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Abstract

This quarterly report documents significant achievements in the Enhanced Practical Photosynthetic CO_2 Mitigation project during the period from 10/2/2001 through 1/01/2003. As indicated in the list of accomplishments below our current efforts are focused on evaluating candidate organisms and growth surfaces, preparing to conduct long-term tests in the bench-scale bioreactor test systems, and scaling-up the test facilities from bench scale to pilot scale.

Specific results and accomplishments for the first quarter of 2003 include:

Organisms and Growth Surfaces:

- Additional thermal features with developed cyanobacterial mats, which might be calcium resistant, were found in the West Thumb area of YNP. New samples were isolated and are being cultured in glass tubes.
- We checked the motile ability of 8.2.1 Synechococcus s.c. (10) and 3.2.2 Synechococcus s.c. 6. It was found that unicellular isolates 8.2.1 Synechococcus s.c. (10) and 3.2.2 Synechococcus s.c. 1 are phototaxic. Isolate 3.2.2 Synechococcus s.c. 1 currently consists of two populations: one population appears to be positive phototaxic, and second population appears negative phototaxis to the same level of light. This means that the character of screen illumination should be uniform and reasonable for cyanobacterial cells.
- The aeration of growth media with 5 % CO_2 in air stimulates cyanobacterial growth 10-20 times over that with air alone. It is possible the rate of the stimulation of cyanobacterial growth in CRF will be higher because cyanobacteria will be grown as a biofilm. We plan to increase the concentration to 15 % CO_2 in air.
- We are continuing the organizing of our collection of the thermophilic cyanobacteria isolated from Yellowstone National Park. During this reporting period we transferred about 160 samples and discarded about 80 samples with weak growth in standard media as BG-11, D or DH. As result of this work we currently have 13 unialgal cultures of thermophilic cyanobacteria.
- We are screening the cultures to measure the effects of Omnisil on the growth of 2.1 (III) *Mastigicladus laminosum*, 8.2.1 *Synechococcus* s.c.10, 1.2 s.c. 6 *Chlorogleopsis* spp. and 3.3.2 *Synechococcus* s.c.1. It was found to date that only 1.2 *Chlorogleopsis* s.c.6 was able to grow in batch culture in the presence of Omnisil. At the moment we have no explanation for the toxic effect of Omnisil, if in fact it is merely spun silica. Nonetheless, we started the selection of Omnisil resistant clones among several cyanobacterial isolates. This process may require several months.

Bioreactor support systems and test facilities:

- A series of tests was run to demonstrate that the initial mass of algae loaded into the CRF-2 system can be accurately determined based on a statistical sampling procedure. Results from the series of tests indicate that the sampling method can be used to reliably estimate the initial algae mass for a CRF-2 test with about 3% uncertainty.
- Survivability tests for Sc1.2(2) on Omnisil using drilled-hole header inserts are currently underway. Numerous system problems have delayed the testing, but we are hopeful that the debugging of the system is now complete and the current test is proceeding well. Full test results will be provided in the next quarterly report.

- Initial tests of both “drilled hole” and “pressure shim” inserts for the integrated wetting/harvesting screens were successful. Both designs showed good flow characteristics and there was no appreciable clogging noticed during and after the test. Test results for flow rate vs. pressure for three header insert designs showed that the performance of the 0.02” shim and the drilled hole design are very similar, and thickening the shim to 0.025” has the expected effect of reducing the flow at a given pressure for the shim design, but the basic pressure-flow relationship retains the same form.
- Since the header inserts will likely be manufactured from stainless steel, leaching tests were carried out to find the tolerance of the organisms to SS 316. The organism SC1.2 was very tolerant to SS 316 and it had no effect on the growth of the organism.
- The pilot-scale bioreactor construction and debugging is continuing on schedule. We are currently waiting for results from the CRF-2 before finalizing the system design.

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Results and Discussion

Task 1.0. Evaluate and rank component and subsystem level alternative design concepts
Subtask 1.1 Investigate critical properties of alternative photosynthetic agents (cyanobacteria)

Report from the researchers at Montana State

Isolation of photosynthetic agents

Our review of the thermal features in Yellowstone National Park (YNP) suggests that we should investigate the possibility that the West Thumb area could be a source of thermotolerant cyanobacteria useful in this project. The first sampling was made at a thermal feature, shown on Fig. 1.

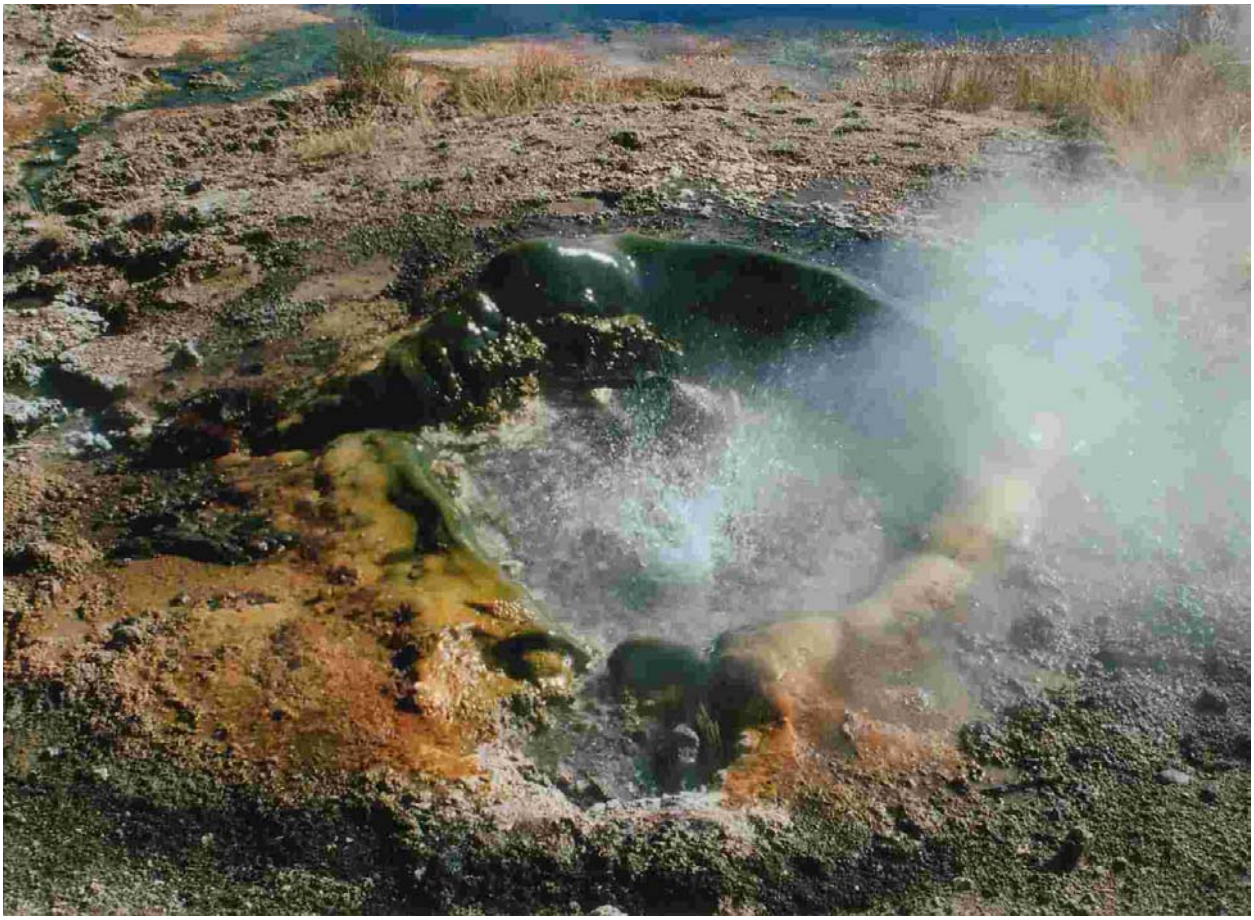

Fig.1. Yellowstone National Park miniature geyser

This feature has the shape of small crater with a miniature geyser (or fountain) inside the crater. The height of this geyser is about 30 cm. The temperature of erupted water is 75^{o}C, pH 8.4. This crater is surrounded with very strong cyanobacterial mat, which has a rampart shape. The thickness of this "rampart" reaches up to 25 cm in some places. We have measured pH inside of

this cyanobacterial mat (at a depth of about 5 mm) and found that it was between 9.75 – 9.84 despite that the water had pH about 8.4. The surface of this mat has a lot of slime.

We have isolated two samples from this mat. The microscopical analysis of this mat showed up that this one is composed with filamentous cyanobacteria, probably *Phormidium*. Interestingly, living filaments were also found at 5 cm depth. We are introducing these isolates into culture, using 3 media: BG-11, D, and DH.

Fig. 2 shows the second place of sampling. It is small thermal feature (a pool) with water pH ~ 6.68 and temperature ~ 42^{o}C.

Fig. 2 Cyanobacterial mat in small pool

The surface of this small pool is covered with floating cyanobacterial mat embedded with the pieces of calcite. Of particular interest is that we have observed a more obvious calcification of this mat in August, 2002 (Fig.3 on next page).

Macroscopical observation of this cyanobacterial mat shows a laminated structure. Microscopical analysis suggested that this mat is composed of several species of filamentous cyanobacteria and unialgal *Synechococcus* (at least by microscopy). Samples from this site are also being cultured. The wall growth exhibited in these sites suggests that the organisms growing in these biofilms may be of particular utility in colonizing the omnisil membranes in the CRF.

We have a stable culture of 8.2.1 *Synechococcus* s.c. (10). *Synechococcus* is unicellular species. Sample 8.2.1 has been isolated from Angel Terrace (Mammoth Hot Springs) characterized with elevated concentration of CO_2 and dissolved calcium in underground waters. After isolating of general culture of *Synechococcus* spp we streaked this culture on hardened BG-11 medium with 1.5 % agarose. After 2 months of incubation of streaked culture in humid chamber we picked 10 single clones of *Synechococcus* from agarose surface and inoculated them in liquid BG-11 medium (0.5 mL). After 1.5 months exposure the inocula grew somewhat and they have been transferred in glass tubes with 1.5 mL of fresh BG-11 medium. The clone # 10 appeared to give the best growth and this was transferred to a flask with fresh medium. Currently we have stable culture of this isolate and are using it in light level/growth experiments. The above gives an indication of the steps needed in the isolation and selection of an organism potentially useful in the CRF. Just because an organism is slow to grow on initial isolation does not obviate its utility in the CRF.

Fig. 3. Visual image of mat calcification

We checked the motile ability of 8.2.1 *Synechococcus* s.c. (10) and 3.2.2 Synechococcus s.c. 6. The ability of cells to migrate has importance in this study. It is known that filamentous cyanobacteria have motile phenotype but coccoid cells are often non-motile. However, Ward and co-authors showed that a thermophilic *Synechococcus* sp. inhabiting a natural microbial mat exhibited negative phototaxis. The screen/membrane in the CRF facility may have real gradients of light, and probably nutrition. The stimulation of cyanobacterial motility by any environmental factor may consequently decrease the rate of the adhesion of cells to a substratum and the photosynthetic efficiency of a screen.

It was found that unicellular isolates 8.2.1 *Synechococcus* s.c. (10) and 3.2.2 *Synechococcus* s.c. 1 are phototaxic (Fig. 4 and 5). Isolate 3.2.2 *Synechococcus* s.c. 1 currently consists of two populations: one population appears to be positive phototaxic, and second population appears negative phototaxis to the same level of light (Fig 5). This means that the character of screen illumination should be uniform and reasonable for cyanobacterial cells. In opposite case cyanobacterial cells might be non-uniformly distributed on screen surface.

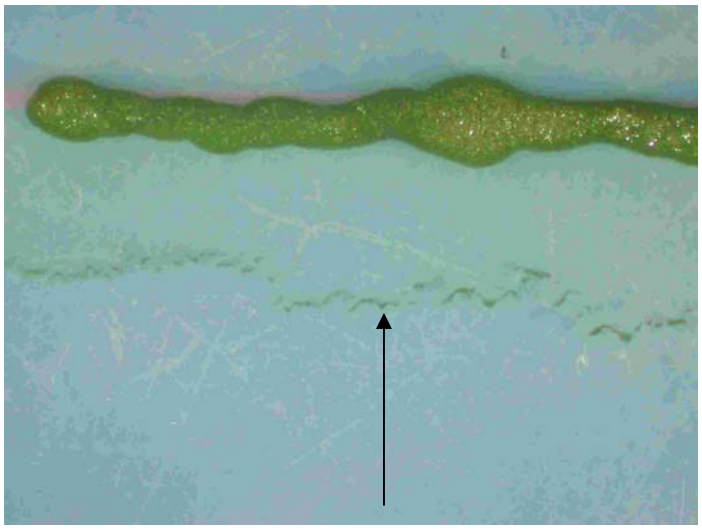

Fig.4. Phototactic population of the isolate 8.2.1 *Synechococcus* s.c. (10). *The arrow also shows the light direction*.

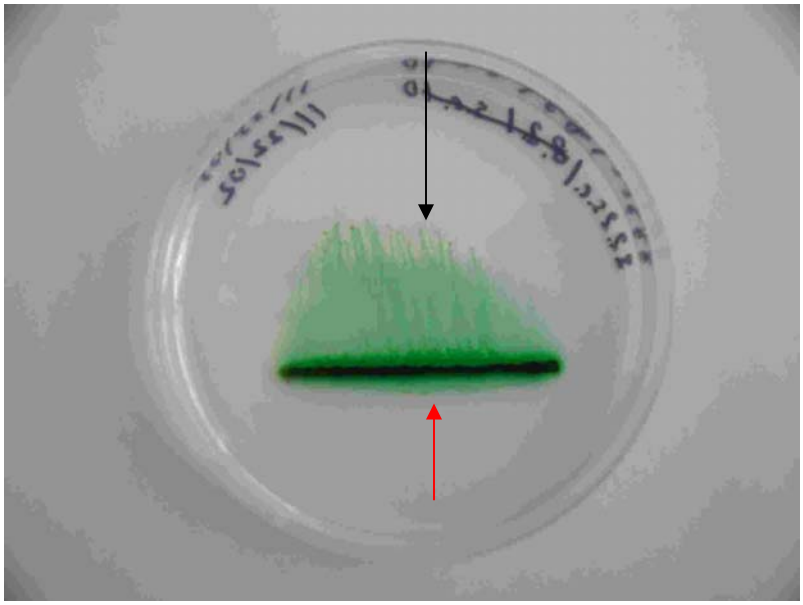

Fig. 5. Two photo dependent populations in the culture of 3.2.2 *Synechococcus* s.c. 1. *Black arrow points to the population with positive phototaxis and light direction, while red arrow points out to the population with negative phototaxis.*

In parallel, we are trying to cultivate 8.2.1 *Synechococcus* s.c. (10) in Ca-BG medium. This isolate grows poorly in batch culture with Ca-BG medium, although it grows well in standard BG-11 medium. It is possible that this is related to a decrease in CO_2/HCO_3^- transport into cyanobacterial cells.

In an experiment to compare the growth of 8.2.1 *Synechococcus* s.c.10 (unialgal isolate from Angel Terrace, where the aqueous environment is saturated with Ca^{2+}, Mg^{2+} and dissolved inorganic carbon-) in sodium- and calcium- containing media under aeration with 5% CO_2 in air. This isolate grows sufficiently well as a batch culture in BG-11 medium but grows poorly in Ca-BG. It is possible that CO_2 aeration could stimulate the growth of this cyanobacterium in calcium medium. After three days this culture was not growing. It is possible that over-aeration could be responsible for this. Thus we decreased the air/CO_2 mix flow rate from 100 ml/min to 40 ml/min and observed a restoration of culture growth in Ca-BG medium. Chlorophyll measurements revealed stable growth of 8.2.1 *Synechococcus* s.c.10 in Ca-BG, but no growth in BG-11 (doubling time was 15 h in early logarithmic stage of growth).

An experiment series has been initiated to compare the growth of several cyanobacteria in media aerated either with regular air (0.04% CO_2) or air/5% CO_2 mixture. The goal was to evaluate the effect of an elevated concentration of CO_2 on cyanobacterial growth in different media. The experiments will separate the effects of mechanical agitation from the bubbling and the direct effect of an increase in the concentration of CO_2. To do this we have increased the size of the glass photo-bioreactor. We are now able to aerate 12 growth tubes either with regular air or with air + 5% CO_2. Specifically, the experiment concerns the effect of enhanced CO_2 concentrations on the growth of 1.2 *Chlorogleopsis* s.c.6 in different media. We prepared 4 variations: i) BG-11 medium (18 mM Na^+) & regular air; ii) Ca-BG (20 mM Ca^{2+}) & regular air; iii) BG-11 and 5% CO_2; iv) Ca-BG & 5 % CO_2.

As it is shown on Fig.6 the aeration of Ca-BG medium with 5 % CO_2 in air leads to stronger acidification of this medium than in the other media.

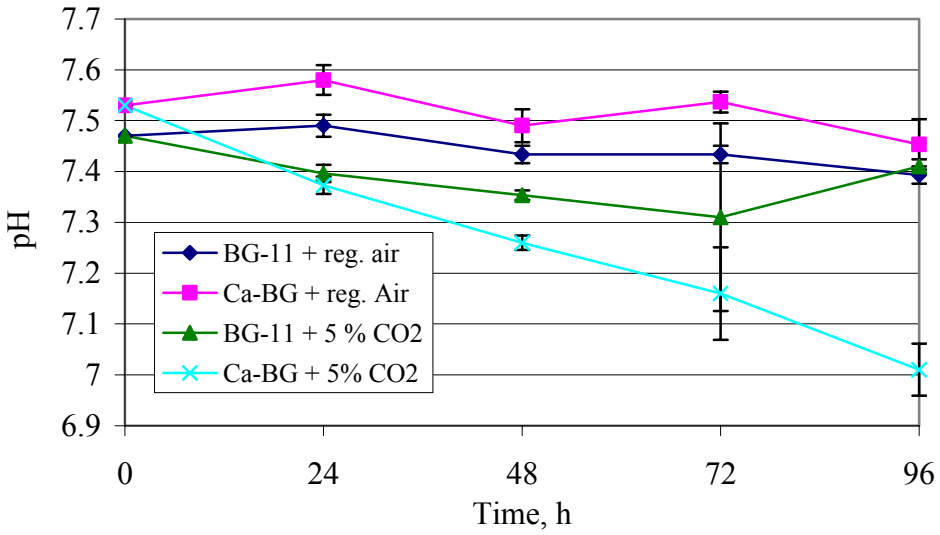

Fig.6. pH dynamics in growing cultures of Chlorogleopsis s.c.6, aerated with different gases

This observation suggests that the level of CO_2 supply was higher than required because the doubling time of isolate 3.2.2 *Chlorogleopsis* s.c. 6 under these conditions was about 13 h during second day of the incubation, as it was observed in regular BG-11 medium (Fig. 7 and 8).

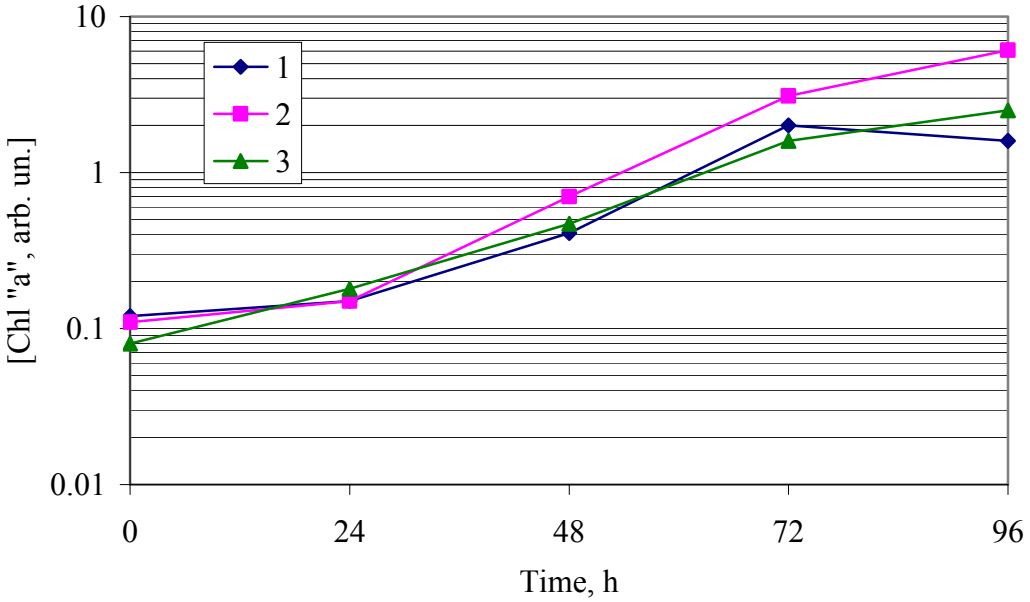

Fig.7. The growth of isolate 1.2.Chlorogleopsis s.c.6 in BG-11 medium aerated with 5 % CO_2 in air (3 single tubes) *Lines 1, 2, and 3 reflects isolate growth in individual tubes. Average doubling time was ~ 13 h during second day of the incubation*

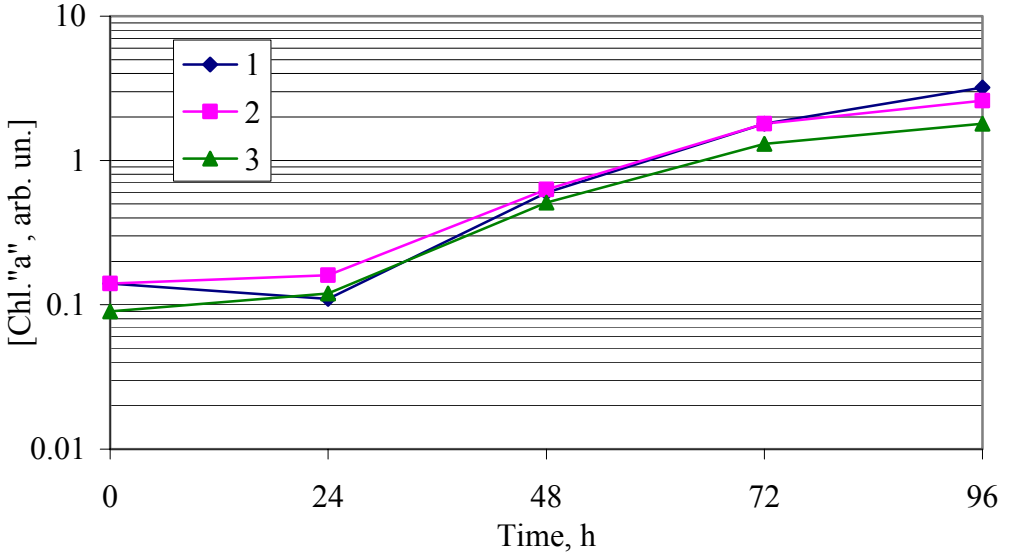

Fig.8. The growth of isolate 1.2.Chlorogleopsis s.c.6 in Ca-BG medium aerated with 5% CO_2 in air (3 single tubes) *Lines 1, 2, and 3 reflect isolate growth in individual tubes. Average doubling time was ~ 13 h during second day of the incubation.*

We believe that repression of cyanobacterial growth after 48 h is related to the observation that isolate 1.2 *Chlorogleopsis* s.c. 6 generates flocks of biomass in the late logarithmic phase. Besides, the rate of cell adhesion to tube walls was also increased at the late logarithmic phase, especially in Ca-BG medium. As the result the rate of single cell illumination significantly decreased and doubling time of cultures increased. Nonetheless, cyanobacterial biomass increased up to 60 times in tube No 2 with BG-11 medium, and up to 30 times in tubes 2 and 3 with Ca-BG medium.

Isolate 1.2 *Chlorogleopsis* s.c. 6 in different media aerated with regular air grew poorly (Fig. 9 and 10). Cyanobacterial biomass increased just up to 3 times under aeration with regular air. On the basis of these observations we conclude that the aeration of growth media with 5 % CO_2 in air stimulates cyanobacterial growth 10-20 times over that with air alone. It is possible the rate of the stimulation of cyanobacterial growth in CRF will be higher because cyanobacteria will be grown as a biofilm. We plan to increase the concentration to 15 % CO_2 in air.

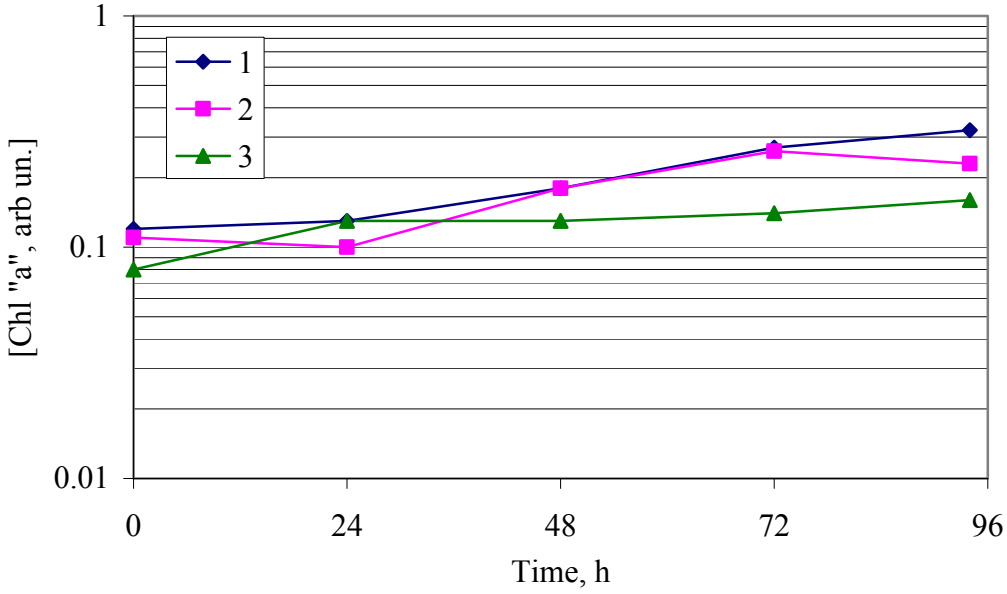

Fig. 9. The growth of isolate 1.2.Chlorogleopsis s.c.6 in BG-11 medium aerated with regular air (3 single tubes) *Average doubling time was ~ 30 h.*

Preservation of photosynthetic agents

We are continuing the organizing of our collection of the thermophilic cyanobacteria isolated from Yellowstone National Park. During this reporting period we transferred about 160 samples and discarded about 80 samples with weak growth in standard media as BG-11, D or DH. As result of this work we currently have 13 unialgal cultures of thermophilic cyanobacteria. They and their basic properties are listed in Table 1.

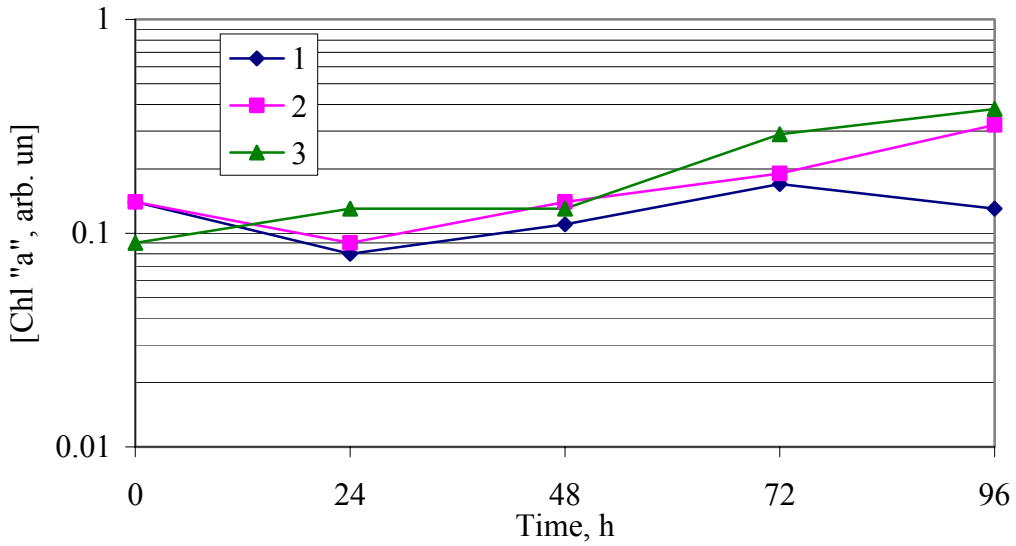

Fig.10 The growth of isolate 1.2.Chlorogleopsis s.c.6 in Ca-BG medium aerated with regular air (3 single tubes) *Average doubling time was ~ 45 h.*

Table 1: Details of the Current Unialgal Cultures at Montana State University. December 2002.

#	Code	Putative name	Place of isolation	Growth Temp.	Shape	Growth in Ca^{2+} media	Ability to grow with Omnisil
1.	8.2.1 Phormidium	*Leptolingbia*	Angel Terrace	45°C	Filament	+	n.d
2.	8.2.1 "Big trichomes"	*Lyngbia*	Angel Terrace	45°C	Filament	+/-	n.d
3.	8.2.1 Syncechococcus s.c. (10)	*Synechococcus*	Angel Terrace	55°C	Unicell	-	-
4.	8.1(IV)	*Oscillatoria*	Angel Terrace	45°C	Filament	n.d.	n.d
5.	1.2 s.c. (2)*[$]	*Chlorogleopsis*	Rabbit Creek	55°C	Weak filament	+	+
6.	1.2 s.c. (3)*[$]	*Chlorogleopsis*	Rabbit Creek	55°C	"	n.d	+
7.	1.2 s.c. (6)[$]	*Chlorogleopsis*	Rabbit Creek	55°C	"	+	+
8.	2.1 Fischerella	*Mastigocladus*	Rabbit Creek	55°C	"	n.d.	n.d.
9.	2.1 (III)	*Mastigocladus*	Rabbit Creek	55°C	"	+	-
10.	3.2.2 Low motility fraction Synechococcus s.c. (1)	*Synechococcus*	Rabbit Creek	55°C	Unicell	n.d.	+
11.	4.1 Oscillatoria princeps	*Oscillatoria princeps*	La Duke (Corwin Spring)	30°C	Filament	n.d.	n.d.
12.	5.2 Synechocystis[$]	*Synechocystis*	La Duke (Corwin Spring)	55°C	Unicell	n.d.	n.d.
13.	8.2 BF	*n.d.*	Angel Terrace	45ºC	?	+	n.d.

Notes
n.d. : Not determined
* sent to Ohio University
$ Isolate from wall mat. It means that such organism growth in the atmosphere of hot steam.
- "Weak filament" means that this organism is a chain former.
- Isolates 1.2s.c.(2,3,6) are probably dissimilar because they behave differently with respect to growth pH.
- Isolate 5.2 Synechocystis (#12) requires an elevated concentration of iron in the medium (X50). La Duke Spring is an iron rich site.

Report from the researchers at Ohio University

As reported last quarter, the organisms TR9.4 and 1.2 s.c. (2) continue to be grown in large containers (from 5-gallon to 109 gallon) for use in CRF tests, offline harvesting tests, and eventually the pilot-scale bioreactor tests. Additional organisms (currently identified as organism 3 and organism 4 for simplicity) are also being cultured and are in the process of being scaled up to the 5-gallon containers. These organisms will be available soon for preliminary testing in the bioreactor systems.

A series of tests was run to demonstrate that the mass of algae loaded into the CRF-2 system can be accurately determined based on a statistical sampling procedure. Results from the series of tests indicate that the sampling method can be used to reliably estimate the initial algae mass for a CRF-2 test with about 3% uncertainty.

Survivability tests for Sc1.2(2) on Omnisil using drilled-hole header inserts are currently underway. Numerous system problems have delayed the testing, but we are hopeful that the debugging of the system is now complete and the current test is proceeding well. Full test results will be provided in the next quarterly report.

Subtask 1.2 Design deep-penetration light delivery subsystem

ORNL continues to support the pilot-scale bioreactor development. The full system for the first solar collector has been installed and debugged and is ready for the pilot scale testing.

Subtask 1.3 Investigate growth surface subsystem design

Report from the researchers at Montana State:

We are screening the cultures to measure the effects of Omnisil on the growth of 2.1 (III) *Mastigicladus laminosum*, 8.2.1 *Synechococcus* s.c.10, 1.2 s.c. 6 *Chlorogleopsis* spp. and 3.3.2 *Synechococcus* s.c.1. It was found to date that only 1.2 *Chlorogleopsis* s.c.6 was able to grow in batch culture in the presence of Omnisil. We have repeated this experiment. The important point here is that in the following experiments the growth flasks were covered by window screening to see if the negative effect of Omnisil could be reduced at lower light levels. It was found that the

cultures 3.2.2 *Synechococcus* s.c. 1 and 1.2 *Chlorogleopsis* s.c. 6 were able to grow in the presence of Omnisil and colonized this substratum under moderate illumination. It seems reasonable to screen all cultures for Omnisil "resistance" before embarking on a program of physiological studies. Such studies would become redundant if in fact the cyanobacteria were not able to grow in the presence of Omnisil. Note that in contrast to the enrichments made from Scotch Brite, these isolates were not initially made from Omnisil placed in the field. The mechanical strength of this material did not allow us to leave them in situ. Further, some of the areas we sampled were mineral precipitating regions and thus the substrata quickly became engulfed in the mineral. This is a new batch of Omnisil we are now using and it has been washed as before. The pH of water after autoclaving with Omnisil is close to neutrality, so there is no contamination with acid as was found in earlier batches. At the moment we have no explanation for the toxic effect of Omnisil, if in fact it is merely spun silica. Nonetheless, we started the selection of Omnisil resistant clones among several cyanobacterial isolates. This process may require several months.

Table 2: Results of 3 experiments with Omnisil and new CB isolates.

Strain name	Survival Exp. On 11/17/02*	Coloniz Rate Exp. On 11/17/02*	Survival Exp on 12/04/02	Coloniz Rate Exp. On 12/04/02	Survival Exp on 12/12/02	Coloniz Rate Exp. On 12/12/02
2.1 (III) Mastigocladus	-	-	+/-	-	+/-	-
8.2.1 Trich. Cord Synechococcus s.c. 10	-	-	+/-	-	+/-	-
3.2.2 Syncechococcus s.c. 1	-	-	+	+	+	+
1.2 Chlorogleopsis s.c. 6	+	+	+	+	+	+

* The negative results of the experiment of 11/17/02 might be connected with high level of illumination.

Subtask 1.4 Investigate the use of a hydraulic jump to improve the system's overall CO_2 conversion efficiency

Based on the results collected and reported in previous reports, the experiments in this area have been discontinued. The Phase I experimental evaluation of the hydraulic jump is complete.

Subtask 1.5 Design harvesting subsystem

Our main activities this quarter were again focused on design and experimental work for the integrated screen wetting/ harvesting system, working primarily with Omnisil screens and 1.2. s.c.(2) organisms. We fabricated and tested the "Drilled Hole Design" inserts (Fig. 11). Initially tests conducted using water only showed that the flow across the membrane was even. In tests of the inserts using solution containing organisms (to analyze the behavior under actual conditions) the headers performed well in terms or providing even flow and avoiding clogging.

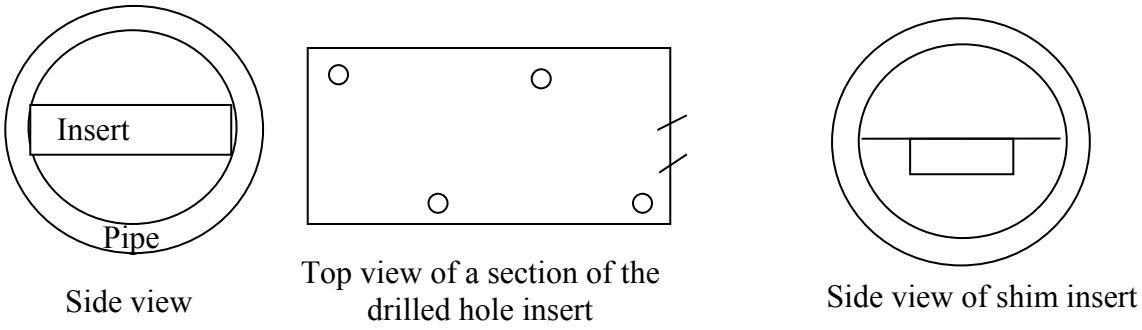

Figure 11: Header pipe flow control inserts

The insert design using shims to function as pressure and flow regulators (Fig 11) was fabricated and tested using 0.015 inch, 0.020inch and 0.025 inch thick shims. This design also showed good flow characteristics and there was no appreciable clogging noticed during and after the test. Overall it appeared that the "Pressure Manifold Design" was less susceptible to clogging than the drilled-hole design, which is an important concern during long term testing.
To gain additional information a comparative test was run to create a flow rate vs. pressure plot for three header insert designs (Fig. 12). Notice that the performance of the 0.02" shim and the drilled hole design are very similar, and thickening the shim has the expected effect of reducing the flow at a given pressure for the shim design, but the basic pressure-flow relationship retains the same form.

The tests described so far could not establish conclusively the header to be used for long-term tests. Tests were planned to analyze the performance of the "Drilled Hole Design" and "Pressure Manifold Design" with regards to Harvesting. The complete header and frame setups were constructed and were immersed in the 30 gal tank containing organism in order to get the membranes to the "harvest ready" condition. A test matrix was decided for the "Offline Harvesting" test. The test has not yet been done on account of unsatisfactory growth of organisms on the membrane surface. Ultimately, both header inserts will be tested in the CRF-2 to see which performs the best over long durations, and the best performing design will be used for the pilot scale bioreactor screens.

Since the header inserts will likely be manufactured from stainless steel, leaching tests were carried out to find the tolerance of the organisms to SS 316. Previous experience had shown that the organisms were not very tolerant towards Aluminum. The organism SC1.2 was very tolerant to SS 316 and it had no effect on the growth of the organism.

A new header using transparent polycarbonate pipes is also being constructed to allow us to see what is actually going on inside the header. This will enable us to sort out the problems faced with the header design selection.

Subtask 1.6 Quantify properties (higher heating value, elemental composition, volatile content) of dried biomass for potential end-uses. We continue to search the literature and search for potential end uses for the biomass, but experiments in this area are on hold until we get closer to making a final decision for the organism.

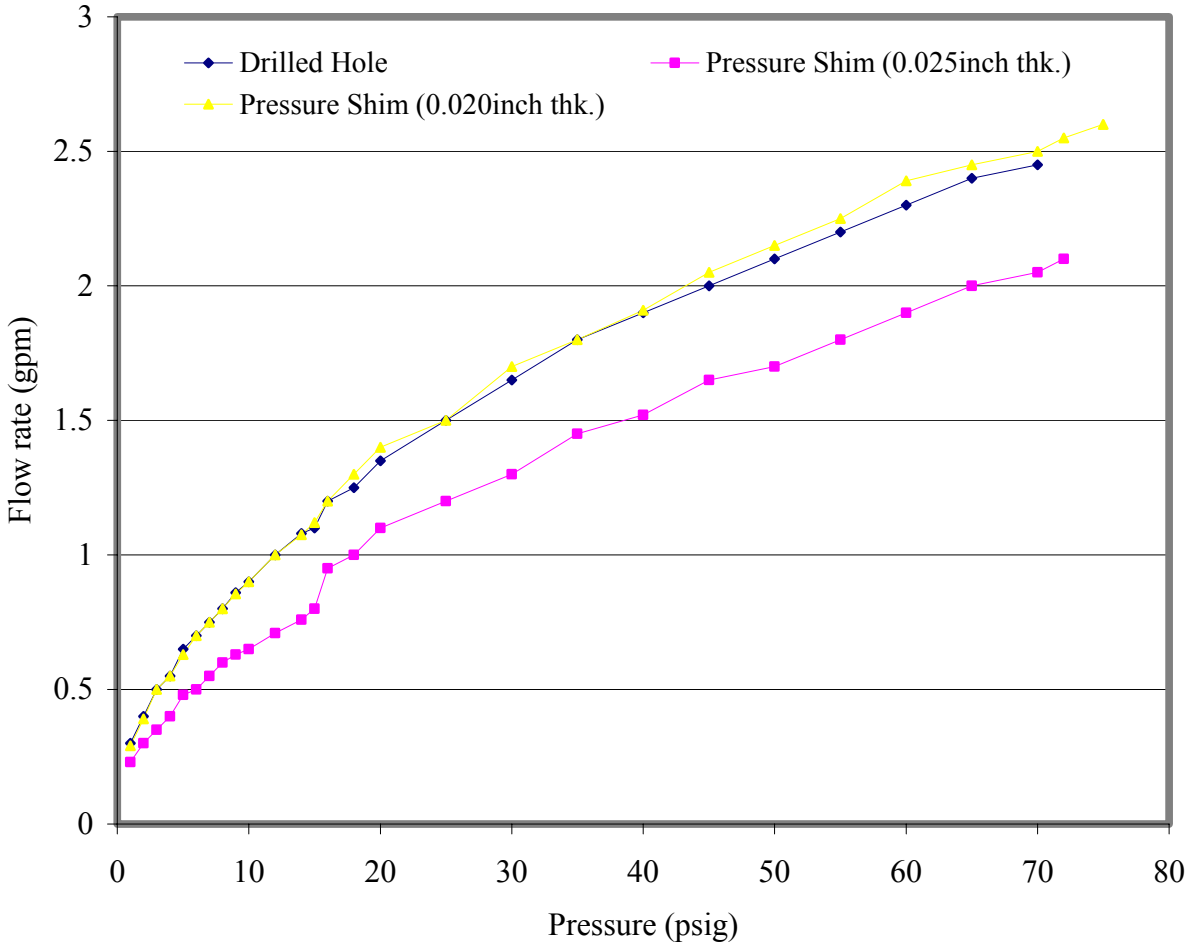

Fig. 12 Header flow characteristics as a function of water pressure

Task 2.0. Evaluate subsystem combinations and select an "optimum" system design

The construction and procedural validation of the larger scale CRF (CRF-2) to be used as the primary test facility for evaluating the subsystem combinations has been completed and initial tests are underway in the system. We should soon be able to run tests in the CRF-2 that will enable us to select the "optimum" system to be tested in the pilot scale bioreactor.

Task 3.0. Implement the optimum system in scaled model

The phase 1 construction of the bioreactor has been completed, including the solar collector and light distribution system. We are now waiting on CRF-2 results in order to be able to finalize the design and construction of the pilot scale system.

Webpage

The web page is running at http://132.235.19.45/DOE . All parties involved in the project have received e-mail instructions and the password to access the information.

Conclusions

As we begin the third year in this three year project we can report that we have made significant progress towards achieving our overall project goals but we have slipped a little behind schedule. We had a very successful project "retreat" on October 26th which included a detailed review of all work currently being done and how it fits in the critical path for completing the project within the next year. All of our test facilities are developed and our test plans and procedures are in place and we hope that in the first quarter of 2003 we will get the results we need from the CRF-2 bioreactor that will allow us to proceed with the pilot scale bioreactor testing.