NOTE

Nodularia spp. (Cyanobacteria) incorporate leucine but not thymidine: importance for bacterial-production measurements

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ABSTRACT: Six strains of *Nodularia* spp., both toxic and non-toxic, were tested for their ability to incorporate leucine and thymidine. All axenic cyanobacterial strains studied showed high leucine incorporation (1.4 to 12 pmol µg chl a^{-1} h^{-1} at a concentration of 212 nM leucine), whereas thymidine was either taken up at a very low rate or not at all (0 to 0.2 pmol µg chl a^{-1} h^{-1} at a concentration of 121 nM thymidine). We therefore recommend using the thymidine incorporation method instead of that of leucine for measuring heterotrophic bacterial production during cyanobacterial blooms.

KEY WORDS: Leucine · Thymidine · Bacterial production · Cyanobacteria

Bacterial production is routinely measured in aquatic ecosystems. The most commonly used methods nowadays are the ^{3}H-thymidine incorporation method by Fuhrman & Azam (1980), and the [3]H- or ^{14}C-leucine incorporation method by Kirchman et al. (1985). The methods have been criticised and subsequently modified, but the principles behind them have not dramatically changed. Azam & Fuhrman (1984) summarised the widely accepted criteria for an exemplary method to estimate bacterial production. They stated that the method used should be specific for heterotrophic bacteria and it should not rely on balanced growth. Furthermore, the growth rates should not be affected by procedures involved in measurements, and if any conversion factors are needed, these should be easy and reliable to produce.

The ^{3}H-thymidine method is based on measuring DNA production and consequently, cell division. Thymidine has only 1 function in a cell, namely participation in DNA synthesis, which makes it a good tool in bacterial production measurements. Several authors have demonstrated that thymidine in nanomolar concentrations is exclusively taken up by heterotrophic bacteria, not by cyanobacteria, algae or zooplankton (Fuhrman & Azam 1982, Bern 1985, Robarts & Wicks 1989, Pedrós-Alió et al. 1993, Lehtimäki et al. 1997). However, it has also been revealed that not all heterotrophic bacteria can incorporate thymidine (Pollard & Moriarty 1984, Davis 1989, Jeffrey & Paul 1990, Robarts & Zohary 1993). In addition, the method relies on using conversion factors when translating the thymidine incorporation into cells created and further, the cells to biomass (carbon and nitrogen) produced. These conversion factors have been shown to vary largely in different environments and there is still controversy as to the best method for generating such factors. In addition to these major drawbacks, questions have also arisen about unspecific labelling after degradation of ^{3}H-thymidine in cells (Pollard & Moriarty 1984, Riemann 1984), proper methods to extract and purify DNA from cells (reviewed by Robarts & Zohary 1993), intra- and extracellular dilution of the isotope (Moriarty 1986, Jeffrey & Paul 1988) and the role of viral DNA synthesis in infected cells (Bratbak et al. 1992).

Because of these uncertainties with the thymidine method, the leucine incorporation method has gained more prevalence in recent years. The [3]H- or, nowadays more commonly, ^{14}C-leucine method is based on measuring protein synthesis in the cells. The main advan-

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Table 1. Cyanobacterial strains used in this study and their observed uptake velocities (avg. ± SD; uptake velocities observed for Baltic coastal seawater [2.4 × 10⁶ heterotrophic bacterial cells ml⁻¹] were 650 pM h⁻¹ for leucine and 175.1 pM h⁻¹ for thymidine). Species identification for *Nodularia* spp. according to Sivonen et al. (1989) and Laamanen et al. (2001). Z8: original growth medium; Z8XS: modified growth medium; /4: ¼ of respective growth medium

Species	Strain	Nodularin production	Geographical origin	Chl *a* (µg l⁻¹)	Growth medium	Avg. max observed uptake velocity (pmol µg chl *a*⁻¹ h⁻¹)	
						Leucine	Thymidine
Nodularia baltica	BY1	+	Baltic Sea (plankton)	67	Z8XS	6.4 ± 0.93	0.16 ± 0.10
Nodularia sp.	PCC 7804	+	Dax, France (thermal spring)	83	Z8XS	9.9 ± 0.98	0.02 ± 0.04
				71	Z8XS/4	2.6 ± 0.52	0.05 ± 0.01
Nodularia harveyana	Hübel 1983/300	–	Baltic Sea (benthic microbial mat)	47	Z8XS	2.1 ± 0.68	0.10 ± 0.06
				62	Z8XS/4	3.8 ± 0.15	0.04 ± 0.03
Nodularia sphaerocarpa	UP16f	–	Baltic Sea (plankton)	105	Z8XS	1.4 ± 0.10	0.02 ± 0.04
Nodularia sphaerocarpa	HKVV	–	Baltic Sea (water)	72	Z8XS	1.7 ± 0.11	0.04 ± 0.03
				66	Z8XS/4	9.4 ± 1.69	0.12 ± 0.04
Nodularia sphaerocarpa	PCC 73104/1	–	Spotted Lake, Canada (alkaline soil)	67	Z8XS	7.5 ± 2.25	0.02 ± 0.20
				58	Z8XS/4	12.1 ± 0.49	0.04 ± 0.03
Microcystis aeruginosa	PCC 7806	+	Braakman Reservoir, The Netherlands (water)	61	Z8	8.4 ± 0.54	0.28 ± 0.12
				61	Z8/4	4.0 ± 0.07	0.15 ± 0.02

tage of the leucine over the thymidine method is that for measuring heterotrophic bacterial carbon production, no experimental conversion factors are needed, as the amount of leucine in bacterial proteins, the amount of protein per cell and the relation of carbon to protein are all constant in marine bacterial assemblages (Simon & Azam 1989). Furthermore, the leucine method is an order of magnitude more sensitive than the thymidine method, thus allowing measurements in low-activity environments, and the isotope dilution can be measured relatively easily (Simon & Azam 1989). Leucine in nanomolar concentrations was also considered to be taken up exclusively by heterotrophic bacteria (Kirchman et al. 1985, Riemann & Azam 1992), until Kamjunke & Jähnichen (2000) reported significant leucine incorporation of axenic culture of the cyanobacterium *Microcystis aeruginosa* strain PCC 7806. They concluded that measurements with leucine in waters dominated by *M. aeruginosa* might yield overestimates of bacterial production. To date, only this one cyanobacterium strain belonging to the unicellular non-heterocystous genus has been shown to incorporate leucine. We tested whether the filamentous, heterocystous *Nodularia* spp., cyanobacteria that bloom annually in the Baltic Sea, also incorporate leucine, and whether thymidine incorporation could be recommended as a method of choice during such a bloom.

Materials and methods. Leucine and thymidine incorporation of 6 axenic nodularin-producing and non-toxic *Nodularia* spp. strains were tested (Table 1). The *Microcystis aeruginosa* strain PCC 7806 was used as a positive control for leucine incorporation (Kamjunke & Jähnichen 2000), and coastal seawater (ca. 2.4 × 10⁶ heterotrophic bacterial cells ml⁻¹) was used as positive control for both leucine and thymidine incorporation. The axenity of strains was confirmed with an epifluorescence microscope (Leitz Aristoplan) after acridine orange staining. The *Nodularia* spp. strains were grown in a modified Z8 medium with salt and without nitrogen (Sivonen et al. 1989), and the *M. aeruginosa* strain was grown in an original Z8 medium without salt and with nitrogen (Rippka 1988 and references therein). In addition, 5 of these strains were grown in media that contained only ¼ of the components of original growth media in order to find out whether possible substrate incorporation was related to nutrient status of the growth media. Cultures were incubated in a programmable growth cabinet (Sanyo) under illumination of 100 µmol s⁻¹ m⁻² on an 18:6 h light:dark cycle. Temperature was 25°C during the daytime and 20°C during the night. Incorporation experiments were done using exponentially growing cultures that were diluted with sterile growth media to about 50 to 100 µg chl *a* l⁻¹. The chlorophyll *a* (chl *a*)

concentrations of the final dilutions (Table 1) were measured from 50 ml samples filtered on glass-fibre filters (GF/F, Whatman). Pigments were extracted (24 h, 4°C) with 10 ml of 94% (v/v) ethanol and determined fluorometrically (Perkin Elmer LS-2).

To evaluate leucine and thymidine uptake rates, each cyanobacterial strain (grown in both nutrient-rich and nutrient-poor media), as well as the control seawater (ca. 2.4×10^6 heterotrophic bacterial cells ml^{-1}), was treated separately (no dual labelling) with 3 concentrations of the 2 substrates (69, 121 and 212 nM for leucine [Amersham, 295 mCi mmol^{-1}]; 13, 30 and 121 nM for thymidine [PerkinElmer Life Sciences, 85.7 Ci mmol^{-1}]. These ranges cover the isotope concentrations most often used in pelagic samples in both oligotrophic and eutrophic environments. Samples of each strain (5 ml; 3 replicates, 1 blank [killed with formalin before adding the isotope]) were incubated with the substrates in autoclaved scintillation vials in darkness at room temperature for 1 h. After terminating the incubations with formalin, the samples were filtered immediately (leucine) or within 20 h (thymidine) in ice-cold conditions on cellulose-nitrate filters (Ø 25 mm, pore size 0.2 μm, Sartorius) that had been briefly soaked in ice-cold 5% (w/v) TCA (trichloro-acetic acid) before use. Sample vials were rinsed with 2 ml of ice-cold 5% TCA and these solutions were added on filters. Then, filtration funnels were rinsed with 2 ml of ice-cold 5% TCA and removed. Cells on the filters were extracted 5 times with 1 ml of ice-cold 5% TCA (Riemann 1984, Børsheim 1990) and then the filters were placed in scintillation vials. To dissolve the filters, 10 ml of scintillation cocktail (Insta-Gel Plus, Packard) was added into vials that were left to stand at room temperature overnight. The next day, the vials were shaken vigorously before assaying the radioactivity in a scintillation counter (Wallac Win-spectral 1414).

Results and discussion. All strains studied incorporated leucine (Fig. 1). The incorporation rate varied between the strains and it was significantly (p < 0.01) higher for *Nodularia* sp. starved of nutrients (nutrient-poor media) than for *Nodularia* sp. grown in abundant nutrient conditions in one case (Strain HKVV), and significantly lower in another (Strain PCC 7804) of the 4 *Nodularia* spp. strains tested. For the *Microcystis aeruginosa*, the incorporation rate was significantly higher in the culture grown in abundant nutrient conditions (Fig. 1). Evidently, most of the strains were not saturated even at the highest leucine concentration used (Fig. 1a–c,e,f). The blank values of cyanobacteria samples were on average 45% of the sample values (disintegrations per minute [DPM]-difference between blanks and samples ranging from 52 to 2685 DPM, average 699 DPM) whereas the blanks were 15% of

the sample values in seawater (DPM-difference between blanks and samples 1792 to 2115 DPM, average 1965 DPM). High blank values may be a result of the isotope adsorption to the cyanobacterial surfaces that often are covered with mucus (Schuster et al. 1998). Adsorption can be expected to be similar in both blanks and samples, and hence blank subtraction provides a reliable elimination of adsorbed (not incorporated) leucine in calculations. Such adsorption might also explain why the cyanobacterial cultures were not saturated even at the high leucine concentrations used. Only after all the adsorption sites are occupied will there be free leucine left for uptake (Schuster et al. 1998). Under such non-saturated conditions internal and external isotope dilution can also be expected to occur, and the measured values may be underestimates of the real uptake capacity.

On the contrary, thymidine was either incorporated at a very low rate (Fig. 2a,c,d,f), or not incorporated at all (Fig. 2b,e), blanks giving higher values than the samples. Some of the strains seemed not to be saturated even at the extremely high thymidine concentration used, although the incorporation rate was very low (Fig. 2a,c,d,f). The blank values were on average 89% of the cyanobacterial sample values, while in seawater they were 9% of the sample. The DPM values after blank subtraction averaged 338 at the lowest and 3334 at the highest incubation concentration when negative values were included, and 555 and 4187, respectively, when negative values were marked 0. The corresponding average values in the seawater sample (ca. 2.4×10^6 heterotrophic bacterial cells ml^{-1}) were 35 979 at the lowest (13 nM) and 119 385 at the highest (121 nM) incubation concentration. The adsorption to mucus has been shown to be even more dramatic for thymidine than for leucine (Schuster et al. 1998), giving a credible explanation to non-saturable uptake kinetics recorded in this experiment.

The leucine incorporation per μg chl *a* varied from 0.95 to 12.4 pmol h^{-1} at the highest incubation concentration (212 nM). Some strains clearly were not saturated and therefore the observed uptake rates cannot be considered maximal. A conservative estimate of the effect of cyanobacteria on the heterotrophic bacterial production measurements using the leucine method can be calculated. In late summer 1990 (1.8 μg chl *a* l^{-1}), heterotrophic bacteria incorporated 90 pM leucine h^{-1} in the Gulf of Finland (Heinänen & Kuparinen 1992). In the Gulf of Finland, late summer cyanobacterial blooms have been shown to reach concentrations of up to 18 μg chl *a* l^{-1} (Heinänen et al. 1995). At such a chl *a* concentration, the leucine incorporation of cyanobacteria (17 to 218 pM h^{-1}) would be equal to or even higher than that of the heterotrophic bacteria. Kamjunke & Jähnichen (2000) calculated the overesti-
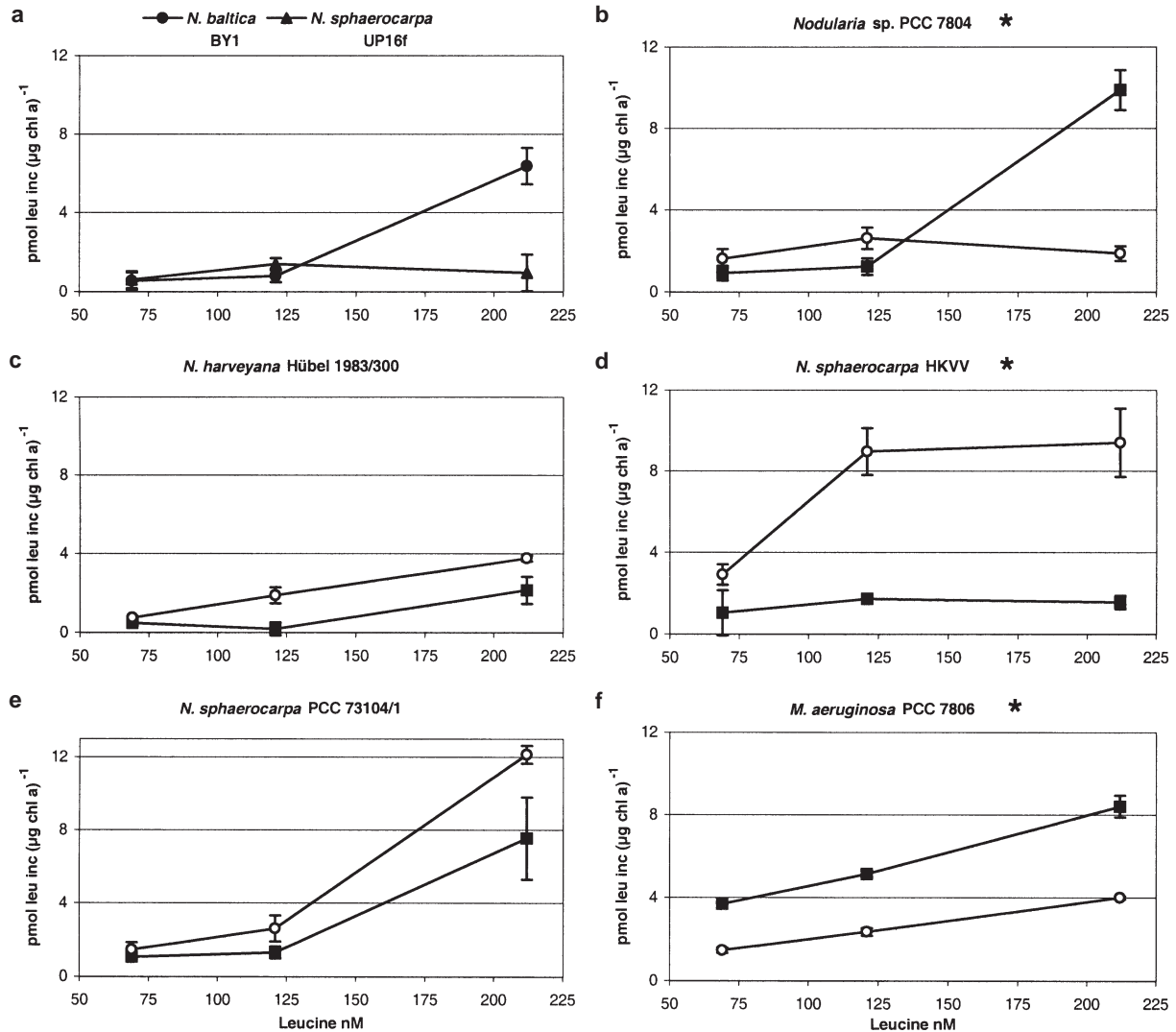
Fig. 1. Cyanobacterial (*Nodularia* spp. and *Microcystis* sp.) leucine uptake velocity (pmol leucine µg chl a^{-1} h^{-1}) at various leucine concentrations. Cyanobacteria grown in nutrient-rich (■) and nutrient-poor (O) growth media. Error bars ± SD. *Significant (p < 0.01) differences in uptake between the cultures grown in nutrient rich and nutrient poor media

mate caused by a freshwater *Microcystis aeruginosa* bloom in a eutrophic lake to be in the same range (20 to 140%). They pointed out that during maximum biomass, cyanobacteria often are already senescent, and therefore less likely to actively incorporate leucine, but that they nevertheless may cause significant overestimation of heterotrophic production. In our experiments the *M. aeruginosa* strain incorporated leucine at a rate 10% of that measured by Kamjunke & Jähnichen (2000). Obviously the growth conditions (nutrients, light, growth phase) have a large effect on incorporation. We measured the cyanobacterial leucine and thymidine incorporation in the dark, as this is the routine method for measuring heterotrophic bacterial production. Kamjunke & Jähnichen (2000) reported

enhanced leucine incorporation by *M. aeruginosa* in the light, and this effect must be kept in mind if light incubations for bacterial production measurements will begin on a routine basis as suggested by Morán et al. (2001).

Kamjunke & Jähnichen (2000) suggested that the leucine uptake by *Microcystis aeruginosa* PCC 7806 could be related to the toxin production, as leucine is one constituent of microcystin-LR. Several strains of *Nodularia* spp. produce the toxin nodularin, which does not contain leucine, and all the *Nodularia* spp. strains tested here incorporated leucine independent from their ability to produce nodularin. Leucine did not seem to be taken up as a source of nitrogen or carbon either as the uptake was both significantly higher
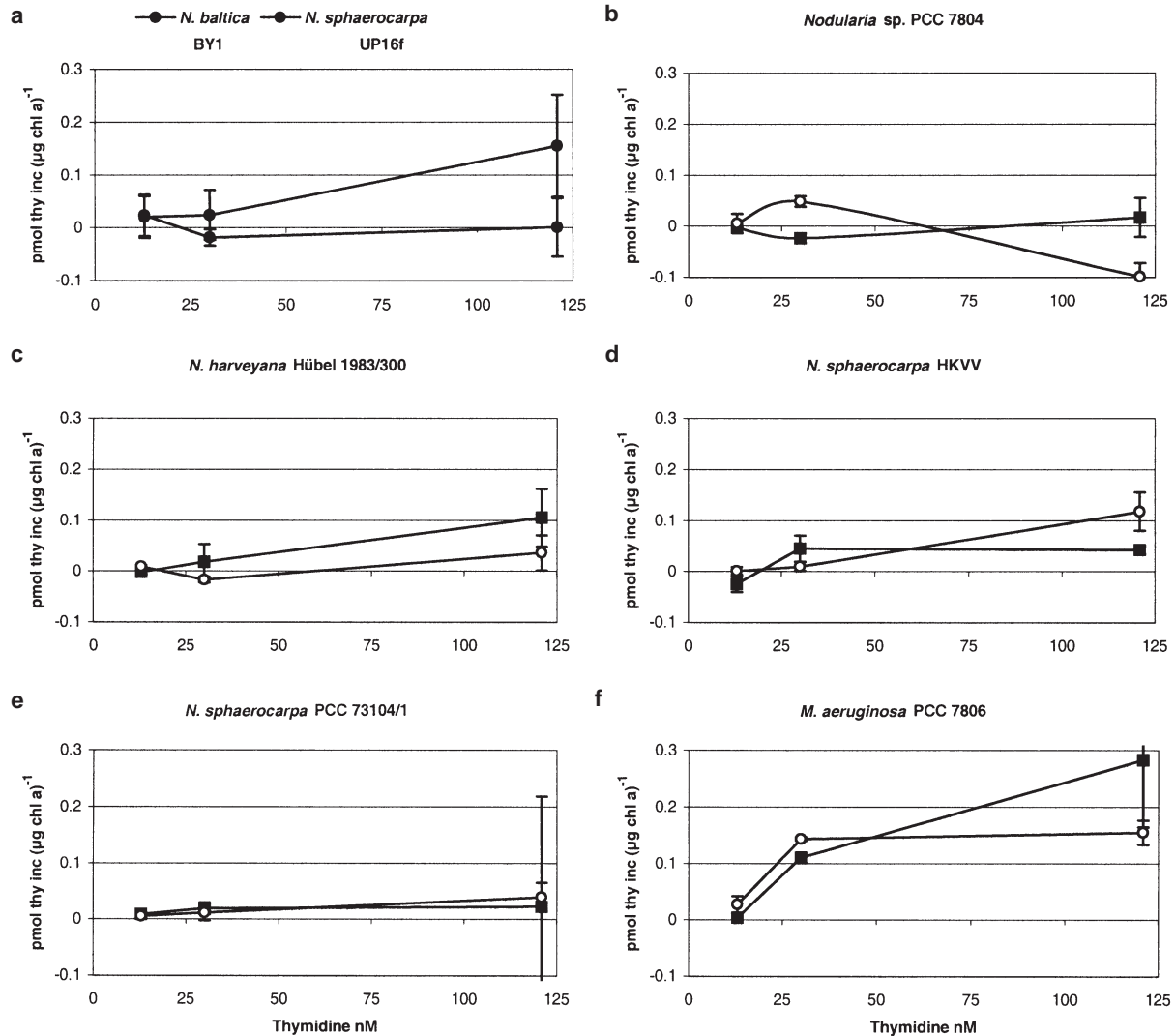
Fig. 2. Cyanobacterial (*Nodularia* spp. and *Microcystis* sp.) thymidine uptake velocity pmol thymidine µg chl a^{-1} h^{-1} at various thymidine concentrations. Cyanobacteria grown in nutrient-rich (■) and nutrient-poor (O) growth media. Error bars ± SD

(*N. sphaerocarpa* HKVV) and significantly lower (*Nodularia* sp. PCC 7804, *M. aeruginosa* PCC 7806) in cultures starved of nutrients compared to those grown in abundant nutrient conditions. We therefore conclude that cyanobacteria incorporate this small amino acid to use it as a building block for other cell products, such as proteins, as do heterotrophic bacteria. So far, the only cyanobacteria shown to incorporate leucine are *Nodularia* spp. (this study) and *Microcystis* sp. (Kamjunke & Jähnichen 2000, this study). Experiments with *Synechococcus* sp. showed no incorporation at a leucine concentration of 24 nM (Torreton & Dufour 1996), nor was incorporation detected in experiments with coccoid cyanobacteria at 0.5 nM concentration (Kirchman et al. 1985). However, more information is needed on potential leucine incorporation rates of

other common cyanobacterial species, such as *Anabaena* spp. and *Aphanizomenon* spp. More experiments using axenic strains should be performed using leucine concentrations relevant to eutrophic environments, as it is possible that leucine is taken up by bloom-forming cyanobacteria other than *Microcystis* sp. and *Nodularia* spp. Obviously, the leucine incorporation method should not be used during cyanobacterial blooms.

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