Phycology Lab – BOT 4404L – Summer B 2001

Lab Manual

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Safety Regulation in the Laboratory

Any work in a scientific laboratory involves delicate and expensive equipment and potentially hazardous chemicals. Whereas thoughtful and conscious working normally minimizes the danger of hazards, safety regulations and measures are required to prevent you and your colleagues from harm.

Safety regulations and instructor's instructions have to be obeyed at all time and immediately. Reckless behavior will result in an immediate suspension from this course without parole and a final grade of F. Do always remember that by not obeying the safety regulations and instructions, you not only endanger yourself but also your colleagues!

All students are expected to be familiar with the following regulations prior to the class:

- Any consumption of food or drinks and using any form of cosmetics is strictly prohibited inside the lab.
- Smoking is strictly prohibited inside the lab.
- Prior to the use of any equipment, make yourself familiar with the equipment or ask the instructors.
- Do not try to run or play with instruments not used in the class. Remember that this course is taught in a research lab, not in a teaching lab. Inexperienced and faulty handling of equipment does not only result in costly damage and repair, it also interrupts ongoing research activities.
- While handling and working with hazardous chemicals (acetone, formaldehyde), wear a lab coat, safety goggles and disposable gloves.
- If you are using glass pipettors, never use them with your mouth if transferring hazardous chemicals such as acetone or formaldehyde. Use a pipettor ball.
- Keep all bottles of hazardous material closed.
- Do not handle any open fire inside the lab. The lab contains highly flammable chemicals.
- Discard any glass objects only in the trash bin dedicated for sharps.
- Never discard hazardous chemicals into the sink. Ask your instructor for disposal.
- Prior to any work in the lab, make yourself familiar with the location and use of emergency exits, fire extinguisher, emergency shower and eye shower. Emergency phone numbers are posted at both entrances to the lab.
- Keep all water samples and chemicals away from electronic equipment. Water samples are only to be handled on benches along the wall.
- Turn off all cell phones and beepers prior to the class. No cell phones, beepers or other wireless communication devices will be tolerated during the class. No use of headphones is allowed in the lab! They may prevent you from hearing distress calls from your colleagues.
- Never ever hesitate to ask your instructors. Asking is always better than running into problems.
- Work always consciously to *prevent* harm rather than to cure harm.

Diversity of the Algae

Algae span from microscopic forms barely larger than a bacterium (0.4 µm) to huge kelp forests that grow to 50 m height. This first class shall introduce into the morphological diversity of algae, using life cultures and preserved specimen of micro- and macroalgae.

Microalgae

Prior to the study of life cultures, ask your instructor for the species name of the culture. Place a small drop of culture in the center of a glass microscope slide by a disposable transfer pipette. Use pipettes only once to avoid contamination of other cultures. Transfer filamentous algae by forceps, grabbing a few filaments

and spreading them out over the center of the microscope slide. Cover with a cover slip and study life specimens under the light microscope.

Take note of general cell shape and size and observe their swimming behavior. Then add a small drop of Lugol's iodine solution to one side of the cover slip and let the solution diffuse into the preparation. Wait 5-10 min to let the fixative work and observe details of cell morphology. In chlorophytes, take special attention to pyrenoids within the plastids; the starch in the pyrenoids shall stain dark blue by the iodine solution. On white paper, prepare drawings of your specimen by soft (HB) pencil. Mount all your drawings into your lab journal and include the species name next to the drawing.

From the cultures of filamentous cyanobacteria, take some filaments by pipette or forceps and place them into a glass scintillation vial containing 5 ml seawater. Add a drop of formaldehyde solution from the dropping bottle and let sample stand for 10 min. Then add 4 drops of methylene blue, let stain for another 10 min. Transfer stained filaments onto the center of a glass microscope slide and cover with cover slip. Examine under the light microscope for volutin (cyanobacterial P storage) and add their location to your drawing of the specimen.

Remember to dispose of glass microscope slides and transfer pipettes only into the dedicated glass/sharps container. Do never dispose of these items into the normal trash bins.

On permanent preparations, species names are listed on labels aside the cover slip. Take note of the species names and study the cellular morphology of your specimen. Prepare drawings and include them in your lab journal.

For demonstration purposes, a phytoplankton sample filtered onto a black polycarbonate filter of 0.2 µm pore size will be placed under the Zeiss Axioskop epifluorescence microscope. Epifluorescence microscopy is used to study and quantify small phytoplankton that is hard to observe or count under a normal light microscope (see page 5). The unstained sample will exhibit red chlorophyll fluorescence, and small, coccoid cyanobacteria (*Synechococcus* spp.) will appear orange to yellow by their accessory pigment phycoerythrin.

Macroalgae

Ask your instructor for the name of a given sample of macroalgae. Observe their general morphology within the culture vessel. Then pick one specimen by forceps and place it into a plastic petri dish. Prepare a drawing of your specimen. Place your petri dish under the stereo microscope and study your specimen's morphology in more detail. Add details to your drawing.

From filamentous algae or thin thalli, take a part of your specimen and spread it in the center of a glass microscope slide. If necessary, add a small amount of seawater. Study the cell morphology and arrangement of cells and draw them.

Extraction of Algal Chlorophyll and Pigments

From the life cultures of macroalgae, pick one specimen of green, brown, and red algae. Place each specimen into a cell grinder and add 10 ml of ice-cold 90% acetone by bottle dispenser. Grind specimen until completely homogenized. Fill homogenate into a labeled glass centrifuge tube and store on ice in the benchtop cooler. When all specimens are extracted, place all tubes into the benchtop centrifuge.

Take care to place tubes in holders on opposite side of the centrifuge head to balance the centrifuge. Spin tubes at 70% setting for 5 min. Then remove tubes, one at a time, carefully from the centrifuge and place in tube rack.

Under the instructor's supervision, fill a small volume of the acetone extract into a glass photometer cuvette and place cuvette into the spectrophotometer. A reference cuvette will be filled with 90% acetone.

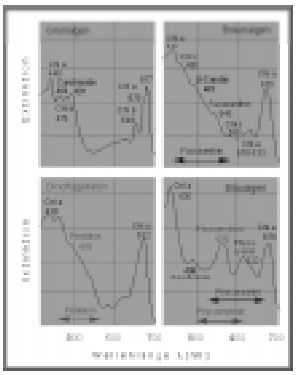

Fig. 1: Absorption spectra of green algae, brown algae, dinoflagellates, and cyanobacteria, 400-700 nm wavelength

The instructor will start a special program on the spectrophotometer to record an absorption spectrum of the pigment extract from 320 to 600 nm wavelength. Since the printer on the spectrophotometer is not in working condition, the spectrum will be photographed from the photometer screen by a Nikon digital camera. Printouts of the spectrum will be made available in the next class.

Empty the sample cuvette into the acetone waste bottle (never discard acetone into a sink!) and fill with your next extract. Another absorp-

tion spectrum will be measured and photographed. Repeat steps for all your extracts.

Mount the photographs of the absorption spectra into your lab journal with the specimen species name aside it. Discuss the differences among the measured spectra and the reasons for the differences (Fig. 1)

Field Sampling in Biscayne Bay

Safety Instructions

All participants in the field trip aboard the pontoon boat must be able to swim. Non-swimmers or students otherwise handicapped or unable to swim cannot go out. Students are expected to sign a liability waiver for FIU prior to the field trip (FIU regulation). All participants are expected to be familiar with the following safety regulations:

- All students aboard have to wear life preservers all the time.
- Wear solid shoes with rubber sole for safety reasons; no open shoes, no flip-flops or other loose shoes, no leather sole, no bare feet aboard; remember that shoes might get wet aboard, so don't bring your Sunday shoes!
- NEVER sit on the reeling!
- Bring rain gear along in case a thunderstorm comes up unexpectedly. Also be aware that waves might come into the boat; so ware cloths that can get wet.
- Bring sun glasses and a hat to protect your eyes and brain from the sun
- Bring sun blocker at least 15+
- Bring at least one quart of water (only water counts, no juice or soda!)
- Operate any equipment only if you are familiar with the equipment and only after the skipper has explicitly allowed deploying equipment (otherwise lines are easily entangled in the prop, destroying the prop and leaving us rowing back home).
- Prior to deploying any equipment overboard, make sure lines are secured to the boat to prevent loss of equipment.
- If interested, you might bring your photo camera and / or binocular. But make sure you have watertight storage for them.

Sampling Procedures

For the Biscayne Bay field study, participating students will be grouped into 3-4 researchteams responsible for sampling and data analysis of one station each. We will visit 3-4 stations in Biscayne Bay and outside the Bay, close to Hallover Inlet. If weather conditions permit, we will visit one blue-water station outside the Bay.

Fig.2: FIU research boat "Southern Star", a 32' fiber glass pontoon boat with two 40 HP Honda outboard engines

Before deploying any instrument, make sure the skipper has positioned the boat properly and has allowed sampling. Deploy all equipment only to the port (left) side of the boat, and only one equipment at a time.

Have your station log ready to take note of all measurements. Notes shall only be made by pencil, because your sheet may become wet, and ink or ball pen is washed out.

At first, note time and station on your sampling log, that measure water temperature and salinity with a YSI probe. Turn the YSI meter on and make sure the pointer settles above the red line. Then turn knob to read temperature. Adjust temperature compensation knob to actual temperature reading, then take salinity reading (take care to take readings from the correct scale!). Salinity is calculated by the YSI meter from measurement of conductivity; since conductivity is temperature-dependent, correct temperature setting on the YSI meter is important for correct salinity data. Note all readings in your station log. Turn the YSI meter off, pull the probe back into the boat and store it in a bucket with seawater.

Take your water sample from ca. 1 m below the water surface by the water sampler (Fig. 3). Lower the water sampler to sampling depth, then release the messenger weight from the boat. The messenger weight will hit the release mechanism of the water sampler, which will close on both sides. Pull the sampler back into the boat, fill your sample in a 1 L polycarbonate bottle and store the bottle in the closed cooler.

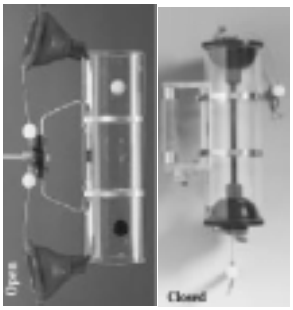

Fig. 3: water sampler, open and closed

Deploy the 20 µm plankton net with a sample bottle attached to the

end. The plankton net will be towed for ten min by slowly moving the boat in circles. Watch the net at all times and inform the skipper immediately if the net comes close to the boat props. After 10 min, pull the net back into the boat and fill the net sample into a storage bottle containing formaldehyde to preserve the sample for later analysis. Wash the net without the end bucket 2-3 times by dipping into the water. Also wash the end bucket and re-attach it to the end of the net for the next deployment.

Sample Handling after the Boat Trip

To preserve the samples for further analyses in the next class, several procedures must be applied directly after the cruise.

The first procedure is to label all your bottles with your group number or station number. Store the formaldehyde-fixed plankton net samples in the dark refrigerator at 4°C.

From your water sample, fill 40 ml into a Falcon plastic centrifuge tube with blue lid. Add 4 ml formaldehyde, shake smoothly, and store in the dark refrigerator at 4°C in the provided tube rack. These samples will serve for later flow cytometric analysis and fluorescence microscopy.

From your water sample, fill another 50 ml into a Falcon plastic centrifuge tube with blue lid. Add 2 ml of Lugol's iodine solution, shake smoothly, and store with the formaldehyde-fixed sample at 4°C. These samples will be used for the inverted microscope (Utermöhl counting). The instructors will use these samples to prepare the Utermöhl settling chambers 24 hrs prior to the next class, so that your samples are ready to be inspected and counted with the inverted microscope in the next class.

From your water sample, measure 500 ml in a cylinder and filter on 25 mm diameter Whatman GF/F glass fiber filter. Use the 6-fold PVC filtration manifold and fill approximately 150 ml into the filtration funnel. The vacuum on the pump gauge shall not read more than –300 mbar to prevent cell damage during filtration (which can lead to underestimation of phytoplankton chlorophyll). Refill the funnel before the filter runs dry. Let the filter run dry at the end of filtration. Under applied vacuum, lift the GF/F filter carefully with forceps and place filter on the bottom of a 20 ml glass scintillation vial with the filtered sample up. Close vial and store in the freezer at –20°C until chlorophyll extraction during next class. Make sure again that all your vials and tubes are properly labeled, using labeling tape and ball pen.

Analyses of field samples

Chlorophyll Analysis

Place your GF/F filter from the glass vial stored at –20°C into a homogenizer (filtered surface up) and add 10 ml of 90% acetone by bottle dispenser. Carefully grind filter in acetone until fully homogenized. Take care not to spill any acetone and wear lab coat and goggles. Fill all acetone with filter debris into a labeled glass centrifuge tube and store on ice in bench cooler.

Warning: Label all vials and tubes with label tape and ball pen. Do not use marker, because acetone might dissolve your labeling if drops are running along the vial or tube surface.

When all groups have extracted their chlorophyll filters, all centrifuge tubes are placed in a desktop centrifuge. Make sure to balance the centrifuge by placing tubes into opposite holders of the centrifuge head. Spin tubes at 70% setting for 10 min. Remove one tube at a time carefully and decant supernatant into your glass scintillation vial. Take care to leave pellet in tube.

Chlorophyll concentrations will be determined by a Turner 111 fluorometer. Your instructors will explain its operation. Fill your chlorophyll extract into a fluorometer cuvette so that the cuvette is ¾ full. Make sure the fluorometer is set to zero by the blind tube, then remove blind tube and place cuvette into fluorometer. Take your reading and note in your lab journal. Convert the fluorometer reading (value between 0 and 100) to chlorophyll concentration ($\mu g\ l^{-1}$) by the provided conversion factor F:

$$\text{Chl.a}\ [\mu g\ l^{-1}] = \text{Reading} \times F$$

The conversion factor was previously estimated by comparing fluorometer readings to chlorophyll concentrations calculated from spectrophotometer readings. Each fluorometer setting (1, 3, 10, 30) has its own conversion factor, so note your fluorometer setting with your original reading in your lab manual.

Depending on the chlorophyll concentration in your extract, chlorophyll can also be measured on a spectrophotometer. A fluorometer has a much higher sensitivity, though, and in low production systems the chlorophyll concentration in extracts of filtered seawater samples is often too low to be estimated by spectrophotometry unless large volumes of water are filtered. The use of fluorometers is, therefore,

largely preferred in open ocean and tropical waters because the lesser volume required to be filtered presents a significant time saving without loss of sensitivity.

For spectrophotometric chlorophyll determination, place your extract into a photometer glass cuvette. Usually cuvettes of 5 cm length are used for chlorophyll analyses (but are not available on present equipment at FIU) because a longer path length as compared to normal 1 cm cuvettes results in higher absorption and, therefore, higher sensitivity of the analysis. Measure absorption of your extract at wavelengths of 750, 663, 645, and 630 nm; 750 nm is used as a correction for any particulate material in your extract; 663, 645, and 630 nm are the absorption maxima for chl. *a*, *b*, and *c*, respectively. Chlorophyll *a* in units of µg l^{-1} is calculated as:

$$\text{Chl.a} = \frac{11.87\times E_{663\text{-}750} - 1.54\times E_{645\text{-}750} - 0.08\times E_{630\text{-}750}}{V \times L}$$

with $E_{663\text{-}750}$ absorption at 663 nm corrected for absorption at 750 nm (E_{663}-E_{750}), etc; V filtered sample volume [l], L length of the cuvette [cm].

Utermöhl Counting

The inverted microscope technique was introduced in 1931 by the German limnologist Utermöhl. He designed an assortment of sedimentation tubes of known volume (typically between 10 and 100 ml), which are placed on top of a special microscope slide with a shallow sample chamber. The bottom of the sample chamber is made of a thin glass plate, not thicker than a normal cover slip.

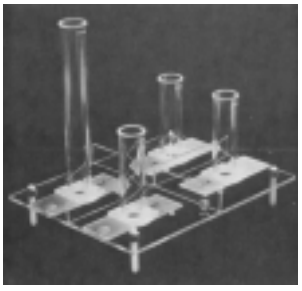

Fig. 4: Utermöhl settling chambers for inverted microscope counts of phytoplankton, settling chambers (tubes) mounted

The water sample is filled into the sedimentation chamber (tube) and a cover glass is positioned without any air bubble at the top of the tube. The assembly is allowed to stay for 24 hrs on a leveled surface. During this time, all plankton cells sediment to the bottom of the assembly. Fixation by Lugol's iodine solution rather than formaldehyde helps plankton sedimentation because the iodine increases the density (specific weight) of the plankton cells.

Prior to analysis, the settling chamber (tube) is slit aside carefully by another, rectangular glass plate that will cover the bottom chamber. No air bubble shall arise in the bottom chamber. The water in the settling tube is discarded. The bottom plate is placed on an inverted microscope, on which the objectives are below the microscope stage rather than above.

At low (20x objective) magnification, scan the sample and note what forms of phytoplankton are present, and which forms are dominant. Try to identify the abundant forms and include sketch drawings in your lab journal. Decide on which forms are abundant enough for quantitative counting. Change to 40x objective and inspect smaller forms in the same manner.

For counting, switch back to the 20x objective and move the microscope stage so that you start at the top end of the plankton chamber. The eye piece of the microscope contains a measuring scale (Fig. 5). Imagine vertical lines from both ends of the graticule scale; count all cells within the view field between the two imaginary lines. Then move microscope stage one view field further and repeat counting for the next view field, etc. until you reach the other side of the plankton chamber. Always keep track of your counts.

Fig. 5: Microscope eye piece graticule with horizontal micrometer scale

Switch to the 40x objective and move microscope stage to a random place in the center of the chamber. Count smaller cells in a view field defined by the edge of the eye piece view and take note of your counts in your lab journal. Repeat counting for a total of 10 random view fields.

For each species/form, compute the total number of cells counted. Convert these numbers to cell numbers per liter by conversion factors (F) provided by your instructors:

N [cells l^{-1}] = Count × F

Epifluorescence Microscopy Counting

The study of small phytoplankton of less than 2 µm in size was virtually impossible until the introduction of epifluorescence microscopy to marine science in 1979. These small algal cells, referred to as picoplankton, are too light to settle in the Utermöhl settling chambers and too small to be quantitatively recognized in normal light microscopy. Although the existence of picophytoplankton was known by electron microscopy studies of water samples, this technique did not

lend itself for routine water sample analysis. High abundance of small, phototrophic flagellates of ca. 5 µm in size was already demonstrated by the German oceanographer and biologist Hans Lohmann in 1902. He termed this group of organisms "nannoplankton", which later became the nanoplankton, the size range of cells 2-20 µm in size. A further problem posed the differentiation of small phytoplankton (phototrophic) cells from similarly sized heterotrophic cells such as bacteria and small heterotrophic flagellates.

It was only after epifluorescence microscopy was introduced and routinely applied that the enormous contribution of picophytoplankton in open ocean and warm-water systems was realized. In the tropic ocean, about 80% or more of the total phytoplankton biomass is contributed by cells less than 2 µm in size. The phototrophic cyanobacteria *Synechococcus* spp. (ca. 0.5-0.8 µm) and *Prochlorococcus* spp. (ca. 0.4 µm) are the most abundant picophytoplankton, but eukaryotic algae contribute to picophytoplankton as well.

A major part of marine primary producers were, thus, not recognized in studies prior to the early 1980's. The realization of the ubiquitous and abundant occurrence of nano- and picosized phytoplankton led to a new evaluation of the role of the microbial food web (bacteria, photo- and heterotrophic pico- and nanoplankton, ciliates), known as the concept of the "microbial loop" postulated by Farouq Azam (Scripps, San Diego) and colleagues in 1984. Whereas the biology, ecology and physiology of *Synechococcus* and *Prochlorococcus* are meanwhile well studied, the taxonomy and physiology of picoplanktonic eukaryotic algae is still largely unknown.

The principle of epifluorescence microscopy is to filter a known volume of water sample onto black Nuclepore® polycarbonate filters with 0.2 µm pore size. In contrast to glass fiber filter (GF/F) or acetate membrane filters, which act like a sponge, polycarbonate filters have a smooth and plane surface on which all cells are retained. Cells can further be stained by, for example, DNA or protein dyes while on the filter. The filters are then placed under an epifluorescence microscope, in which the light comes from above the filter (in contrast to transmitted light in normal light microscopy). The light source, usually a 50W mercury lamp, is filtered through light filters to provide excitation light of known wavelengths to excite molecules within the cells to fluoresce. Cells are recognized in the microscope by their characteristic fluorescence.

Under blue light (450-490 nm), chlorophyll will fluoresce red. Phycoerythrin, an accessory pigment present in cyanobacteria and cryptophytes, will fluoresce yellow; by the combination of their red chlorophyll and their yellow phycoerythrin fluorescence, these cells will appear orange under the epifluorescence microscope. Heterotrophic flagellates, ciliates and bacteria, which lack chlorophyll, will emit no fluorescence. These organisms can be made visible by protein staining with proflavine or DTAF, which produces a green fluorescence. Algal food within ciliate food vacuoles might be seen by the faint red chlorophyll autofluorescence of the prey, though. Despite being presently assigned to the cyanobacteria, *Prochlorococcus* spp. lack phycoerythrin and exhibit only red chlorophyll fluorescence. These cells are hard to count under the epifluorescence microscope, because their chlorophyll fluorescence is extremely week and fades within less than a minute under the blue light microscope excitation. Flow cytometry (see below) is the only reliable way to count these smallest phototrophs.

Epifluorescence microscopy is also used to count bacteria, either by staining with DAPI or acridine orange. Both dyes stain DNA. Acridine orange produces an orange fluorescence under blue light excitation, whereas DAPI produces a blue fluorescence under UV light excitation. One problem with acridine orange is the differentiation of big bacteria and *Synechococcus* spp., both exhibiting a similar fluorescence.

Since this lab course is focused on the phytoplankton, we will not apply a fluorescent dye. From the formaldehyde-fixed sample stored at 4°C, filter 10 ml onto black 0.2 µm Nuclepore filters at no more than –300 mbar vacuum on the 6-fold filtration manifold. Let the filter run dry. Under applied vacuum, remove the filter funnel and pick the black Nuclepore filter by forceps. Take a labeled microscope slide, breath three times on it to moisten it, and place the filter in the center of the slide, slowly letting it slide down from one side. Place a drop of non-fluorescent Cargile® immersion oil in the center of the filter and let a cover slip glide on it. The oil will spread over the filter by the weight of the cover slip alone. Such slides can be stored at –20°C for several months without loss of fluorescence.

When you have your slide prepared, contact your instructor to mount the slide on the Zeiss Axioskop fluorescence microscope. He will add a drop of immersion oil on top of the cover slip and place your filter under the 100× oil objective.

First take an overview look at what cells you can see on your filter (different fluorescence colors, cell sizes and shapes). Discuss with your instructor which cells are probably which species or groups of phytoplankton. Take note of your observations and your discussion with your instructor in your lab manual.

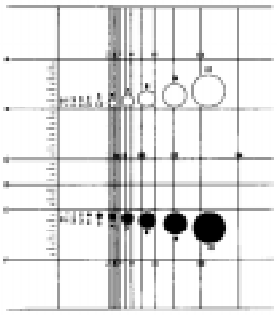

Fig. 6: New Porton G12 eye-piece graticule

Then count your cells. While counting, distinguish 2 or 3 groups of cells, e.g. orange fluorescent *Synechococcus*, red fluorescent small eukaryotes, red fluorescent big eukaryotes. In one of the eye-pieces, the Zeiss microscope has a New-Porton G12 grid (Fig. 6). Adjust the eye-piece so that you can see the grid in focus. Use the circles on the grid to differentiate "small" and "big" (e.g. all smaller than circle 6 are "small"). Count all cells in a grid and take note of the counts (Tab. 1). The statistical counting error decreases with the number of cells counted. Counting of ca. 300 events is required for a ±10% counting error. The lab class does not allow for enough time to achieve this counting precision, though. In total, count 10 different grids randomly distributed on the filter by slowly moving the slide by the microscope mechanical stage.

Table 1: Record of cell counts by epifluorescence microscopy

Field No.	Synechococcus	Small Eukaryotes	Big Eukaryotes
1	20	7	3
2	23	11	6
…	…	…	…
10	19	6	5
Sum	C	C	C
10^3 ml^{-1}	N	N	N

For each group of organisms, convert your counts to cell concentration:

$$N\ [10^3\ ml^{-1}] = (84.0892 \times C) / (GF \times V)$$

with C = sum of counted cells; GF = total fields counted; V = filtered sample volume [ml]. The conversion factor of 84.0892 reflects the share of the area of one New-Porton grid in relation to the total filter area (inner diameter of the filter funnel), divided by 1000 to express cell concentration in 10^3 cells ml^{-1}.

Algae Mating Experiments

Isolated cultures of + (female) and – (male) strains of the green algae *Cosmarium* sp. (Desmidiaceae) and *Oedogonium* sp. were provided by Carolina Biological Supply Co. Two to three days prior to the class, your instructors initialize the cultures for the mating experiment.

Culture material from both the + and – strains are poured into a plastic petri dish that is placed inside a plastic box acting as "greenhouse" container. Water is filled into the "greenhouse" container, avoiding any water to enter the petri dish. One Alka-Seltzer® tablet is placed into the water outside the petri dish as carbon dioxide generator. The "greenhouse" container is closed and placed in the light incubator at 20°C and 16:8 hours light / dark cycle.

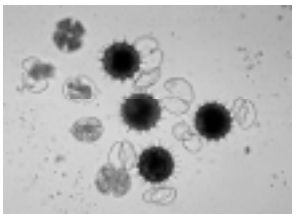

Fig. 7: Zygote formation in the green algae Cosmarium

Cosmarium is a common phytoplankton alga found in rivers and lakes as well as other water bodies. The cell is composed of two semi-cells, constricted in the middle. This region is termed the isthmus and is the site of the nucleus. The outer portions of the semi cells contain massive chloroplast with pyrenoids. The outer cell wall of each semi-cell is very ornate and is species specific. Both asexual and sexual reproduction occurs. Fig. 7 depicts sexual reproduction with the formation of zygospores (zygotes). The gametes have gone from the parental cell walls through pores and have fused in a region midway between the parental walls. The zygote has formed a very ornate wall.

Pairing and first fusion usually begin to occur within 48 hours. Large, dark, spiny-walled zygotes can easily be seen with the four empty semicell walls still in place. The progress of the mating may be observed by placing the petri dish under a stereo microscope. For detailed examination, try to capture some mating stages

and zygotes from the petri dish under the stereo microscope by a disposable glass pipette. Place specimens on microscope slide, cover with cover slip and study under the compound microscope. Take drawings of your specimens into your lab journal.

The zoospores and sperm that are produced by *Oedogonium* have a ring of flagella that is unique to the algae. In *Oedogonium*, life cycles are somewhat complex. There are plants that produce both eggs (oogonium) and sperm (antheridium) (Fig. 8), produce two plants, one producing sperm and the other producing eggs, and a third that produces three plants, one producing eggs, one producing androspores and a third in which the androspores produce a dwarf male plant (Fig. 9).

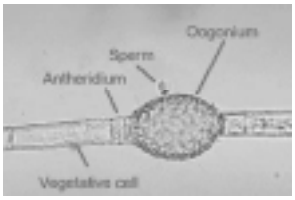

Fig. 8: Oogonium and antheridium formation in the green alga Oedogonium; dioecous life cycle form

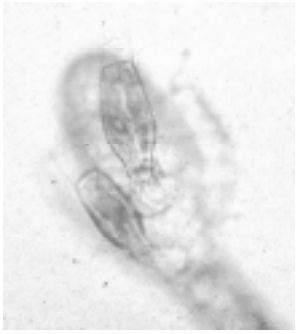

Fig. 9: Dwarf males of Oedogonium

The style of new wall formation is also unique. A ring of hemicellulose first forms in the anterior end of the cell (Fig. 10). This is followed by nuclear division, migration of the new nuclei, and an expansion of the hemicellulose ring. The last phase is a form of cytokinesis.

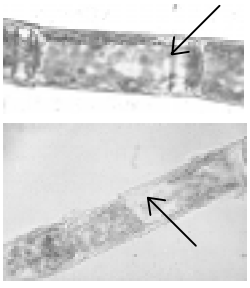

Fig. 10: Cell wall formation in Oedogonium. Hemicellulose rings form at left side of the cell (upper panel), which further expand upon nuclear division (lower panel)

Oogonia and antheridia can usually be found in great quantity 48 to 72 hours after mating. There will often be zoospore production at the same time as mating. These large zoospores and young germlings can be seen at the edges of the petri dish under the stereo microscope. For detailed examination, try to capture some mating stages and zygotes from the petri dish under the stereo microscope by a disposable glass pipette. Place specimens on microscope slide, cover with cover slip and study under the compound microscope. Take drawings of your specimens into your lab journal.

Productivity versus light experiment

Background

Estimating primary production, i.e. the photosynthetic capacity, of phytoplankton is a major task in marine and freshwater ecology. The primary production (units of µg C l^{-1} h^{-1}) determines the energy and carbon input into the pelagic food web and constraints production of higher trophic levels (protozoa, zooplankton, fish).

Three principle methods are today applied to estimate primary production in aquatic systems:

- *In-situ* measurements: Water samples are filled in bottles that are incubated in the field at the depth of sampling for 4-24 hrs;
- *Simulated in-situ* measurements: Water samples are filled in bottles that are incubated in the lab or aboard ship in incubators providing ambient temperature and light conditions; light conditions for samples from different depths are usually achieved by wrapping bottles into neutral density filters of known absorption. Light profiles of the sampling station are required.
- *Productivity versus light* measurements: Each sample is exposed to a range of light intensities and photosynthesis is measured at each light intensity. Light profiles of the sampled field station are required. The primary productivity at any given light depth in the field is computed from the production versus light relationship.

Whereas *in-situ* measurements are generally the least unbiased approach, it is often not possible to perform such incubations over extended periods. This is a particular problem on larger research vessels accommodating the needs for several working groups; often, the ship cannot afford to stay on a given station for the incubation time required for *in-situ* measurements (several hours).

Different techniques are available to measure primary production in any of the three incubation methods:

- ^{14}C method: A known activity of radioactive $H^{14}CO_3^-$ is added to the sample bottles; dark bottles are used as correction for any non-

photosynthetic carbon uptake (recent techniques replace the dark bottle by bottles treated with photosystem inhibitiors). After incubation, the sample is filtered onto 0.2 µm membrane filters and the radioactivity on the filters is measured by liquid scintillation counting. This method is quite sensitive but produces radioactive waste. Special work training and certification is required.

- ^{13}C method: Works in the same way as the ^{14}C method, but the stable isotope ^{13}C is used instead of the radioactive ^{14}C. Sensitivity is lower than with ^{14}C due to the limitation of today's mass spectrometers, and the substrate $H^{13}CO_3^-$ is fairly expensive.
- Oxygen method: The production of oxygen by photosynthesis is measured either by start and end point analyses (oxygen titration) or continuously by oxygen probes. Recent oxygen probes became very sensitive but are not suited for *in-situ* measurements yet. Oxygen production by the phytoplankton is converted to carbon production by a conversion factor, the *photosynthetic quotient* (molecules oxygen produced per molecule CO_2 assimilated), usually a value of 1.2. The oxygen method has inherent problems in that (a) the photosynthetic quotient may vary among phytoplankton groups or with environmental conditions; (b) respiration of heterotrophic organisms during the incubation mask part of the oxygen production, so that oxygen measurements present the net primary production (photosynthesis minus respiration).

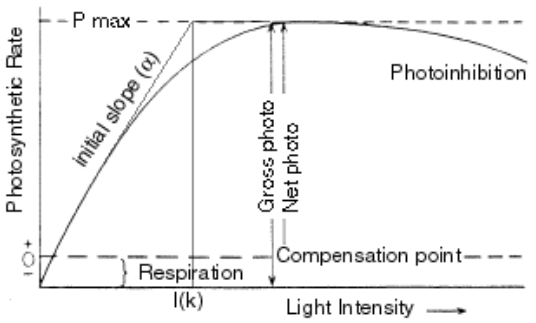

Fig. 11: Productivity versus light curve with characteristic parameters, P_{max}, α, I_k

Productivity versus light intensity curves (short P/I curves) also provide valuable insight into the phytoplankton physiology as they provide characteristic, numeric parameters (Fig. 11). To establish these parameters, productivity is usually normalized to chlorophyll *a* or total carbon in the sample, so that values are comparable among differently concentrated samples.

The maximum productivity (P_{max}) of the sample describes the photosynthetic capacity of the specific community or species. More light does not further enhance photosynthesis, and often higher light intensities cause decreased productivity. This phenomenon known as light inhibition is related to damage to the photosystem by too high light intensities. They may be reversible or become irreversible.

The linearized slope of the P/I curve, α, is also known as the *quantum yield* ($\Phi = \Delta P / \Delta I$). The quantum yield describes how efficient low light intensities are used for photosynthesis. Shade-adapted plants exhibit a higher α than high light adapted plants, whereas they mostly have a lower P_{max}.

Another parameter to characterize the physiology and light adaptation of algae from P/I curves is I_k. I_k is a combination of P_{max} and α; it is constructed in the P/I curve as the vertical intercept on the light axis from the intercept of α and P_{max}. Shade-adapted plants exhibit a lower I_k.

Experimental Design

Each "research group" is provided with one sample, either a field sample or a phytoplankton culture. Fill the provided incubation bottle almost full with your sample. To lower the oxygen concentration, your incubation bottle is briefly bubbled with nitrogen gas (ask instructor for how long). Lowering the oxygen concentration in your incubation bottle shall prevent inhibition of oxygen production (photosynthesis) by oversaturation of the sample water. Final oxygen concentration should be ca. 50%. Top up your incubation bottle with your sample material and insert the oxygen probe without air bubbles.

Place your incubation bottle in front of a slide projector (light source). Different light intensities are achieved by neutral density filter tubes of known absorption (noted on the tubes) that are placed over the incubation bottle.

The oxygen probe measures O_2 concentration in units of ml/l, and readings are plotted on a XY chart recorder. It is important that you note the time setting (chart advance) in your notebook. Also note the amplification setting, i.e. how many millimeters on the chart (Y scale) correspond to how many ml O_2/l. You will need this information for later calculations.

Start your experiment with the dark tube and record changes in O_2 concentration over 10 min. Photosynthesis will be inhibited in the dark, respiration will prevail, and oxygen production will have a negative value! After 10 min, change the dark tube for the light tube with the lowest number (numbers on tubes correspond to light transmittance in % of incident light). Mark your chart on the recorder when the light tubes are switched. Record O_2 concentration for another 10 min and switch to the next tube; etc. For light tubes of high transmittance, 5 min recording time might suffice.

After all light intensities, filter a known volume of your sample onto Whatman GF/F glass fiber filters, extract and measure chlorophyll *a* as described on page 4.

Data Analysis

From your XY chart recording, calculate oxygen production for each light intensity by computing the linear slope from the beginning to the end of illumination at this intensity.

$P1 = \Delta O_2$ (ml/l) / t (illumination time; min)

Convert P1 to oxygen production per hour:

$P2 = P1 \times t/60$ [ml O_2 l^{-1} h^{-1}]

Convert P2 into oxygen production in units of mg O_2 per liter and hour:

$P3 = P2 \times 1.4291$ [mg O_2 l^{-1} h^{-1}]

Convert oxygen production to carbon production:

$P = P3 \times 1000 \times 0.375 / 1.2$ [µg C l^{-1} h^{-1}]

with 0.375 being the mass ratio of carbon to oxygen (12 mg C / 32 mg O_2); 1.2 being the photosynthetic quotient; factor of 1000 to convert mg into µg.

For comparison with data from the other research groups, normalize your production for all light intensities by your measured chl.*a* concentration:

$P^* = P$ / Chl.a (µg l^{-1}) [µg C h^{-1} (µg chl.a)$^{-1}$]

Plot all your P* values against light intensity on a letter-size millimeter paper. From your graph, extrapolate the characteristic parameters P_{max}, α, and I_k. Mount your plot in your lab journal and note the above parameters. Also get the parameters from the other "research teams" and discuss the results in terms of light adaptation.

Nutrient Limitation in Biscayne Bay

Background

In the field, the growth of organisms may be limited by the availability of essential resources to fuel the energy and metabolic demands of growing and dividing cells. For phytoplankton, such limiting resources can be light as the primary energy resource to fuel photosynthesis or nutrients. Among nutrients, nitrogen and phosphate are the major limiting resources in marine and freshwater systems. For diatoms, silicate is another potentially limiting nutrient, because diatoms require silicate for frustle formation upon cell division.

Deep mixing during winter storms reduce the light availability to phytoplankton particularly in temperate and polar seas. Light decreases exponentially with water depth, and by deep mixing, the average light energy a single cell experiences over a day is decreased. Integrated over the light period of a day, deep mixing can cause algal cells to experience less light, thus less energy input, than required for active growth. Light limitation is less likely in clear, shallow waters and in tropical oceans, which are mostly stratified and where mixing is shallow.

Carbon dioxide is another essential resource for phytoplankton growth as it presents the major carbon source for biomass formation by photosynthesis. Whereas CO_2 can become limiting to photosynthesis in terrestrial plants and freshwater systems, the carbonate reservoir of seawater mostly prevents limiting CO_2 concentrations.

There are special regions in the oceans where other essential elements are limiting phytoplankton growth. For example, iron has been shown to limit phytoplankton production in the Southern Ocean around Antarctica, but also in the central Pacific Ocean. Such elements that are required for cell growth and division but occur in minor concentrations are referred to as micronutrients, in contrast to macronutrients such as nitrogen (N), phosphorus (P) and silicate (Si).

Whereas most freshwater systems are P-limited, most marine systems are N-limited. In coastal areas and estuaries, the influence of land and rivers can shift nutrient limitation to P as well. Studies in the late 1980's revealed that Biscayne Bay phytoplankton is primarily P-limited. Our experiments shall revisit this hypothesis and reveal the degree of nitrogen or phosphorus limitation at different stations within and outside the Bay.

Experimental Design

The principle of nutrient limitation experiments is to incubate sample volumes from the

same station at identical conditions (light, temperature). Nutrients are added in surplus to all but one (control) sample, and algal growth is followed and compared to the control. If algal growth is nutrient limited, it will be higher in the nutrient amended sample(s) than in the control (Fig12).

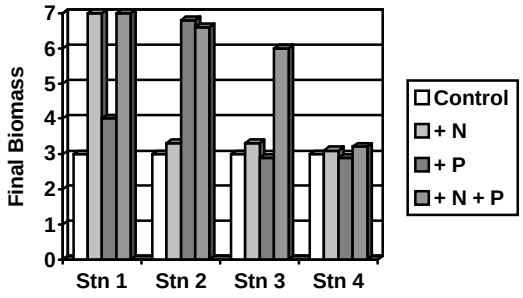

Fig 12: Nutrient enrichment experiments. Stn 1 is N limited, Stn 2 is P limited, Stn 3 is N/P co-limited, Stn 4 is neither limited by N or P.

Whereas in such experiments, the growth curve of the samples is recorded normally by daily sampling, the time frame of this lab class does not allow for daily sampling. Therefore, we will compare the final biomass or cell yields of the different treatments. Experiment results shall be plotted by each group as in Fig. 12.

Procedures

From your sample, fill 5 1-L Erlenmeyer flasks with 500 ml of sample water. Label each flask as "control", "N", "P", "NP", "Si", respectively. Add nutrients to each flask according to the below scheme (nutrient stock solutions will be provided):

Control	no addition
N	1 ml nitrate solution
P	1 ml phosphate solution
NP	1 ml nitrate + 1 ml phosphate
Si	1 ml silicate solution

Close all flasks with aluminum foil to prevent contamination and excess water evaporation and place your labeled flasks in the light incubator. Make sure that all flasks are placed on the same shelf and at the same distance from the door (light source).

From the original sample, measure 500 ml and filter onto a Whatman GF/F glass fiber filter under –300 mbar vacuum for later chlorophyll analysis. Place your filters in labeled glass scintillation vials with the filtered (green) surface up and store vials in the freezer at –20°C.

Fill another 10 ml from your original sample into another labeled glass scintillation vial and add 1 ml of formaldehyde solution. Shake smoothly and place vial into dark refrigerator at 4°C for later sample analysis by flow cytometry.

In the next class, retrieve your culture flasks from the light incubator. Fill 10 ml of each flask in labeled glass scintillation vials and add 1 ml of formaldehyde. From each flask, also measure 400 ml, filter onto Whatman GF/F filter under –300 mbar vacuum, and place filter in labeled glass scintillation vials.

One after the other, place the GF/F filter in a homogenizer (filtered side up) and add 10 ml ice-cold 90% acetone by bottle dispenser. Grind the filter and fill the acetone extract with ground filter debris into a labeled glass tube. Place glass tube on ice in benchtop cooler. Keep your labeled scintillation vials for later use! After all filters are extracted, place all 6 glass tubes into the centrifuge and spin for 5 min at 70% setting. Then carefully remove one tube from the centrifuge and decant the supernatant carefully into the corresponding scintillation vial. Close vial and put on ice. Repeat for all tubes.

Warning: Label all vials and tubes with label tape and ball pen. Do not use marker, because acetone might dissolve your labeling if drops are running along the vial or tube surface.

Chlorophyll extracts will be measured in the Turner 111 fluorometer. One after the other, fill your chlorophyll extract from the vial into the fluorometer cuvette and note your reading. Convert fluorometer readings to chl.a in units of $\mu g\ l^{-1}$ by the provided conversion factor.

Bring all six formaldehyde-fixed samples to the FACSort flow cytometer. Under instructor's supervision, perform cytometric cell counting of all samples after choosing the appropriate gate settings. To allow all groups to perform their measurements within the class time, printouts with the results of your cytometric measurements will be provided at the end of the class or the beginning of next class.

Analyze both chlorophyll concentrations and cell counts in comparing the final numbers of each flask with the initial numbers. Plot increase or decrease of phytoplankton biomass and cell number (final – initial value) against the sample treatment as depicted in Fig. 12. From the cytometric histograms, deduct changes in phytoplankton composition, if discernible, and describe these changes in your lab report.