PHYSIOLOGICAL STUDIES OF TWO STRAINS OF *SYNECHOCOCCUS* FROM FLORIDA BAY

by

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Cyanophycin is a nitrogen storage compound produced by some cyanobacteria, but not believed to be synthesized by marine *Synechococcus*. Two strains of *Synechococcus* (95021415 S1 and 95032005 WR 22 B6) from Florida Bay were tested for cyanophycin production. Strain 95021415 S1 did not produce cyanophycin. Strain 95032005 WR 22 B6 produced cyanophycin when growth was phosphorous-limited and under conditions of nutrient sufficiency when growth was inhibited by chloramphenicol. Growth of the two strains under different conditions of temperature and irradiance was also investigated.

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Introduction

Florida Bay is a unique ecosystem located between the southern coast of Florida and the Florida Keys; it is bounded on the northern side by the Everglades and on the southern side by coral reefs, and includes vast sea grass beds and many unique species of plants and animals. Since 1990, blooms of **cyanobacteria*** and **diatoms** have occurred regularly in Western and Central Florida Bay (Florida Bay News, 1996). It has been hypothesized that the cause of these blooms is the **eutrophication** of Florida Bay due to increased agricultural and human waste input into the watershed. Florida Bay is one of many habitats that has experienced eutrophication through the draining of swamps for development (Myers and Ewel, 1990).

Roughly 80% of Florida Bay (1800 square kilometers) forms the open-water portion of the Everglades National Park. It is greatly influenced by land use practices within the park and elsewhere in the Florida peninsula because most of Florida Bay's freshwater supply flows out to the bay through the freshwater marsh and the rest of the park (McIvor et. al., 1994). The inflow of fresh water to Florida Bay has decreased in recent years due to diversion of water for urban use and/or flood control, resulting in an increase in the salinity of Florida Bay (McIvor et. al., 1994). Much of the aquifer which underlies Florida drains into Florida Bay and mixes with seawater from the Gulf of Mexico, forming an environment of dilute seawater (Myers and Ewel, 1990).

As farming in south Florida increases, so does the input of agricultural

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*Terms in boldface are defined in the glossary at the end of the thesis.

wastes and pesticides into the aquifer and, ultimately, into Florida Bay. Similarly, the human population of Florida is increasing rapidly, and with new housing developments and encroachments onto previously undeveloped land comes more pollution of Florida's waters in the form of sewage, pesticides, thermal pollution, and a host of other factors (Myers and Ewel, 1990). Sewage and drainage from septic tanks generally increases nutrient levels, particularly of nitrogen. This excess of nitrogen and other nutrients required for growth favors **primary producers** which can store nitrogen until other nutrients become available.

The main anthropogenic inputs to Florida Bay consist of phosphorous and nitrogen (Lapointe and Clark, 1992). It has been hypothesized that these inputs consist of alternating phosphorous-rich agricultural inputs and nitrogen-rich sewage and waste water inputs. If this theory is true, organisms with the ability to store nitrogen and/or phosphorous could be at an advantage compared to organisms that do not form storage compounds because the level of nutrients and the ratio of nutrients relative to one another fluctuates greatly in the environment of Florida Bay. Organisms that are able to store nutrients will be better able to utilize pulses of nutrients because each cell has a certain quota of each essential nutrient that is necessary for cell division; if the cells can store nutrients, they are better able to fill all of their nutrient quotas even during nutrient pulses. These organisms can utilize more nutrients per cell than other organisms because they have stored excess nutrients. Therefore during a pulse they can utilize as much of the "pulsed" nutrient as they can combine with their stored nutrients in order to meet their nutrient quotas, so they can divide more than cells without stored nutrients.

The dominant forms of cyanobacteria in the Florida Bay algal blooms belong to the genus *Synechococcus*, which includes a large number of the different types of unicellular cyanobacteria. *Synechococcus* species are generally coccoid (spherical) or rod-shaped, unicellular organisms that divide in a single plane; they range in size from approximately 0.8 µm to 3.0 µm. While little is known about the physiology of the species and genotypes of *Synechococcus* that occur in Florida Bay, many cyanobacteria are capable of forming intracellular inclusions for the storage of nitrogen, phosphorous, glycogen, and other compounds (Allen et. al., 1984). Cyanophycin is one such storage product (Allen et. al., 1984). Cyanophycin is organized in the form of a chain of aspartate with arginine branches (Simon and Weathers, 1976). The formation of cyanophycin is induced by the depletion of nutrients other than nitrogen, and the main function of cyanophycin is probably to store nitrogen (Allen et. al., 1980). Cyanophycin is a cellular inclusion that was once thought to be found in all cyanobacteria, but it has since been shown that some species of cyanobacteria do not synthesize cyanophycin (Newman et. al., 1987). In this paper, I compare the physiology of two strains of *Synechococcus* isolated from Florida Bay and present data which suggest that at least one of them is capable of producing cyanophycin.

Experimental Design

In this project I attempted to determine whether or not cyanophycin is present in one strain of the *Synechococcus* tested by a comparative experimental approach. I studied this strain as well as another strain of *Synechococcus* which does not appear to produce a significant amount of cyanophycin.

Materials and Methods

Strain Identification

Isolates were obtained from the Marine Microalgae Research Culture Collection at the Florida Marine Research Institute of the Florida Department of Environmental Conservation in St. Petersburg, Florida, and represent the only two pure cultures of *Synechococcus* from Florida Bay that are available at present. Both strains contain phycocyanin as their primary accessory pigment and are blue-green in color when in culture. Strain 95021415 S1 (referred to here as strain A) is rod-shaped with a length:width ratio of approximately 2:1 and a width of approximately 1 µm. Strain 95032005 WR 22 B6 (referred to here as strain B) is spherical, with a diameter of 2.0 to 2.5 µm.

Culture conditions and growth measurement

Cells were grown in 25 mm borosilicate glass tubes containing approximately 30 mL of culture medium. Each tube was inoculated with between 0.5 and 1.0 mL of stock culture. Growth was monitored using a **fluorometer** after calibration with cell count data (Fig. 1; Appendix A). Unless otherwise noted, cells were grown in f/2 medium made from aged Coos Bay water with a salinity of approximately 32 parts per thousand. Cells were incubated in constant temperature incubators with illumination provided by Cool White fluorescent bulbs on a 14:10 light:dark cycle, except as noted below.

Instantaneous growth rates of each culture were calculated from cell count or fluorescence data according to the equation:

$$\mu = (0.69)(\log_{10}X_{T1} - \log_{10}X_{T0})(0.301)^{-1}(T_1 - T_0)^{-1}$$

where X_{T1} = cell count or fluorescence at time "1" and X_{T0} = cell count or

fluorescence at time "0". T_1 and T_0 were points in the growth curve where changes in cell number or fluorescence were loglinear with time (Madigan et. al., 1997, A-5). The instantaneous growth rate for each culture was then multiplied by 24 to obtain a daily growth rate.

The effects of temperature and irradiance on the growth rates of each culture were investigated in a series of experiments. **Growth curves** for each experiment were created using fluorometer readings and, in some cases, cell counts. The temperatures used were 18°C, room temperature (~20°C), and 26°C. The different irradiances used were 100 μEinsteins/m^2sec, 166 μEinsteins/m^2sec, and very low, variable light (less than 10 μEinsteins/m^2sec). Cultures incubated at room temperature (~20°C) received incident light from a north-facing window and occasional room light, thus irradiance levels were low and variable. Irradiance levels were measured with a QSL-1000 light meter (Biospherical Instruments Inc., San Diego, California).

Cyanophycin experiments

The basic design of the cyanophycin experiments involved growing each strain for a few cell divisions and then adding chloramphenicol, an inhibitor of protein synthesis, to the culture. It was intended to add the chloramphenicol during **exponential phase** because f/2 is a phosphorous-limited medium, and therefore cells will run out of phosphorous before they run out of nitrogen; it has been shown that cyanophycin production can be induced by phosphorous limitation (Allen et. al, 1980). If cells were allowed to continue growing into **stationary phase** before addition of chloramphenicol, cyanophycin production could be induced via phosphorous

limitation within the culture without the addition of chloramphenicol.

Each cyanophycin experiment was initiated by inoculating tubes of medium with stationary phase culture that had been previously been growing at the assay temperature (26 °C for both strains) and the same irradiance at which the cells would be grown during the assay (100 μEinsteins/m^2sec for both strains). All cultures were grown in f/2 medium as described above; cells were grown in 30 mL borosilicate glass tubes instead of large-volume flasks due to the limited amount of space available for culture growth and also because it is convenient to measure growth by fluorometry using these tubes (the tubes can be placed directly in the fluorometer). Twelve to fourteen tubes of medium were inoculated at the beginning of each experiment. After a few days of growth, 10 μg chloramphenicol per mL of culture was added to half of the cultures (6 or 7 tubes) in each experiment, except in the nitrogen limitation experiment, where 1 μg of chloramphenicol was added per mL culture. Stock solutions of chloramphenicol (.05 g chloramphenicol/10 mL Nanopure water) were prepared from Sigma water-soluble chloramphenicol (catalog # C-3175). Chloramphenicol inhibits ribosomal synthesis of proteins and has been shown to induce production of cyanophycin (Allen and Hawley, 1983). The cultures were grown in the presence of chloramphenicol for 48 hours before harvesting.

The cultures not treated with chloramphenicol were allowed to continue growing under the same conditions as before and were harvested at the same time and assayed by the same procedure as the chloramphenicol-treated cells. The cultures that did not receive chloramphenicol were used as controls; if these cultures did not produce cyanophycin under conditions that

supported their growth and the cultures that received chloramphenicol did produce cyanophycin, it is verified that chloramphenicol induces formation of cyanophycin and that the cells do not make cyanophycin constitutively under the growth conditions tested.

The colorimetric assay protocol outlined in Allen (1988) and Messineo (1966) was followed, with modifications, in order to ascertain the presence of cyanophycin in strains A and B (Appendix B). This approach involves separation of cyanophycin from other cell components by repeated washes with water and detergent followed by extraction of the isolated cyanophycin with HCl. This isolation is possible because cyanophycin is not soluble in either water or detergent, but most other cell components are soluble in either water or detergent and are therefore removed in the washing procedure. The purified cyanophycin extract is then assayed by measurement of the amount of arginine in the extracted material. A standard curve for arginine is prepared and used to quantify the amount of arginine in the cell extract. When an estimate of cell abundance in the harvested material was available, this value was used to calculate the concentration of cyanophycin-bound arginine on a per cell basis; otherwise, the concentration was calculated based on the total culture volume assayed.

The colorimetric assay is used to measure the amount of arginine in a sample. This assay can be used to determine the amount of cyanophycin in a sample because cyanophycin is composed of equimolar amounts of arginine and aspartate; therefore if there is a large amount of cyanophycin in a cell, there should be a large amount of arginine in the cell as well (Simon and Weathers, 1976).

Nitrogen limitation experiment

This experiment involved a slightly different approach to the induction of cyanophycin synthesis. It differed from the procedure described above in that cells grown in nitrogen-limited medium were used. The low-nitrogen medium has 10% of the concentration of nitrogen found in f/2 medium; all other nutrient levels are identical to those found in f/2 medium. $NaNO_3$ was used as the nitrogen source for all media used. In this assay, 2.142 mg of $NaNO_3$ (in solution) was added to the cultures at the same time as the chloramphenicol addition. The same amount of nitrogen was added to the cultures that did not receive chloramphenicol. The colorimetric arginine assay for this experiment was conducted as part of the October colorimetric arginine assay; this assay also included two experiments conducted with cells grown in regular Coos Bay f/2 medium.

Results

Growth characterizations

The growth rates of strain A and strain B were similar to each other at all irradiances and temperatures tested (Tables 1 & 2). Of the three temperatures tested, the preferred growth temperature for the cyanophycin experiments was 26°C for both cultures, since the cultures grew too fast at 18°C to make experimental manipulation convenient to schedule before the cells were well into stationary phase. The growth rate constant for strain A at 26°C was approximately 0.64296 per 24 hours under 100 µEinsteins/m^2s of light and 0.60636 per 24 hours under 166 µEinsteins/m^2s of light (Table 1). The growth rate for strain B at 26°C and 100 µEinsteins/m^2s was 0.51712 per 24 hours; at 166 µEinsteins/m^2s the growth rate was 0.60489 per 24 hours

(Table 2). Neither strain grew at 20°C. The two irradiances tested did not appear to have a very substantial affect on the growth rates of the cultures.

Cyanophycin Experiments

In some of the colorimetric arginine assays chloramphenicol appeared to induce cyanophycin production, while in others the cells without chloramphenicol appeared to make more cyanophycin than the cells that were "induced" by the addition of chloramphenicol. In the May experiment, all three B reps were induced to make cyanophycin through the addition of chloramphenicol, because the amount of arginine per mL culture was much higher for the cultures that had been treated with chloramphenicol than for those that had not received chloramphenicol (Fig. 2). Strain A had very low levels of arginine when compared with strain B and there was very little difference between the chloramphenicol treatment and the no-chloramphenicol treatment in strain A. I originally planned to assay cyanophycin production in both of the strains; however, this and other preliminary experiments indicated that strain A produced only baseline amounts of cyanophycin, if any. Growth curves for the May experiment show that all cells were in late exponential phase when chloramphenicol was added (Figs. 3 & 4).

In the experiment begun on 21 Sept. (cells grown in complete f/2 medium) high levels of arginine were measured in extracts from all cultures, whether or not chloramphenicol was added. In fact, more arginine was produced (both per mL culture and per cell) by extracts of cultures with no chloramphenicol added than in the extract from cultures to which chloramphenicol was added. In the experiment begun on 2 Oct., more arginine was produced in the extract from cultures that had been grown with

chloramphenicol than in the extract from cultures with no chloramphenicol (Fig. 5). Growth curves from these two experiments show similar response in the cultures with added chloramphenicol as compared to cultures that did not receive chloramphenicol (Figs. 6 & 7). These curves also show that the culture inoculated on 21 Sept. was well into stationary phase when assayed, while the culture inoculated on 2 Oct. was in late exponential/early stationary phase when assayed.

Throughout all of the experiments, the readings for 0.5 mL of extract were approximately half of the readings for 1.0 mL of the corresponding extract. In both arginine assays the extract, both before and after treatment, was optically clear and not clouded with extraneous cell material. In both arginine assays the standard curve dilutions were dark orange and clear when containing high amounts of arginine and clear and colorless when containing no arginine. After conversion of the arginine in the experimental samples to its colored form, the extracts were clear in various shades of orange, with no clouding.

Nitrogen limitation experiment

The cells grown in nitrogen-limited medium (14-Sep in Fig. 5) produced no cyanophycin when nitrogen was added without the addition of chloramphenicol, but did produce cyanophycin when chloramphenicol was added with the nitrogen. These cells were in stationary phase when nitrogen and chloramphenicol were added, but the addition of nitrogen brought all of the cells (both those that received chloramphenicol and those that did not receive chloramphenicol) back into exponential phase (Fig. 8).

Discussion

Growth characterizations

At 18°C strain B appeared to be slightly light-limited at 100 μEinsteins/m^2s as the growth rate under these conditions was lower than the growth rate at 18°C and 166 μEinsteins/m^2s. There was not sufficient data available to draw any conclusions about light limitation in strain A at 18°C. At 26°C both strains were light saturated, as the growth rates were approximately the same for both irradiances at this temperature. The lack of growth in both strains at 20°C was most likely due to the extremely low light that they received in this environment, since it is unlikely that the cells would grow well under higher irradiance at 18 and 26°C but not at 20°C.

Cyanophycin experiments

In the May experiment (Fig. 2) the cells were in exponential phase when chloramphenicol was added (Figs. 3 & 4); in this experimental design, cells that were induced to make cyanophycin through the addition of chloramphenicol would be expected to produce a large amount of cyanophycin, while the cells that did not receive chloramphenicol should not produce much cyanophycin since they had not been induced either by reaching stationary phase (running out of phosphorous) or by exposure to chloramphenicol. If the cells did produce cyanophycin, the arginine per mL culture for 0.5 mL of extract should be half of the arginine per mL culture for 1.0 mL of extract.

The results of this experiment were as predicted by the experimental design; the amount of arginine per mL culture was much higher in the cultures that had received chloramphenicol than in the cultures that did not receive chloramphenicol. Additionally, the 0.5 mL samples gave readings

that were lower than the readings for the corresponding 1.0 mL samples; in the cultures that did receive chloramphenicol, the 0.5 mL readings were approximately half of the 1.0 mL readings. A likely reason for the 0.5 mL readings not being exactly half of the 1.0 mL readings in the cultures that did not receive chloramphenicol is that these readings show primarily "background" arginine that was not incorporated into cyanophycin. Alternatively, these cells could be producing a low amount of cyanophycin at all times, which would also lead to a baseline reading of arginine, even when cells were not induced to form cyanophycin.

The cells in the experiment begun on 21 Sept. were in stationary phase when assayed (Fig. 6). Thus it would be expected that both the cells with chloramphenicol added and the cells with no chloramphenicol would produce a high amount of cyanophycin, because f/2 is a phosphorous-limited medium. In this phosphorous-limited medium the cells were induced to enter stationary phase by depletion of phosphorous, and had available nitrogen to make into cyanophycin throughout stationary phase. They simply could not divide because there was no phosphorous, which is required in the synthesis of membranes and for energy metabolism. The results of the experiment begun on 21 Sept. were as expected; both the cells that had received chloramphenicol and the cells that had not received chloramphenicol made a large amount of cyanophycin, which is consistent with the theory that phosphorous-limited cells in stationary phase have copious nitrogen available for incorporation into cyanophycin.

The cells in the experiment begun on 2 Oct. were in early stationary phase when the assay was conducted (Fig. 7). Under these conditions, the addition of chloramphenicol should induce some production of cyanophycin,

because the cells were not completely into stationary phase and therefore had not completely run out of phosphorous. Thus the cells would not have started to use the available nitrogen to make cyanophycin, and some of the cells in the culture would not have reached stationary phase yet and would still be using all of the available nutrients, including nitrogen, for cell division. The results of this experiment are similar to those expected; a very high amount of cyanophycin was produced in the cells that received chloramphenicol relative to the cells that did not receive chloramphenicol.

Based on these results for the arginine assay, it cannot be clearly stated that strain B is induced to produce cyanophycin by the addition of chloramphenicol, since the cultures both with and without chloramphenicol produced cyanophycin in the experiment begun on 21 Sept. However, strain B definitely does produce cyanophycin; it is possible that these cells produce cyanophycin constitutively. Based on these experiments strain A produces very little or no cyanophycin, even when induced.

Nitrogen limitation experiment

In the nitrogen limitation experiment (14-Sep in Fig. 5), the cells were in stationary phase when chloramphenicol and nitrogen were added. When nitrogen was added, the culture returned to exponential phase; the culture with no chloramphenicol and the culture with chloramphenicol showed an almost identical pattern of return to exponential phase upon the addition of nitrogen (Fig. 8). The low amount of nitrogen in the medium for this experiment insured that the cells could not make cyanophycin while they were in stationary phase; there was no excess nitrogen in the medium for the cells to make into cyanophycin, thus in this experiment the medium was nitrogen-limited, instead of phosphorous-limited as in the other

experiments. Therefore the first opportunity for the cells to make cyanophycin was when nitrogen (and chloramphenicol in half of the tubes) was added to the medium. From these experimental parameters I would expect that a high amount of cyanophycin would be produced by the cells that received both nitrogen and chloramphenicol, because these cells would not be able to use the nitrogen to make anything but cyanophycin. No cyanophycin would be produced by the cells that received nitrogen only, because these cells would be using all available nitrogen to recover from the low-nitrogen conditions. The results of this experiment were as expected; the cells that received only nitrogen produced a very low amount of arginine (so low that it appeared negative when plotted against the standard curve), and the cells that received both nitrogen and chloramphenicol produced a high amount of arginine and presumably cyanophycin (Fig. 5).

Overall conclusions

My finding that cyanophycin can be produced by bloom-forming cyanobacteria has many ramifications for the health of Florida Bay. Any cell living in this pulse-eutrophied environment that is capable of storing nitrogen is at a competitive advantage. If these cells can take advantage of nitrogen pulses by producing cyanophycin, they could theoretically break down this cyanophycin and use the nitrogen at a later time when other cells are not able to grow due to low ambient nitrogen. This would cause large blooms of the cells that can make cyanophycin, choking out many other organisms in Florida Bay.

The dominant organisms in Florida Bay blooms are cyanobacteria and diatoms. Presumably these cyanobacteria and diatoms compete for nutrients, and the resulting blooms are dominated by the organism that can utilize the

available nutrients most effectively. Cyanobacteria that are able to make nutrient storage compounds such as cyanophycin have a competitive advantage over diatoms, which are not able to produce cyanophycin. These cyanobacteria would be able to flourish during periods of high phosphorous and low nitrogen because they can break down cyanophycin and use it as a nitrogen source to continue to divide when other organisms cannot because nitrogen concentrations in the environment are low relative to phosphorous concentrations.

The knowledge that these cells make cyanophycin could enable humans to control these blooms in a variety of ways, primarily by limiting nitrogen input into Florida Bay. This remediation strategy would not have visible effects for quite some time, however, because the cyanophycin-producing cells would have to use up all of their cyanophycin before the low-nitrogen conditions would be effective in keeping them from dividing.

Although the abundance of strains A and B in blooms in Florida Bay is not known, these are the only *Synechococcus* isolates available from Florida Bay, and it is known that organisms like these strains are involved to some degree in cyanobacterial blooms in Florida Bay. Characterization of these strains, especially with respect to nutrient storage capabilities, contributes to the body of research on *Synechococcus* and cyanobacteria as a group. This study has made an important contribution to the body of work on *Synechococcus* ; prior to this study, no *Synechococcus* had been shown to make cyanophycin.

Appendix A - fluorometer calibration

Growth curves were necessary to ascertain when the cells should be harvested for the arginine assay. To make growth curves without counting an inordinate amount of cells, a fluorometer was used to measure the fluorescence of each culture at the same time every day or every other day. At the same time as the fluorescence was measured, a small, measured amount of each culture was filtered and the filter was placed on a slide for counting under a microscope. The number of cells per mL of culture was calculated based on the number of cells counted on the slide, the amount of culture filtered, and the size of the filter. This number was then multiplied by the total volume of culture to find the total number of cells in the culture. These data were used to generate Figure 1, which shows that the two curves (fluorescence versus time and cell number versus time) are relatively parallel (when cell number was regressed against fluorometer reading, $r^2 = 0.9257$), and therefore fluorescence is a good indicator of cell number for this strain.

Appendix B - arginine assay

The protocol detailed in Allen 1988 (modification of Simon and Weathers, 1976) was used as a guide for the colorimetric arginine assay. Colorimetric assays are widely used to determine the presence and amount of many different compounds. The idea behind the colorimetric assay is simple; a standard of whatever material is being measured in the experiment (arginine in this particular experiment) is obtained and diluted into different concentrations (for example, 1 mL, 2 mL, 3 mL, 4 mL and 5 mL of the original solution, each brought to a volume of 10 mL, giving five different known concentrations of the standard). This standard is then converted to a colored compound and the optical density of this compound is measured by a **spectrophotometer**; the differences between optical densities in the different dilutions can often be seen with the naked eye as well, and are proportional to concentration of the material being assayed. In this case, the chemical conversion to a colored compound is accomplished by the addition of 2,4-Dichloro-1-Napthol; in an alkaline solution, 2,4-Dichloro-1-Napthol forms a red complex with arginine when an oxidizing agent is present. This complex is made up of the guanidino group of arginine and napthol that has been previously oxidized to a semiquinone-quinone form (Messineo, 1966) This dilution series generates a standard curve based on numerical values for the optical density of each dilution.

To estimate the cyanophycin content of an solution, the sample in question is converted to a cell extract containing only the materials being assayed. The cells are crushed, then this slurry is subjected to treatments that remove everything but the desired component. In this case, all that should remain in the sample is cyanophycin granule polypeptide, which is not

soluble in either water or detergent, but is soluble in HCl (see Materials and Methods). The cell extract is then converted to a colored form using the same procedure that was applied to the standard, and the optical density is measured spectrophotometrically. The value obtained from this sample can be plotted on the standard curve to determine the amount of the standard present in the sample, or substituted into a regression equation derived from the standard curve. Each sample was divided into two parts, one using 0.5 mL of the extract and one using 1.0 mL of the extract. The part using 0.5 mL of extract was brought to a volume of 1.0 mL using 0.1N HCl (no HCl was added to the 1.0 mL part). This was done to confirm that the test was assaying the presence of arginine; if the presence of arginine was indeed the cause of high readings, the readings for 1.0 mL would be twice the reading for 0.5 mL. It is important to note that the colorimetric assay was only used to measure the amount of arginine present in these cells.

Preparation of cell extract for arginine assay

1. Pool replicate culture tubes by transferring to 250 mL centrifuge bottle, mix, subsample for cell count (subsampling not done in all experiments), add Nanopure water until bottle is full (to prevent bottle rupture in centrifuge)

2. Harvest via centrifugation at 27,000 x*g* (13,500 RPM, r_{max}= 137.0 mm, JA-14 rotor in Beckman J2-21 centrifuge) for 30 minutes

3. Discard supernatant. This supernatant is essentially medium with dissolved nutrients and/or other dissolved material produced during cell growth.

4. Store in -80°C freezer until ready to crush

5. Resuspend pellet in ~500µL Nanopure water

6. Crush suspended pellet in mortar and pestle while freezing with liquid nitrogen, transfer to 35 mL centrifuge tubes (in assays done before August 1998, 2 mL centrifuge tubes were used)*

7. Wash pellet in ~30 mL of Nanopure water, centrifuge at 27,000 x*g* (15,000 RPM, r_{max}= 108.0 mm, SS-34 rotor in Sorval RC-2 centrifuge) for 20 to 30 minutes

8. Pour off & discard supernatant (contains water-soluble cell components such as phycobiliproteins)*

9. Repeat water wash and centrifugation

10. Wash pellet with 8-10 mL of 1% Triton-X detergent, centrifuge as in step 7

11. Pour off and discard supernatant (contains detergent-soluble cell components such as lipids)*

12. Repeat Triton-X wash, discard supernatant, do 2 more water washes as in step 7, discarding supernatant after each centrifugation*

13. Extract pellet with 1.0 mL 0.1N HCl

14. Let stand 30 minutes

15. Centrifuge as in step 7

16. Pour off and SAVE supernatant (extract)

17. Repeat steps 13, 14, 15, 16 - should have ~2.0 mL of extract

18. Arginine assay according to the modified Sakaguchi reaction of Messineo (1966)

* = pellet may be stored in -80°C freezer indefinitely at this point

Glossary

cyanobacteria - prokaryotic cells (see **prokaryote**) which carry out photosynthesis and produce oxygen; cyanobacteria also contain chlorophyll *a* and phycobiliproteins (light-harvesting pigments)

diatoms - unicellular **eukaryotes**; the only algae that have been shown to exhibit gliding motility; members of the class Bacillariophyceae with a mineralized cell wall that produces an "exoskeleton" or "frustule" of silica

eukaryote - an organism with cells having a unit membrane-enclosed (true) nucleus and usually other organelles. Unicellular eukaryotes may be photosynthetic (obtaining energy from light, i.e. diatoms) or heterotrophic (obtaining energy from the oxidation of organic compounds).

eutrophication - increasing the amount of nutrients in a habitat

exponential phase - a period during the growth cycle of a population in which population size increases at an exponential rate (logarithmic growth)

fluorometer - a machine that measures the amount of light that a material emits when excited by a certain wavelength of light

growth curve - graph of the number of cells (measured using cell counts, fluorometer readings, etc.) in a culture versus the amount of time that the culture has been growing; used to determine the growth phase of a culture

phytoplankton - unicellular organisms usually ranging in size from 0.8 µm to ~100 µm per cell - generally photosynthetic drifters, live in marine or freshwater environments (includes cyanobacteria, diatoms, many other algae), cells may be solitary or colonial in growth habit

primary producer - an organism that generally uses light or organic material as an energy source to reduce carbon from an inorganic form (CO_2) to organic compounds available for metabolism by itself &/or other organisms

prokaryote - an organism lacking a nucleus and other membrane-enclosed organelles, usually having its DNA in a single circular molecule

spectrophotometer - a machine that measures the amount of light that can pass through a substance (optical density)

stationary phase - a period during the growth cycle of a population during which population size neither increases nor decreases (no net growth)

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Table 1. Growth rates for strain A. low = low, variable irradiance, med = 100 μEinsteins/m^2sec, high = 166 μEinsteins/m^2sec, NG = no growth, - = no data collected. Med and high irradiance under Cool White fluorescent bulbs as described in Materials and Methods.

temperature	irradiance		
	low	med	high
18 degrees	-	-	0.24326
20 degrees	NG	-	-
26 degrees	-	0.64296	0.60636

Table 2. Growth rates for strain B. low = low, variable irradiance, med = 100 μEinsteins/m^2sec, high = 166 μEinsteins/m^2sec, NG = no growth, - = no data collected. Med and high irradiance under Cool White fluorescent bulbs as described in Materials and Methods.

temperature	irradiance		
	low	med	high
18 degrees	-	0.28881	0.19188
20 degrees	NG	-	-
26 degrees	-	0.51712	0.60489

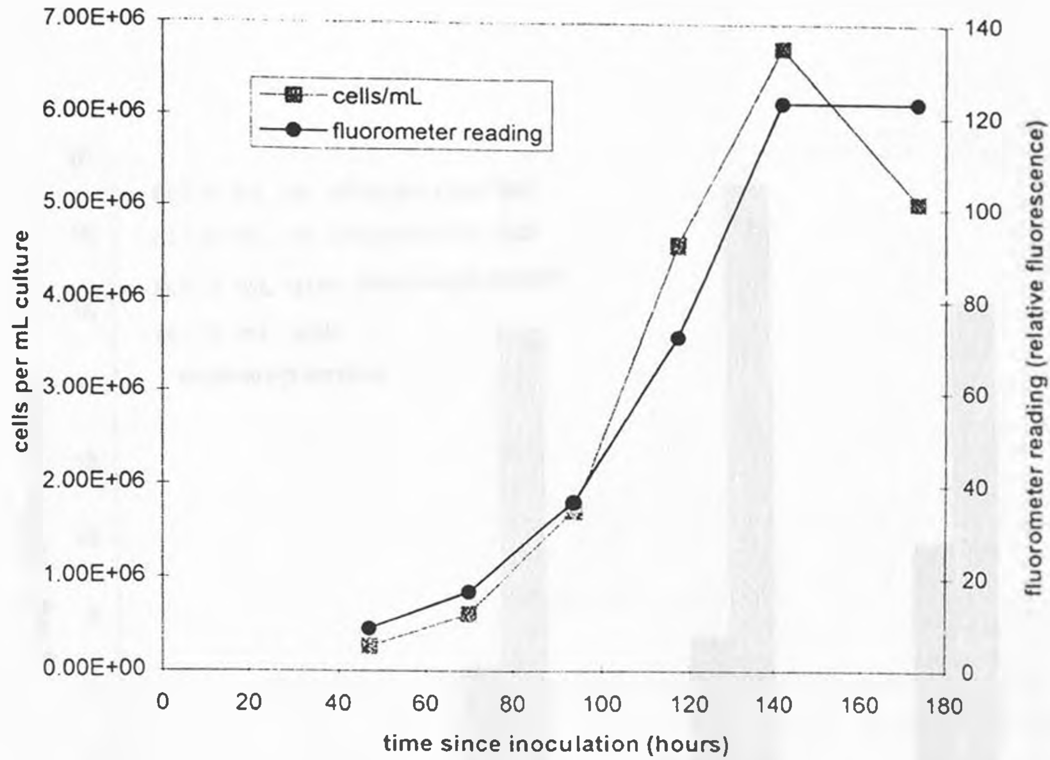

Fig. 1. Comparison of fluorometer reading with cells per mL culture. Values are for strain B at 26°C and 100 μEinsteins/m^{2}sec, in Coos Bay f/2 medium as described in Materials and Methods.

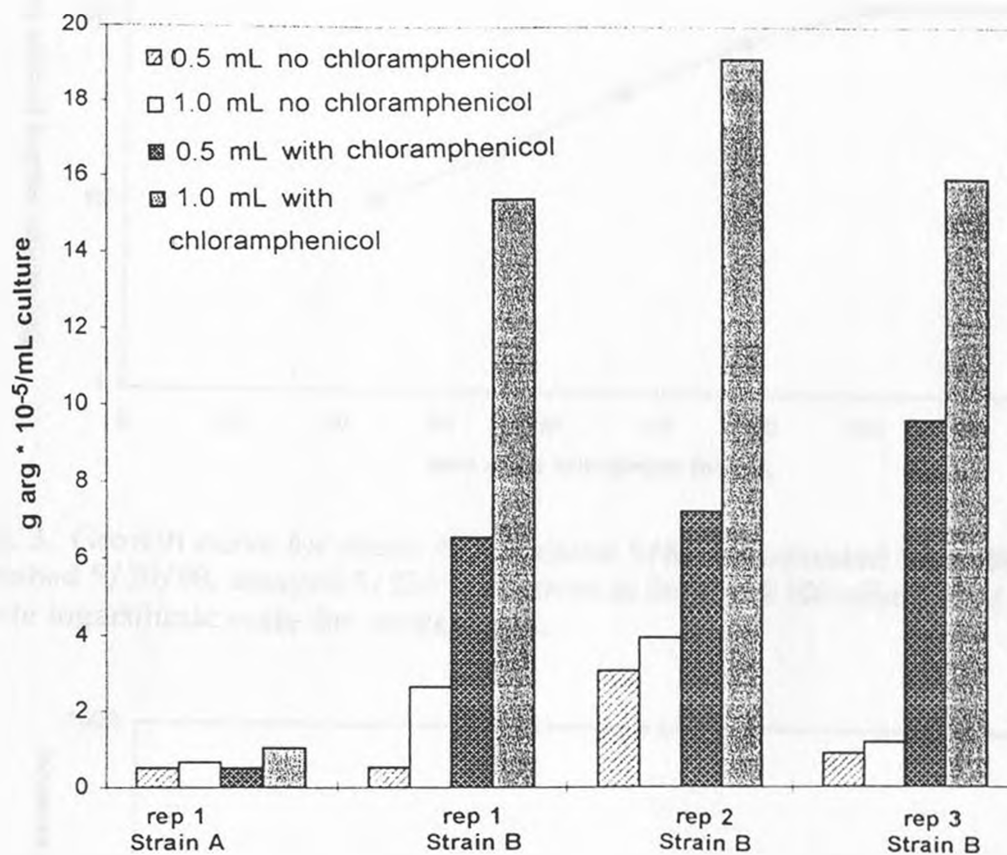

Fig. 2. Arginine per mL culture for assay conducted 5/23/98. Note that values are shown for 0.5 and 1.0 mL of extract; values for 0.5 mL of extract in strain B are approximately half of the values for 1.0 mL of extract.

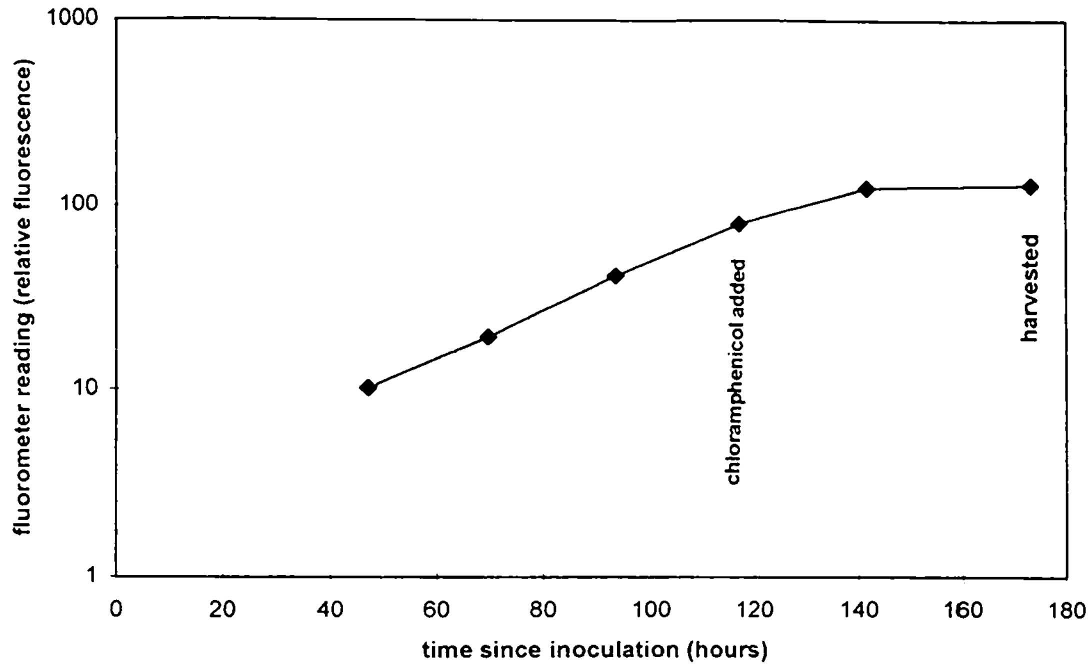

Fig. 3. Growth curve for strain A inoculated 5/8/98, harvested 5/15/98, crushed 5/20/98, assayed 5/23/98. Grown at 26°C and 100 μEinsteins/m^{2}sec. Note logarithmic scale for vertical axis.

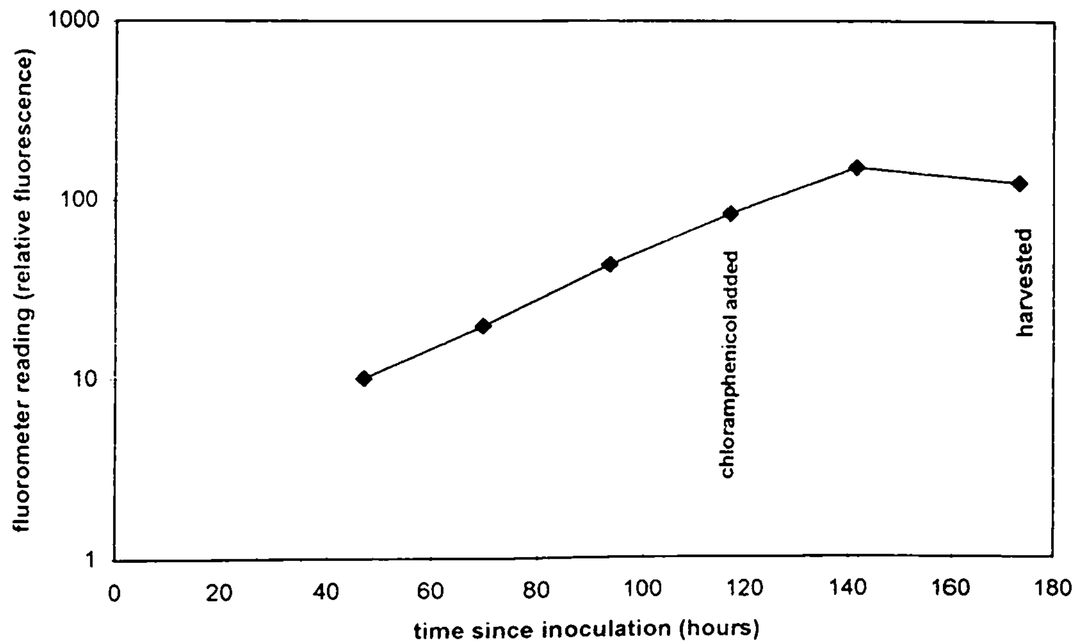

Fig. 4. Growth curve for strain B inoculated 5/8/98, harvested 5/15/98, crushed 5/20/98, assayed 5/23/98. Grown at 26°C and 100 μEinsteins/m^{2}sec. Note logarithmic scale for vertical axis.

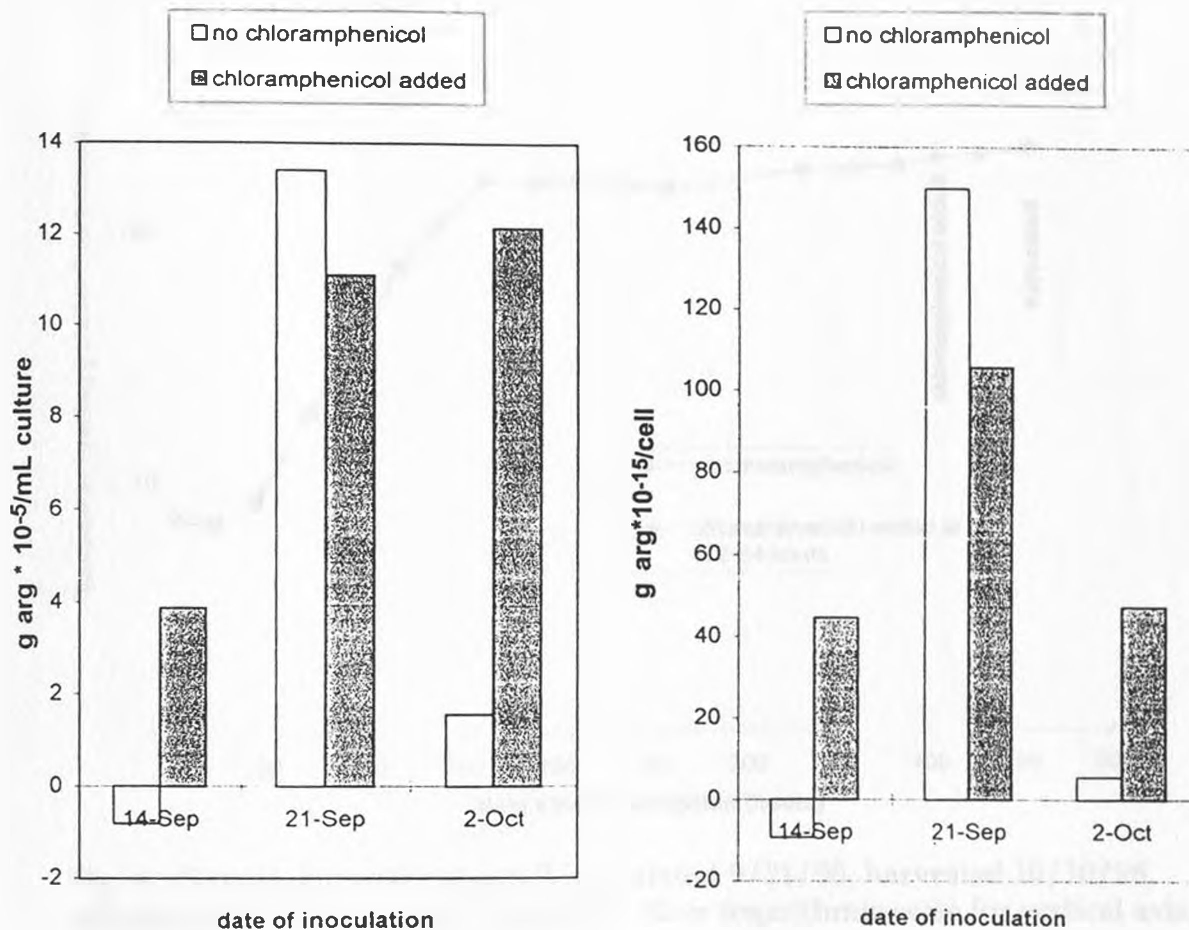

Fig. 5. Arginine per mL culture and per cell for assay conducted 10/20/98. Values shown are for 1.0 mL of extract; values for 0.5 mL of extract were approximately half of the 1.0 values as in Fig. 2. After harvest at the end of each experiment, cells were frozen until analysis. Arginine assays for all three experiments were performed on October 20, 1998.

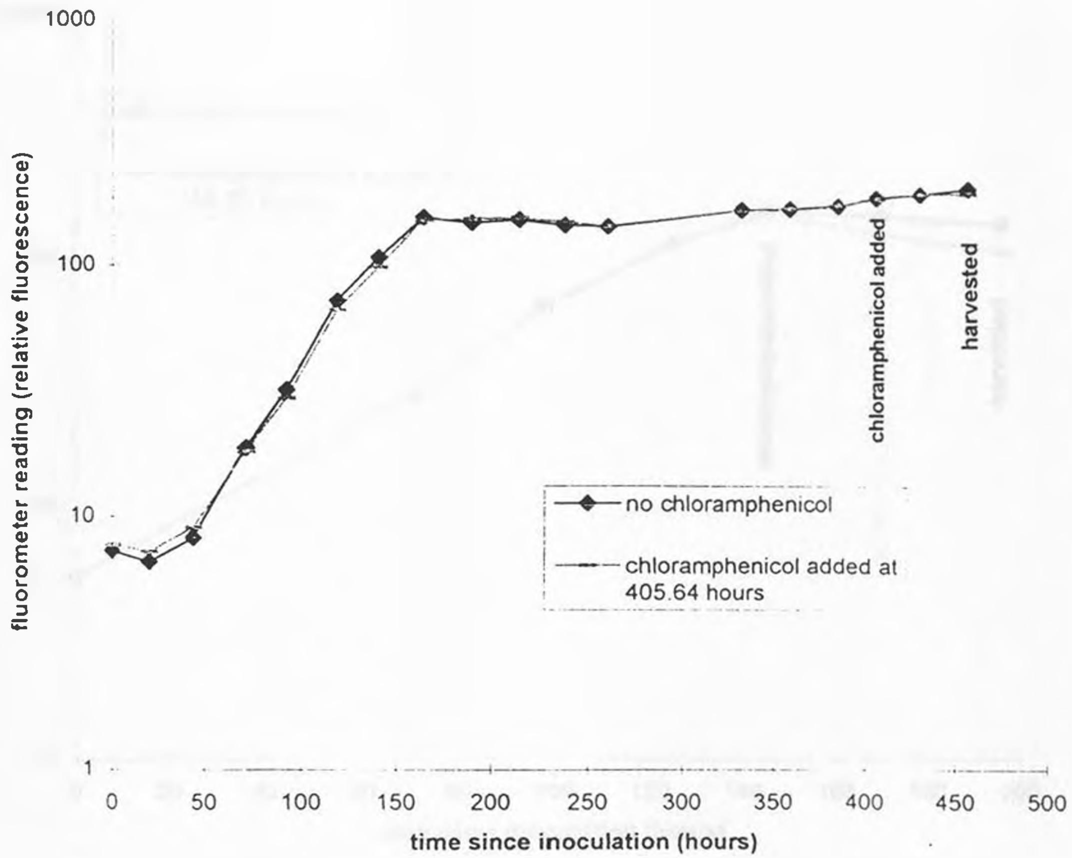

Fig. 6. Growth curve for strain B inoculated 9/21/98, harvested 10/10/98, crushed 10/15/98, assayed 10/20/98. Note logarithmic scale for vertical axis.

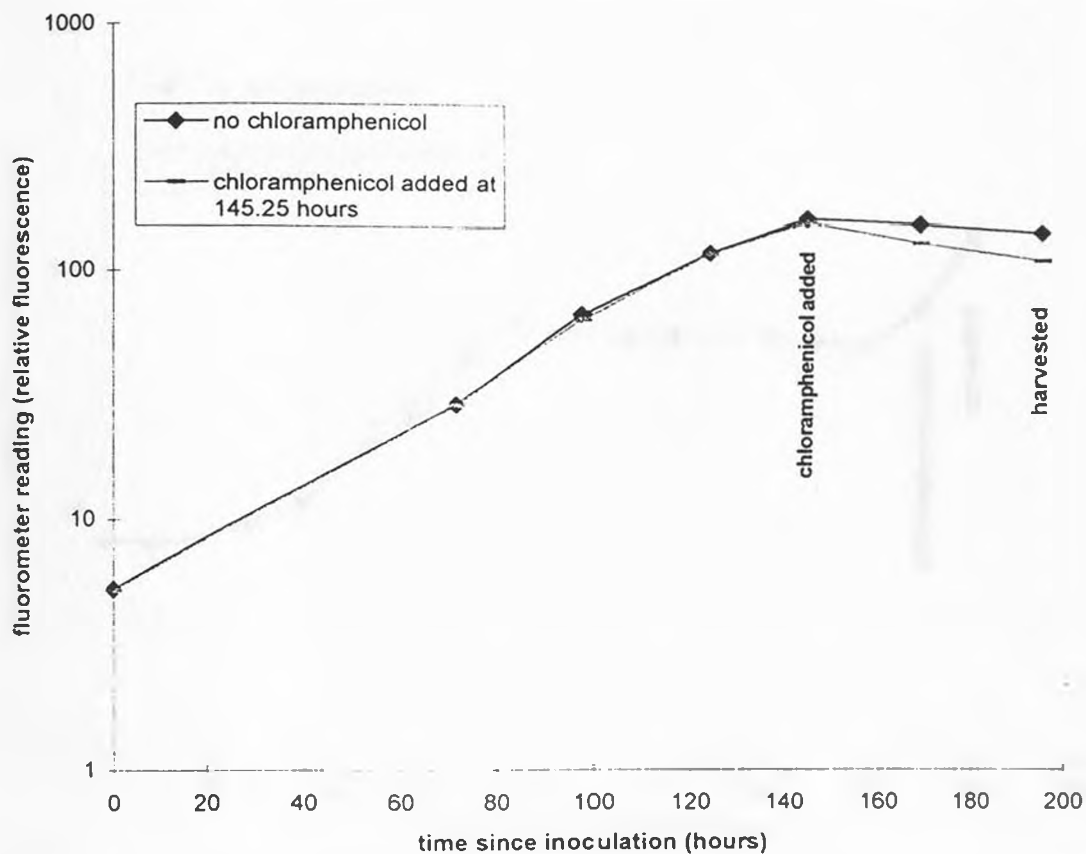

Fig. 7. Growth curve for Strain B inoculated 10/2/98, harvested 10/10/98, crushed 10/15/98, assayed 10/20/98. Note logarithmic scale for vertical axis.

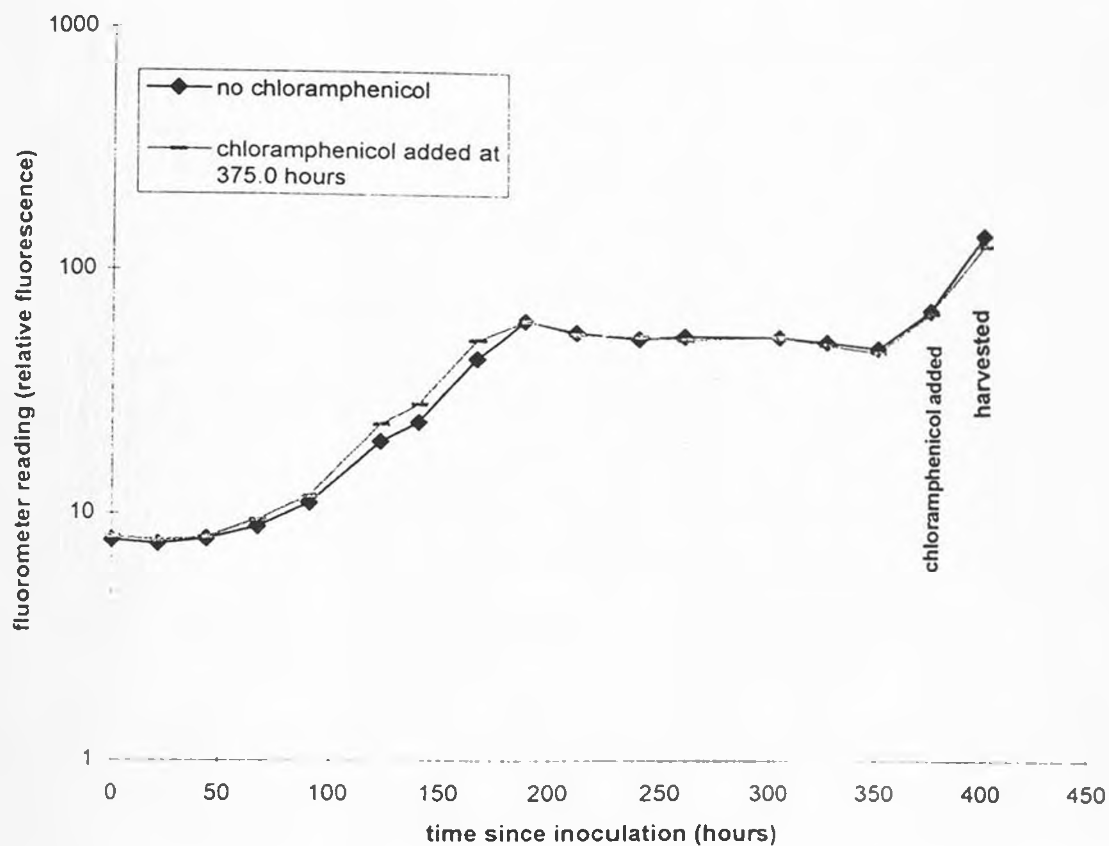

Fig. 8. Growth curve for strain B inoculated into nitrogen-limited medium 9/14/98, harvested 9/30/98, crushed 10/15/98, assayed 10/20/98. Nitrogen was added to all cells (with chloramphenicol and without) at the same time as the chloramphenicol addition. Note logarithmic scale for vertical axis.