Oishi 1985; Orr and Jones 1998; Rivasseau et al. 1998) and in a highly eutrophic lake (S. Jähnichen, unpubl. data). Leucine is a constituent of microcystin LR and one component in its biosynthesis (Rinehart et al. 1994). Therefore, exponentially growing and microcystin producing *Microcystis* show a higher specific incorporation rate of leucine.

Heterotrophic bacterial production can be overestimated due to leucine uptake by *Microcystis* if the water sample contains considerable numbers of this cyanobacterium. The assumptions for the calculation of bacterial production (e.g., 7.3% molar percentage of leucine in protein, 63% protein content of dry weight; Simon and Azam 1989) applied to heterotrophic bacteria cannot be used for the calculation of a specific bacterial production of *Microcystis.* But we suggest the production overestimated by the leucine uptake of *Microcystis* should be corrected for this nonbacterial production. By using the equations of Simon and Azam (1989) and an isotope dilution of 2, this would result in a *Microcystis* overestimation of 0.043 mg C (mg Chl a)$^{-1}$ h^{-1} for exponentially growing *Microcystis* (at 30 nM and using hot TCA extraction) and 0.006 mg C (mg Chl a)$^{-1}$ h^{-1} for *Microcystis* in stationary growth phase (at 50 nM and using cold TCA extraction), respectively. Assuming a *Microcystis* biomass of 100 mg Chl a m^{-3} in a highly eutrophic lake (e.g., Robarts et al. 1994 for total cyanobacteria), an overestimation of 4.3 mg C m^{-3} h^{-1} and 0.6 mg C m^{-3} h^{-1} would result from *Microcystis* in exponential and stationary growth phase, respectively. A heterotrophic bacterial production of about 3 mg C m^{-3} h^{-1} (that is 422 mg C m^{-2} d^{-1} at 6-m lake depth; Robarts et al. 1994) would be seriously overestimated due to the additional leucine uptake by *Microcystis* in the first case (by 143%) while the overestimation would be rather low in the second case (by 20%). However, the high value of exponentially growing *Microcystis* represents a maximum estimation that is rarely achieved in lakes since *Microcystis* does not grow exponentially all the time and particularly not during blooms at maximum biomass. Furthermore, specific leucine uptake should be lower in large *Microcystis* colonies than in single cells. Therefore, the actual leucine uptake by *Microcystis* in lakes should be in the range of the lower value. Nevertheless, the leucine method may not be suitable for bacterial production measurement in highly eutrophic waters dominated by cyanobacteria.

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