CarboSchools

Carbon Dioxide Fertilization of Marine Microalgae (*Dunalliela sp.*) Cultures

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Aim:

This experiment was designed to illustrate the importance of carbon dioxide for microalgal growth in the aquatic environment (in this case, the green microalga, *Dunalliela sp,* in seawater). It should be emphasized beforehand, that the conditions simulated in this experiment do not represent the natural environment, since so far microalgae in the oceans have not been shown to be CO2 limited.

Materials:

200 ml - Polycarbonate Erlenmeyer Culture Flasks with Polyethylene covers
Pipettes
Vacuum Filter apparatus
Vacuum Pump
Filter Paper
GFF Filters
Cotton pads
Straws
Culture water (may be pond or lake water, Full strength seawater 35 psu or Baltic seawater 15 psu)
Nutrients for algae culture (for seawater f2 Medium) with NO3, PO4, Si, Vitamin mix, Metal Mix
Stock culture of freshwater or marine microalgae

If you are doing chlorophyll concentration analysis, it will be a little bit more complicated because you will need:

Spectrophotometer or a fluorometer
refrigerated centrifuge
homogenizer
Acetone

Procedure:

Simple Version: (Please see notes for teachers for improvisations)

1. Filter the culture water first with the paper filters and then filter sterilize it with the GFF filters. Filter sterilization is optional, this is necessary to minimize bacterial and foreign algal contamination.
2. Prepare a common culture for all the treatments. In 1 liter filtered culture water add the nutrients. In our case, 1ml of the NO3, PO4, Si and Metal mix stock solutions and 500ul of the Vitamin mix. (*This may be available in the partner research institute*)
3. Depending on the density of the stock algal culture, add about 5ml of it to the prepared culture medium.
4. Fill the culture flasks (properly washed and rinsed with filtered culture water) up to the 150 ml mark with the prepared algal culture. You can decide what treatments you would like to make. For example:
   Treatment 1 (T1): CO2 limited - flask covered tightly
   Treatment 2 (T2): CO2 fertilzed - flask covered with a cotton wad with a straw inserted in the middle for CO2 introduction by blowing
   Treatment 3 (T3): Control - flask covered with a cotton wad no CO2 fertilization
5. Make 3 replicates for each treatment. *(At this stage the flasks can be prepared in the classroom and the students can take the covered flasks home)*.
6. Place the flasks on a bright place but no direct sunlight. Treatments 1 and 3 are to be shaken at least twice daily. For treatment 2, air should be blown into the culture for 30 – 60 seconds twice a day.

More complicated version: (For older students)

1. It is imperative to filter sterilize the culture water. After filtering the water, the medium can be further prepared as described in the simple version.
2. Pipette the algae stock culture into the prepared medium. Get samples (about 20 ml) of the prepared culture for *chlorophyll a* determination at Day 0 (D 0). This will be filtered using a GFF filter. The filter will be placed in a special plastic tube, stoppered and then frozen for later processing.
3. Divide the prepared culture into the flasks and proceed as described in the simple version. Take samples as described above every 3days from the different treatments for *chlorophyll a* analysis. For T1, much care should be done that not too much CO2 from the air outside the flask gets into it. Depending on the density of the culture, 5-10 ml samples will be sufficient so that a variable pipette will be needed to get the samples.
4. *Chlorophyll a* determination will be described separately. To save on time and resources, all the samples can be frozen and processed together at one time.
5. After obtaining the chlorophyll concentrations, a growth curve (increase of chlorophyll with time) can be drawn.

Observations: (See pictures)

Day 0 -	No changes yet
Day 4 -	Green tinge in T2.
Day 6 -	Light green in T2. Green tinge in T1 and T3.
Day 10-	Darker green in T2. T3 becomes darker than T1.
Day 13-	Darker green in T2, T3 catching up with T2, T1 lightest green
Day 14-	No more change in T2 and T3(have reached the plateau stage of the growth curve) T1 no change in greenness much lighter than T2 and T3, arrested g growth of the cells

Further Experiment:

To test if the CO2 limitation is reversible, CO2 can now be introduced into T1 by blowing air into it, then closing the flask again.

Observation:

Even after just a day of fertilization with CO2 of T1, visible changes take place. The algae start growing and by the end of the sixth day, it has reached the intensity of greenness of T2 and T3 at Day 14.

Fig 1. From left to right CO2 limited treatment (T1), control (T3) and CO2 fertilized culture (T2). Clear difference can be seen between T2 and the other 2 treatments.

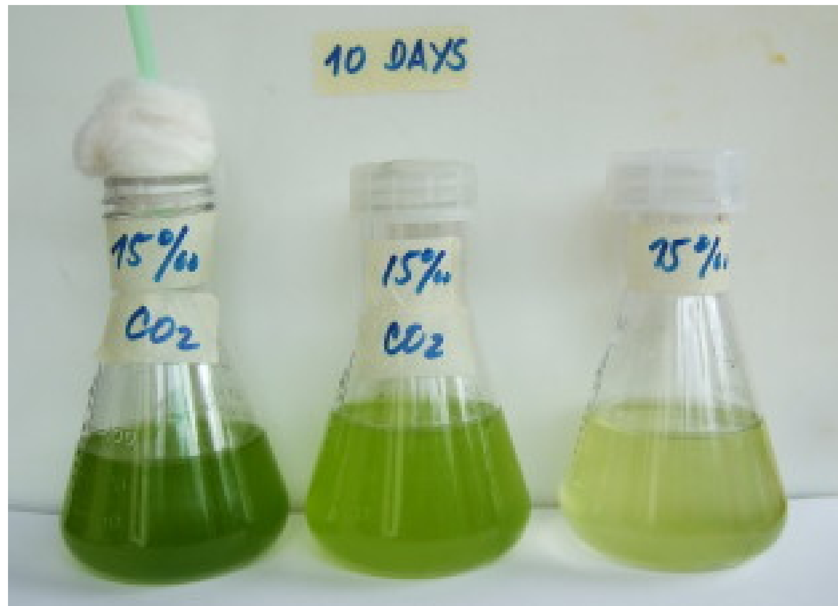

Fig 2. From l. to r. T2, T3 and T1. Clear differences can be observed between the 3 treatments. T2 has may have reached the plateau phase of microalgal growth. T3 is catching up with T2 and no further growth can be observed at T1.

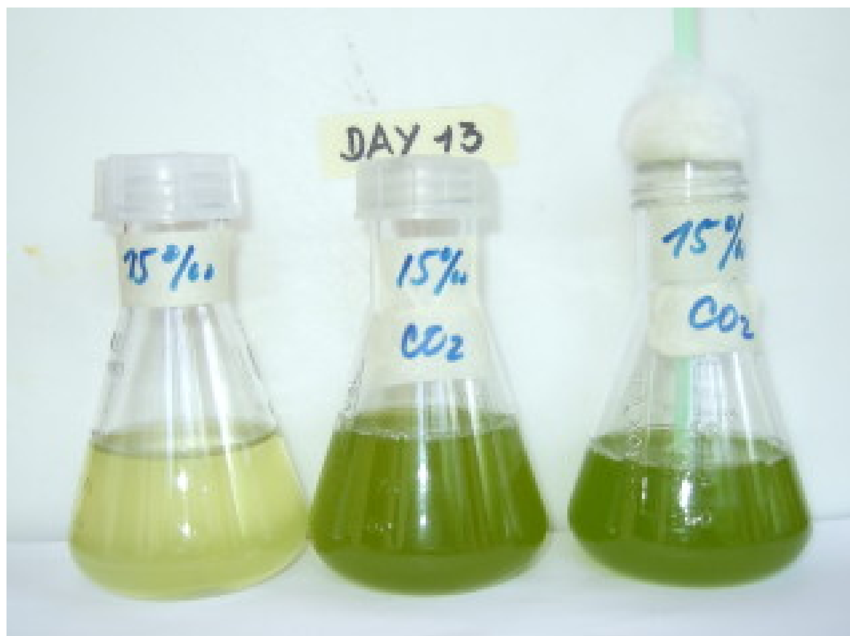

Fig 3. From l. to r. T1, T3 and T2. T3 and T2 are almost identical and T1 still lagging very far behind.

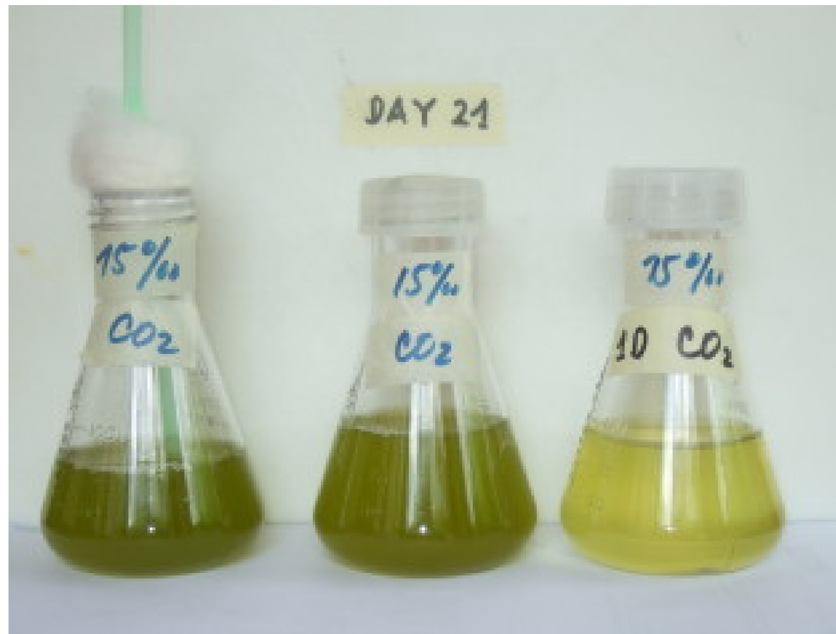

Fig 4. From l. to r. T2, T3 and T1. After 20 days, CO2 was introduced into T1. 1D CO2 means that CO2 has been introduced for 1 day in the T1 flask.

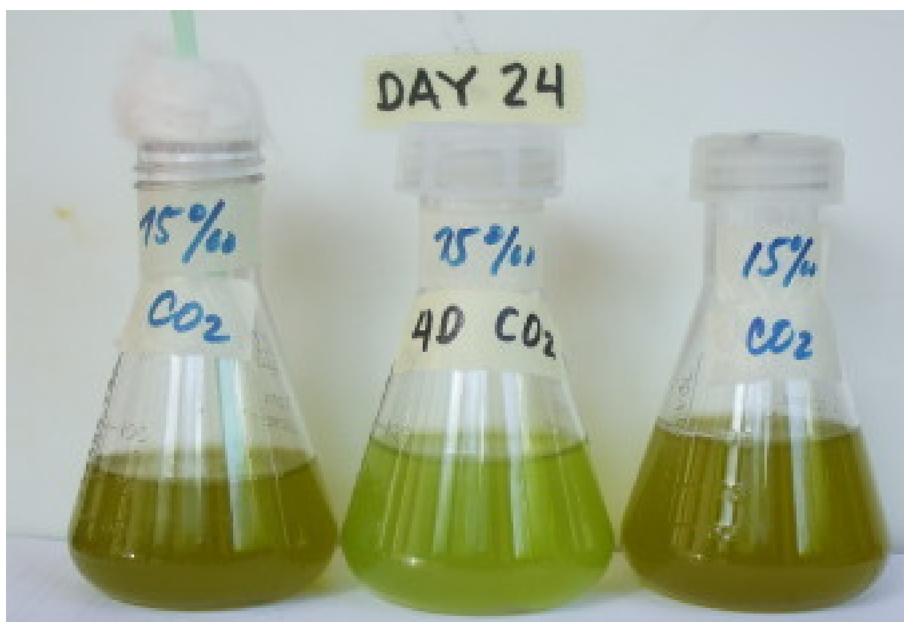

Fig 5. From l. to r. T2,T1 and T3. Whereas the cultures at T2 and T3 are starting to deteriorate as seen from the olive green color, T1 shows a fresh green color indicating younger cells in the culture. Recovery from CO2 limitation occurs fast in T1.

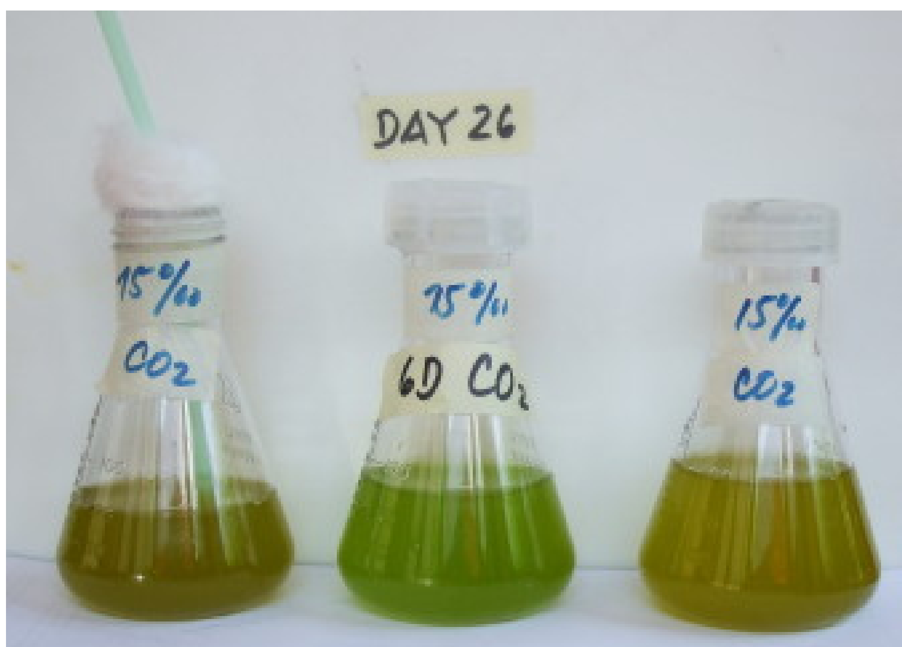

Fig 6. From l. to r. T2,T1 and T3. After 6 days of CO2 fertilization of T1, the culture has reached the plateau stage of the growth curve. T2 and T3 are further deteriorated.

NOTES FOR TEACHERS

1. Advantages of this experiment:

This experiment can be modified to fit different age groups. The method described here was designed for pupils aged 10-14. For older students, a quantitative approach can be done, where the growth of the microalga is not only determined qualitatively (visually) but also quantitatively through analysis of the increase in chlorophyll concentration in a time series sampling mode.

Other advantages are the relatively simple set up which school children can do by themselves alone and the relatively short span of time until the first results can be observed (depending on light and temperature conditions, in about 5 days).

This experiment can be prepared in the lab or in the classroom and the students can do the further steps at home, since the agal cultures are contained in easily transportable leak- proof culture flasks.

This experiment may look complicated at first glance but as soon as you get started up, it is very versatile because you can make and think of your own experiments like testing the effects of light, temperature, nutrients, etc. on algal growth.

2. Some suggestions for improvisations*:

If **seawater is not available**: Mix your own seawater. Salt for preparing seawater is available in your local pet shop.

If **the nutrients are not available:** Use ordinary plant fertilizer. Use one with high nitrate and phosphate content. You must use a higher concentration of the fertilizer like about 5-10 ml for a liter of culture water.

If **algal stock culture is not available:** Use a wild stock or naturally occurring populations. Collect water sample from the field and filter this through a wide mesh strainer to remove debris.

* Take note however, that these improvisations will lead to a longer incubation time, i.e., it will take longer till the first results can be observed. Normally all these can be provided by the partner research institute.

If **fluorometer or spectrophotometer is not available:** Try to ask your partner institute if they can lend you one. Or better still, you can do chlorophyll determinations in a lab at the institute.

Another alternative: If you want to do a quantitative approach to this experiment and no fluorometer or spectrophotometer is available, you can use an ordinary school photometer to measure changes in the turbidity of the cultures. 670 nm (wavelength) may be sensitive enough. This, however, does not give an accurate picture of the growth of the cells.

3. Answers to some questions which may arise:

Why is there growth in T1 even if there was no CO2 added?
Residual CO2 was still in the water and in the air inside the flask. This was sufficient to trigger initial growth of the algae but this could not be sustained because of the absence of CO2.

What is the difference in the growth of the microalgae in T2 and T3?
The growth curve of microalgae in culture is characterized by an initial log phase and at about day 14, the growth enters the plateau stage. With an excess of CO2 with T2, the plateau stage was reached faster than with the control where CO2 was available but not in excessive concentrations.

Why is there no CO2 -limitation in the natural marine environment?
The surface water of the ocean is in constant movement (waves)and contact with the atmosphere. This movement facilitates dissolution of CO2 in the water. Apparently the amount of CO2 dissolved in this manner is enough to sustain phytoplankton growth in the oceans, provided all other requirements are present. Normally there are other factors which limit phytoplankton growth in the oceans. The most common of which are the nutrients which normally come from freshwater inputs or from land masses. Iron has also been shown to limit phytoplankton growth in some parts of the ocean.

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