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# MELiSSA

## Preliminary studies for the development of a closed ecosystem limited to gas exchanges between two partners

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# INTRODUCTION

Europe projects to land an experimental module on moon before 2001, and in this context, we take advantage of the opportunity for a biological experiment which would consist in developing a little ecosystem, limited to gas exchanges (O2 and CO2) between two, autotrohic and heterotrophic partners. The aim of this experiment is to study the kinetics of gas exchanges and of biomass production on moon surface, under gravity reduced to 1/6 of that found on earth.

The very large number of potential autotroph and heterotoph candidates is greatly reduced by technical and biological constraints imposed by the flight ant experimental conditions.

* An important technical constraint is a 34 to 36 days long waiting period in orbit before landing on moon surface, that imposes to select organisms which may be temporarily conserved in resting metabolic conditions and subsequently revitalised.
* Other technical constraints originate in the structure of the bioreactor, in which gas exchanges between the two compartment may occur through an intermediate gas chamber or directly through a single gas permeable membrane, a case where accompanying additional ions and molecules (particularly metabolites) exchanges should be considered. The probable optical method for measurement of biomass formation the probable regulation of the reactor at a single temperature also direct the choice of the biological partners.
* The coupled growth of the two partners on the basis of O2 and CO2 exchanges imposes the choice of an obligate oxygenic photosynthetic phototroph organism, which would grow in the presence of CO2 and light and produce oxygen required by its heterotoph partner, which should be obligate aerobic to avoid fermentative processes and oxygen independent growth.
* In addition, any additional informations concerning possible candidates, such as environment parameters (temperature, light, culture medium, ...), kinetics parameters (growth, μmax, O2, CO2, ...), availability in strain collections, or strain axeny would be welcome.

Our participation to this project consists in a preliminary bibliographic study of these constraints and of possible autotroph and heterotoph candidate for the planned development on the moon of such a simple closed ecological ecosystem limited to gas exchanges between two partners. The first part of this study is devoted to the analysis of the imposed constraints, the second to the research of biological characteristics of possible partners, in order to propose in a third part a limited number of candidates that would be the best adapted for the project.

# PART 1 - ANALYSIS OF TECHNICAL CONSTRAINTS

## 1 - BIOMASS MEASUREMENTS

A simple method required for the measurement of biomass formation in the limited volume and weight allowed for the experiment could correspond to an optical, nephelometric method based on light scattering by particles. This would then imply that the selected organisms would be unicellular (or eventually small filamentous), prokaryotic or eukaryotic microorganisms growing in liquid, culture medium. It would then be critical to ensure a good homogeneity of the culture by efficient stirring, and to avoid the formation of biofilms on glasses in the optical path.

The requirement of controlled culture conditions implies an axenic selected strain.

## 2 - CELL STORAGE AND STABILITY : METHODS OF PRESERVATION

The microorganisms selected for the colonisation of the ecosystem compartments should be maintained in resting conditions and survive during the ~ 35 days long waiting period in orbit before landing. The preservation and storage procedure are therefore of critical importance for the success of the experiment. We selected three methods to temporarily block any metabolic activity for cells storage during this waiting phase, one based on natural spore formation, and two others using cold or desiccation.

### 2.1- Spores and resting cells

Microorganisms have developed different strategies to resist to unfavourable environmental conditions, among which sporulation. The term "spore" is somewhat loosely used in microbiology. It embraces various kinds of prokaryotic and eukaryotic cells involved in vegetative propagation and dispersal (aplanospores and zoospores); and also specialised resting cells, usually with thickened or sculptured walls (hypnospores, akinetes, etc.).

Resting spores are common among terrestrial and freshwater Chlorobionts, cyanobacteria, fungi or bacteria. They are considered to be resting cells resistant to low temperatures and to desiccation and they were observed to remain viable for long periods under such adverse conditions. By comparison with the extensive studies of the spores of fungi or bacteria, however, there have been very few critical investigations of the physical and biochemical factors involved in algal sporulation and germination. The table below shows the Eubacteria resting cells and spores :

| | **endospores** | **exospores** | **myxospores microcysts** | **cysts** | **arthro spores** | **akinetes** |
|---|---|---|---|---|---|---|
| **Resistance to desiccation** | High | High | High | High | High | High |
| **Thermo resistance** | High | High | Moderated | Low | Moderated | Moderated (Variable) |
| **Structure** | cortex (exosporium) | | spore envelope or capsule | exocystorium exine | fibrous sheath | cell differentiation |
| **Genera or group** | *Bacillus Clostridium Desulfato maculum Sporosarcina Thermo actinomyces* | methanotrophs *Methylosinas* | mixobacteria *Myxococcus Archangium Stigmatella* | *Azotobacter* methanotrophs *Methylobacter Methylococcus Methylomonas* | *Streptomyces* | filamentous cyanobacteria |

Some precisions on parameters involved in spore formation are given with the following examples :

**Endospore-forming bacteria** are distinguished by their morphology, relationship to O2, and energy metabolism. The shape and cellular location of endospores are of taxonomic value in distinguishing species within a genus. These organisms are genetically heterogeneous, but ecologically related. All occur primarily in soil; the endospore provides a survival mechanism in the highly variable soil environment. *Bacillus* strains often produce extracellular hydrolases which attack macromolecules. Antibiotics may be produced during sporulation. *Clostridium* species are obligate anaerobes that can only synthesise ATP by substrate-level phosphorylation.

Endospore-forming bacteria produce a unique resting cell called an endospore. They are Gram-positive and usually rod-shaped, but there are exceptions. The two important genera are Bacillus, which are aerobic spore-formers in the soils, and *Clostridium*, which are anaerobic spore-formers of soils, sediments and the intestinal tracts of animals.

**Cyanobacterial resting cells:** Some cyanobacteria of section IV produce resistant forms or akinetes. Cyanobacterial akinetes are considered to be resting cells, normally resistant to low temperatures and to desiccation but rarely surviving to high temperature. Akinetes were found to be restricted to certain genera of filamentous heterocystous cyanobacteria (see cyanobacteria Section VI and V). The structure of cyanobacterial akinetes, well reviewed previously[1] [2], will not be described in detail here.

Akinete development is induced by growth phase, light and extracellular compounds; their differentiation is controlled by energetic aspects of photosynthesis and respiration. On return of conditions favourable for growth, akinetes were seen to germinate, either immediately or only after a resting period

Cyanobacterial akinetes appear to be of interest for our project as a resistant form of the photosynthetic partner, but their use may be limited by the uncertainties concerning the environmental and biological factors involved in their formation (even if phosphate deficiency is an inducing factor in *Cylindrospermum*) and in their germination into vegetative cells. Moreover, axenic strains appear to loose this capacity after several generations in artificial culture medium., and the use of non axenic strains which would have kept the capacity of akinete formation is not compatible with our project.

The use of cyanobacterial akinetes as resistant form of the photosynthetic partner therefore requires preliminary studies that are beyond the scope of the project.

**Auxospores of diatoms :** Among spores of photosynthetic micro-eukaryotes, auxospores of diatoms are particularly resistant and can relatively easily reactivated. However, in cultures of *Melosira Nummuloides* it was not found possible to inhibit auxospore formation altogether, or initiate it in non competent cells, by varying the concentration of nitrate or phosphate within the range which permitted mitotic growth or by modifying the metabolism with sub lethal concentration of 2,4-dinitrophenol. On the other hand, treatment with continuous light of 3400 lux intensity at 24°C inhibited sporulation completely, although it permitted intensive vegetative growth leading to cells with the smallest diameter known for this species[3].

In conclusion, in most of spore forming microorganisms, the environmental or physiological parameters that induce spore formation or germination are poorly understood; and

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[1] Nichols, J.M., Carr, N.G., 1978, Akinetes of cyanobacteria, In C.H. Chambliss and J.C. Vary (eds.), Spores, VII, American society for microbiology, Washington D.C., pp. 335-343.

[2] Nichols, J.M., Adam, D.G., 1982, Akinetes, In N.G. Carr and B.A. Whitton (eds.), The biology of cyanobacteria, Blackwell Scientific Publ., Oxford, pp. 387-412.

[3] Erben, K, 1962, Sporulation In RA Lewin (Ed.), Physiology and biochemistry of algae, Academic Press, New York, pp.701-710.

small variations in environmental conditions are susceptible to induce sporulation and to disturb the experiment. The choice of spores as resting cells therefore does not appear to be appropriate for our project.

## 2.2- Desiccation and drying

Many microorganisms (spore-forming fungi, cyanobacteria, streptomycetes) can be preserved by drying (25 to 40°C) the spores or the organisms on the surface of various sterilised inert solid substrates, such soil, silica gel or glass beads[4].

If applicable to our project, this relatively simple method would be particularly suitable to conserve large amounts of microorganisms in limited volume. However, informations on technical and environmental conditions for successful treatment of most of possible candidates are not available in the literature and therefore would have to be experimentally determined if this option was retained.

## 2.3- Cryopreservation

**Freezing** is the simplest used method for preserving microorganisms. Cultures are frozen with temperatures ranging from -5 to -20°C. The viability of many microorganisms can be maintained for weeks by this procedure. However, this method does not allow a total recovery of living cells upon reactivation of many microorganisms (micro-algae and cyanobacteria are examples).

**Cryopreservation** refers to the storage of a living organism at ultra-low-temperature such that it can be revived and restored to the same living state as before it was stored. Indefinitely long storage times require that the organism be maintained below the glass transformation temperature of aqueous solutions, approximately -130 °C, the temperature at which frozen water no longer sublimes and recrystallizes. Storage in liquid nitrogen is often used.

In any case, microorganisms (bacteria, fungi[5], ...), as all other living cells, suffer severe osmotic stress and/or ice crystal damage during the freezing and thawing processes. The most effective known ways to minimise these potentially lethal effects are to add a cryoprotectant compound to the culture prior to its freezing for storage, and to control the transient cooling and warming rates during preservation.

**Lyophilisation** : After being frozen the material is freeze-dried through the sublimation of ice[6]. The procedure consists of the following three stages : freezing, sublimation and desorption. Lyophilisation is particularly useful when the preserved cultures are in need of transport since there is usually no need to refrigerate lyophilised cultures. Freeze-dried cells should be conserved for long term under vacuum.

These methods of preservation provide varying degrees of success with different species of microorganisms, and neither results in 100% recovery of the preserved cells. When it is vitally important that cultures are not lost, it is advisable to use two methods in parallel. If desiccation may be used without important risks for the conservation of prokaryotic cells, storage in liquid nitrogen and freeze-drying techniques have become standard methods for the long-term maintenance of many micro-organism cultures because they are the best documented and they provide the best chances of success.

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[4]Chang, LT, Elander, RP, , Long-term preservation of industrially important microorganisms, In : pp.52-53.

[5] Smith D., 1984, Maintenance of fungi. In Kirsop B.E. and Snell J.J.S. (eds). Maintenance of microorganisms. A manuel of laboratory methods. Acad. Press. pp. 109-130.

[6] Kirsop B.E.,1984, Maintenance of bacteria freeze-drying. In Kirsop B.E. and Snell J.J.S. (eds). Maintenance of microorganisms. A manuel of laboratory methods. Acad. Press. pp. 109-130.

## 2.4 - Procedures and technical aspects of cryopreservation and Lyophilisation

(see annex for the specific case of cyanobacteria)

**Characteristics of the used strains :** The selected strains should be obtained from a culture collection, with its characteristics of axeny, and with the possible methods of preservation (frozen, freeze-dried or storage in liquid nitrogen). Bacteria are easily stored with any one of these methods, but oxygenic photosynthetic microorganisms (micro-algae and cyanobacteria) may be susceptible to some of these preservation methods.

**Freezing of cultures :** Cultures should be harvested at the end of logarithmic phase or beginning of stationary phase because they survive to cryopreservation better than those harvested at other phases of growth[7]. Transfer the liquid culture into a cryovial, pellet the cells and discard the supernatant. Alternatively the culture can be grown directly as a lawn on a tiny agar slant prepared within the cryovial[8]. Resuspend the pelleted cells in a small volume (some ml) of the cryoprotectant solution containing half-strength culture medium and 5% methanol or 8% DMSO. Alternatively, if the culture is growing on an agar slant, transfer 1 ml of cryoprotectant solution above the slant.

**Storage in liquid nitrogen or Lyophilisation :** freezing and freeze-drying techniques have become standard methods for the long-term maintenance of micro-organism cultures (see [9][10]).

Storage at low temperature implies that cells frozen in liquid nitrogen would be maintained at a maximal temperature of -10°C with any device during the duration of the flight before revitalisation. Moreover, the frozen cell sample should be diluted to limit the toxic effects of cryoprotectants and bring in contact with the separated liquid culture medium only at the onset of the experiment. This complicated method increases weight and volume.

Lyophilisation appears more adapted to the project because it satisfy to the weight and volume constraints and because this method does not need additional procedures to preserve the viability of the cells. Protectants such as proline may be used.

**Revitalisation of cultures :** The revitalisation process is the step most susceptible to failure and it ii improved by limiting possible stresses, . It is generally best to resuspend the cells stored in liquid $N_2$ or on lyophilised disks in a small volume of the appropriate medium (no more than 5 ml) followed by incubation at room temperature (20-25°C) without illumination for a couple of day. The viability of the cryopreserved photosynthetic micro-organism depends on the slow or rapid warming of the sample. However, significantly higher levels of viability were always observed when the algae were thawed rapidly[11]. Both liquid nitrogen freezing and Lyophilisation result in cellular killing, magnitude level depending on the species of phototrophic or heterotrophic microorganisms being used.

The culture may then be placed in liquid growth media under normal growth conditions. The viable cells of photosynthetic microorganisms should begin normal growth within 1-2 days in light, although they are especially susceptible to damage by excessive light intensity, and in this respect, it may be necessary to determine the optimal duration of a dark period after thawing and before illumination of the photosynthetic cells. A few cyanobacteria, such as *Synechococcus* PCC 7002, may be directly resuspended in medium and revitalised at 39°C under light illumination.

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[7] Day, JG, De Ville, MM, 1995, cryopreservation of algae, In : Day JG and McLellan MR (eds), Methods in molecular biology, Cryopreservation and freeze-drying protocols, Humana press in., 38 : 21-30.

[8] Bodas, K., Diller, K.R., Brand, J.J., 1995, *Cryo-Letters*, 16:267-274.

[9] Perry, FP, 1995, Freeze-drying and cryopreservation of bacteria, In : Day JG and McLellan MR (eds), Methods in molecular biology, Cryopreservation and freeze-drying protocols, Humana press in., 38 : 81-89.

[10] Holm-Hansen, O, 1973, Preservation by freezing and freeze-drying, in Stein J, ed., Hand book of phycological methods : culture methods and growth measurements, CUP, Cambridge, UK, pp. 173-205.

[11] Canavate, JP, Lubian, LM, 1997, Effects of slow and rapid warming on the cryopreservation of marine microalgae. *Cryobiology*, 35:143-149.

**Remarks :** It is important to respect the following points : Freezing and thawing must be performed in dim light, the storage culture medium should be diluted and cells should be stored at constant temperature and in darkness.

**Special case of photosynthetic microorganisms :** Photosynthetic microorganisms are the most susceptible to cryopreservation

* Cyanobacteria may be stored in liquid nitrogen, and Lyophilisation is easier for unicellular than for filamentous species[12]. Starr *et al.*[13] have been able to cryopreserve in liquid nitrogen virtually all of the approximately 200 strains of cyanobacteria in the UTEX collection of algae. This includes unicells, branching and unbranching filamentous species, marine and freshwater species, and those with heterocysts and akinetes. They also have successfully stored several photosynthetic mutants of cyanobacterial species and eukaryotic algae. The required procedures are straightforward and inexpensive, but require attention to a few details. Brand[14] describes procedures that cryopreserve nearly all of cyanobacterial strains (see Annex 1).

Cryopreservation of *Arthrospira platensis* PCC 8005 which is used in the MELISSA project is difficult, with 50% success only, with a revitalisation period of about 2 months. Improving the method would require a 2 - 3 months preliminary study. Nevertheless, strains of *Spirulina* and *Arthrospira stored in liquid nitrogen a*re available in the Institut Pasteur collection (CCAP).

* Photosynthetic micro-eukaryotes are mainly stored in liquid nitrogen, with viability over 50%.

**Viability :** The viability of cells varies from 10 to 90% depending on the used method and on the susceptibility of the strains. It is therefore necessary to determine for each of the selected strains the proportion of cells surviving the selected preservation method, in order to define the minimal cell concentration of the stored inoculum that would allow a successful experiment.

Moreover, one should take care to the dead or lysed biomass which could be oxidised and consume and divert the oxygen required for respiration by the heterotrophic partner, and the addition of antioxidants in the culture medium should be considered. The chemical oxygen demand (COD) of the culture medium containing this dead biomass, and the biological oxygen demand (BOD) of cells for respiration should therefore be determined in order to evaluate the amount of antioxidant to be added to the culture medium (for example, vitamins A and E for lipids, vitamin C for hydrosoluble compounds). Another possibility would be to use an heterotrophic partner a strain able to degrade and to use for growth this dead biomass. Nevertheless, in that case, the exact composition of the culture medium and the subsequent growth parameters would not be known with precision.

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[12] Corbett, LL, Parker, DL, 1976, Viability of lyophilized cyanobacteria (Blue-green algae). *Apl. Envir. Microbio.*, 32 : 777-780.

[13] Starr, R.C., Zeikus, J.A., 1993, *J Phycol* 29 (supp).

[14] Brand J. J., 1996, Cryopreservation of cyanobacteria, *Cyanonews*, 12 (2)

# PART II - THE BIOLOGICAL CONSTRAINTS: NUTRITION, GROWTH AND METABOLISM

The selection the microorganisms for the colonisation of a gas equilibrated artificial ecosystem requires knowledge of their nutritional and growth characteristics. Their choice will be therefore largely based on their specific growth rate or their generation time, and some specificity of their metabolism, particularly of their secondary metabolism will also have to be taken into account, since secreted molecules may exert inhibitory effects and disturb culture conditions.

## 1- NUTRITION

### 1.1- Energetic and nutrient needs

**1.1.1 - Major nutritional types of microorganisms :** the three nutritional requirements that are quantitatively the most important for microbes are a carbon substrate, an energy source and an electron donor. These together with the electron acceptor are governed by the enzymes in the cell.

The carbon source available to the microbes can either be organic or inorganic. Those microbes that require organic compounds as either their sole or principal carbon substrates are classified as heterotrophs, whilst those that require inorganic carbonaceous compounds are classified as autotrophs. Microbes that utilise light as their energy source are described as phototrophs and those that obtain energy form the oxidation of either organic or inorganic compounds are described as chemotrophs. Microbes that utilise organic compounds as their source of electrons are described as organotrophs, whilst those employing inorganic electron sources are described as lithotrophs.

Minerals are found in the environment as macro elements (N, S, P), micro elements Mg, Cl) and oligo elements. In addition, auxotroph microorganisms require growth factors (.organic substances) that they are unable to synthesise while prototrophs do not.

The tableau below describes and gives examples of the major ways in which micro-organisms use chemicals and energy sources in the environment for nutrition and growth (see table below).

| Major nutritional types | Sources of energy ; energetic substrate ; Carbon source | Representative microorganisms |
|---|---|---|
| **Photo-litho-autotrophy** | light energy ;<br>inorganic H/ e- donor ;<br>$CO_2$ | Algae, Prochlorobacteria,<br>Purple and Green sulphur bacteria<br>Cyanobacteria |
| **Photo-organo-heterotrophy** | light energy ;<br>organic H/ e- donor ;<br>organic C ($CO_2$ may be used) | Purple and Green non sulphur bacteria |
| **Chemo-litho-autotrophy** | inorganic chemical source ;<br>inorganic H/ e- donor ;<br>$CO_2$ | Sulphur-oxidising bacteria,<br>Hydrogen bacteria, Nitrifying bacteria,<br>Iron bacteria |
| **Chemo-organo-heterotrophy** | organic chemical source ;<br>organic H/ e- donor ;<br>organic C | Protozoa,<br>Fungi,<br>most non photosynthetic bacteria |

In our project, it would be preferable to define a common culture medium for the two selected partners, particularly in the case of two compartments separated by a semi permeable membrane. The culture medium of the photosynthetic partner then should be completed with the organic compound(s) required by the heterotroph.

**1.1.2 - Metabolic types :** the coupled growth by gas exchanges in this artificial ecosystem implies the partners to be selected among the photo-litho-autotroph for the oxygen producer and among the chemo-organo-heterotroph for the oxygen consumer.

**Obligate oxygenic photolithotrophs** : Carbon dioxide is the source of cell carbon during the growth of photosynthetic microorganisms. In obligate photoautotrophic growth, light energy is converted into the chemical energy of ATP and $NADPH_2$, most of which is used to convert $CO_2$ into reduced carbon compounds. The rate of autotrophic $CO_2$ fixation and its products are regulated by a variety of factors (e.g. light intensity and wavelength, nutrient supply, $O_2$ and $CO_2$ levels).

**Aerobic chemoorganotrophs** : their energy source is the oxidation of chemical compounds. The electron donor is a single organic molecule for some species (glycine for *Diplococcus glycinophyllus*) or numerous substrates (90 different types for *Pseudomonas*). The electron acceptor is oxygen, and any fermentative process should be discarded.

## 1.2- Physico-chemical factors affecting metabolism and growth rate

This part is concerned with the effects of various physico-chemical factors on growth and physiology. It is important to appreciate at the outset that the effects of external factors show complex interactions and that an optimum level of one factor under certain conditions may be sub-optimum under other conditions. A good example of this is the finding that the optimum temperature for growth of photosynthetic microbes may vary depending on the light intensity employed. Responses also vary depending on the physiological state of the experimental material and whether it is non adapted, adapting or adapted.

**1.2.1 - Oxygen** : Heterotrophic microorganisms vary in their need for, or tolerance to oxygen. In fact, microorganisms can be divided into several groups depending on the effect of oxygen, as outlined in table below.

**Oxygen relationships of microorganisms**

| Group | Relationship to $O_2$ | Type of metabolism | Example |
|---|---|---|---|
| **Obligate aerobes** | Required | Aerobic respiration | *Micrococcus luteus* |
| **Facultative aerobes** | Not required, but growth better with $O_2$ | Aerobic, anaerobic respiration, fermentation | *Escherichia coli* |
| **Microaerophilic** | Required but at levels lower than atmospheric | Aerobic respiration | *Spirillum volutans* |
| **Air tolerant anaerobes** | Not required, and growth no better when $O_2$ present | Fermentation | *Streptococcus pyogenes* |
| **Obligate anaerobes** | Harmful or lethal | Fermentation or anaerobic respiration | *Methanobacterium formicium* |

In the planned ecosystem, growth of the photosynthetic autotroph and the heterotroph partners will be coupled by oxygen and CO2 exchanges. The selected heterotoph micro-organism therefore should be a strict aerobe in order to avoid growth in the absence of oxygen by fermentative processes.

Growth of many aerobes requires extensive aeration because $O_2$ is only poorly soluble in water and the $O_2$ used up by the organisms is not replaced fast enough by simple diffusion. What would be the chosen configuration of the bioreactor (2 or 3 chambers), it should be continuously stirred.

Light,, hydrogen ion concentration, osmotic effects and water activity, mechanical factors (turbulence, hydrostatic pressure) are not dealt with in detail here because is well considered by biochemical process engineers.

**1.2.2 - Temperature** : It exerts a strong effect on growth rate, and species growing in the same range of temperature should be selected to avoid the technical problems of control of heat transfers from one compartment to the other.

Since temperature conditions of the planed experiment are not yet defined, it would be advisable to select microorganisms growing in a large range of temperature (10 - 40°C), with an optimum at 20-25°C (psychrotrophs mesophiles).

| Category | $T_{min}$ (°C) | $T_{opt}$ (°C) | $T_{max}$ (°C) | Organism |
|---|---|---|---|---|
| Mesophiles | 5-10 | 30-37 | 40-43 | many cyanobacteria, *Spirulina, Synechococcus, synechocystis* |
| Psychrotrophs | -5 | 20-25 | 35 | *Pseudomonas, Cytophaga, Flavobacterium, Aeromonas, Euglena, Chlamydomonas* |

**1.2.3 - Low gravity** : Little information on the effect of low gravity on microorganisms are available biological. The low gravity on moon (1/6 of that on earth) reduces to 169 the atmospheric pressure to 169 hPa, therefore reducing the solubility of gases in liquids. This would be an additional reason to stir the culture medium to increase gas transfers.

**1.2.4 - Invisible radiations** : radiations in space refer generally to those kinds of electromagnetic or particle radiations that can cause possible extensive damage to living tissue. Infrared light (IR), ultraviolet light (UV), x-rays, γ-rays, and beams of fast electrons, protons, a-particles and neutrons all fall within this category. The types of damage differ significantly from one form of radiation to another, but few information on their effects on microorganisms in space are available. The IR and UV radiations is not dealt with in detail here because different methods limit biomass damage.

In their metabolism and in their chromosomes organisation the classes of micro-algae differ as profoundly from one another as they do from others groups of micro-organisms fungi and bacteria. Sensitivity to radiation of micro-algae is in general low; but some cells may survive 2 000 000 rads and continue to grow with vigour indefinitely. Such a degree of resistance, or capacity for recovery, is approached by certain fungal spores and highly polyploid ciliates[15].
Some distinction may be drawn between : i- the action of UV, which may produce genetic or lethal damage, is not markedly influenced by external oxygen, may be stimulatory in small doses, and had various other photochemical effects ; ii- the action of ionising radiation, which has at least partly "indirect" effects, is markedly influenced by the presence of external oxygen and reducing substances, and is destructive only. Visible light, if administered sufficiently soon after irradiation, may partially reverse the effects of UV, and has even some effect on x-irradiated cells.

To summarise this part relative to the nutrient requirements of the selected partners, each chamber of the bioreactor should be colonised by a pure monoculture of dispersed cells growing aerobically in a defined liquid growth medium, in accordance with Monod type kinetics, at similar temperature and under continuous stirring to optimise gas exchanges.

[15]Godward, M.B.E., 1962, Invisible radiations In Lewin R.A. (ed), Physiology and biochemistry of algae, Academic press, New york and london, pp.551-566.

# 2- GROWTH OF MICROORGANISMS

## 2.1 - Microbial growth kinetics

The dominant microbes responsible for the oxidation of biodegradable carbonaceous are chemoheterotrophic bacteria. Such bacteria reproduce by binary fission. When the necessary requirements for bacterial growth are satisfied, the growth rate of a bacterial culture can be expressed as :

$$dx/dt = \mu x$$

where x = microbial cell mass
$\mu$ = specific growth rate coefficient

This equation is acceptable for describing binary fission ; the rate of increase is proportional to the mass of organisms. However, the increase of biomass stops when food is exhausted, and therefore,. the specific growth rate is a function of the concentration of some nutrient.

## 2.2 - Specific growth rates of micro-organisms at 20°C

The table below presents μmax values of a range of prokaryotic and eukaryotic microorganisms. For the sake of comparison, $\mu_{max}$ values measured at the quoted temperature have been corrected (assuming a $Q_{10}$ of 2) to give values for 20°C [16] ; the data are quoted as $\mu_{20°C}$ .

Dealing first with chemo-organotrophic nutrition, it is clear that the highest $\mu_{max}$ values occur in prokaryotes rather than in eukaryotes, with a difference of only about two-fold between the fastest-growing prokaryote (*Escherichia coli*) and eukaryote (*Achlya bisexualis*), when $\mu_{max}$ values are adjusted at 20°C. Complex mediums usually permit faster growth than minimal medium, likely in relation to differences in C (and other resource) availability.

This argument of resource availability may also explain the lowers $\mu_{max}$ values for photolithotrophs prokaryotes and eukaryotes compared to their chemo-organotrophic counterparts, and the fact that prokaryotic $O_2$-evolving photolithotrophs (cyanobacteria) cannot grow faster than the fastest-growing eukaryotic $O_2$-evolving photolithotrophs.

Finally one should notice that these $\mu_{max}$ values normalised at 20°C may be modified are for some microorganisms[17] which are able to modify their metabolism to adapt to large range of temperatures ; in that case, they will have therefore to be recalculated from growth curves in the environmental conditions of the project.

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[16]Raven JA, 1985, Limits to growth, in Borowitzka MA, Borowitzka, LJ, Micro-algal biotechnology, CPU, Cambridge, pp. 331-356.

[17] VanEykelenburg, C, 1979, The ultrastructure of *Spirulina platensis* in relation to temperature and light intensity. *Antonie van Leeuwenhoek*, 45:369-390.

| ORGANISMS | GROWTH MEDIUM | GROWTH TEMPERATURE (°C) | $\mu_{20°C}$ (x $10^6$ $s^{-1}$) |
|---|---|---|---|
| | **Aerobic chemo-organotrophic prokaryotes** | | |
| *Escherichia coli* | complex | 37 | 283 |
| *Escherichia coli* | glucose + salts | 37 | 68 |
| Pseudomonad | complex | 30 | 150 |
| Pseudomonad | complex | 20 | 150 |
| *Cytophaga* sp. | glucose + salts | 20 | 69 |
| | **Aerobic photo-lithotrophic prokaryotes** | | |
| *Synechococcus leopolensis* | salts, CO2, N | 40 | 24 |
| *Synechococcus leopolensis* | salts, CO2, N | 25 | 10 |
| *Synechococcus leopolensis* | salts, CO2, N | 31 | 11 |
| *Synechococcus* sp. | salts, CO2, N | 44 | 15 |
| | **Aerobic obligate chemo-organotrophic eukaryotes** | | |
| *Achlya bisexualis* | complex | 24 | 170 |
| *Achlya bisexualis* | glucose, salts, glutamate | 24 | 77 |
| *Prototheca zopfii* | complex | 25 | 44 |
| *Prototheca zopfii* | propionate + salts | 25 | 45 |
| *Polytomella uvella* | complex | 22 | 31 |
| | **Aerobic photo-organotrophic eukaryotes** | | |
| *C. mundana* var. *astigmata* | acetate, salts, CO2 | 32 | 28 |
| | **Aerobic photo-lithotrophic eukaryotes** | | |
| Small Dinophyceae | salts, CO2, hν | various | 9 |
| Small Bacillarophyceae | salts, CO2, hν | various | 26 |
| *Chlamydomonas reinhardtii* | salts, CO2, hν | 25 | 21 |
| *C. mundana* var. *astigmata* | salts, CO2, hν | 32 | 10 |
| *Chlorella pyrenoidosa* | salts, CO2, hν | 39 | 20 |
| *C. pyrenoidosa* (7-11-5) | salts, CO2, hν | 25 | 17 |

## 2.3 - Growth limitations

**2.3.1 - Growth-limiting nutrient** : living cells require several types of nutrients. These may supply carbon-energy, building blocks for biochemicals, or trace factors such as vitamins or hormones. All cells require relatively large amounts of carbon, nitrogen, sulphur, phosphorus, oxygen, hydrogen, etc. Except in very rare cases in which the recipe for the medium has been carefully adjusted, one nutrient will be exhausted before the others; this is termed the "growth-limiting nutrient". This growth-limiting nutrient is determined by its proportion relative to other components of the culture medium rather than by its absolute amount.

**2.3.2 - Growth inhibition** : growth of microorganisms is frequently affected by the presence of inhibitory compounds in the culture medium, which may originate in the bioreactor feed, or in the excretion of inhibitory products or intermediates by living cells, or in the production of toxic compounds after cell lysis.

**2.3.3 - Co-oxidation and co-metabolism** : the three terms: co-oxidation, co-metabolism and fortuitous oxidation are all used to describe the oxidation and degradation of non-growth substrates by microbes.

Co-oxidation was originally described as the phenomenon whereby the actively growing microbes oxidise a compound, but do not utilise either carbon or energy derived from the oxidation. Co-metabolism is defined as the intracellular biological transformation (oxidation) of a compound in the requisite presence of another metabolically transformable co-substrate, but which is unable to support cell growth. In some cases of co-metabolism, the energy derived from the oxidation of the non-growth substrate may be utilised to fix carbon from the growth substrate. Fortuitous oxidations result from non-specific mono-oxygenase activity.

Such processes have to be taken into account because they may occur in the projected bioreactor in consequence of the presence of dead biomass resulting from the about 50% viability of the cryopreserved cells after revitalisation, and because they may therefore interfere in oxygen utilisation, reducing its availability for cell growth.

**2.3.4 - Biofilms, growth of sessile bacteria :** Their organisation and their metabolism depend on the nature of the surface and of the physicochemical properties of the environment.

Microbial cells possess surface activity that causes them to congregate at interfaces, and to form biofilms. These are formed by complexes associations of microorganisms attached on the surface by an organic matrix, and they may considerably decrease mass transfers. Particularly, the development of biofilms at water/membrane/water or air/membrane/water interfaces retards transfers of nutrient and/or gases which therefore may become rate limiting for growth.

Little is known about the conditions that promote biofilms formation, but it may be reduced by active stirring of the medium. It would therefore be important to estimate the extent of biofilm formation by the selected microorganisms on the used semipermeable membranes during the duration of the experiment and, in addition, to verify that these membranes are not hydrolyses by biofilms.

# 3- EXTRACELLULAR PRODUCTS, SECONDARY METABOLISMS AND GROWTH REGULATORS

Microorganisms produce of a great variety of extracellular substances that often play important roles in microorganisms growth and physiology, as well as in aquatic food chains and ecosystems in general.

## 3.1 - Characteristics of the release processes

The release of simple substances, such as sugars, amino acids and organic acids by exponentially growing cells occurs by diffusion through the cell membrane or by active excretion, whilst larger molecules such as polysaccharides or proteins are excreted by more complex processes.

The rate of production of extracellular products by the above processes depends on physiological (growth stage,...) and environmental factors (pH, light, carbon source...) affecting membrane permeability and intracellular concentrations of metabolites.

## 3.2 - Nature of extracellular products

**3.2.1 - Carbohydrates** : simple and complex polysaccharides are liberated by a large number of taxonomically diverse microorganisms, in variable amounts, depending on the physiological status of the organism and on the microorganisms species. Some photosynthetic micro-organisms may excrete during active growth[18] a considerable fraction (15 to 95%) of the photo assimilated carbon.

**3.2.2 - Nitrogenous substances** : Amino acids and peptides are very common in microorganisms filtrates but in most cases they represent only a small fraction of the total extracellular material. Cyanobacteria liberate very large proportions of assimilated nitrogenous substances into the medium (15-60%), mostly in the form of polypeptides, and several algae release glycoproteins. Other extracellular proteins, or proteins containing substances with unique properties, such as toxins, growth inhibitors or promoters, reproductive factors and enzymes may be released into the medium by some species.

**3.2.3 - Slimes :** microorganisms excrete polysaccharides that are similar in composition to capsular material and that form slimes. Such slimes participate to the flocculation of microorganisms as pellets in which circulation of fluids and molecular diffusion are slowed down. For our project, the strains of microorganisms and/or the culture conditions should therefore be selected for a minimal excretion of polysaccharides in order to limit disturbances in gas transfers and stirring conditions,

**3.2.4 - Organic acids** : Glycolic acid is commonly liberated by micro-algae in aerobic conditions,. particularly when $CO_2$ limits photosynthesis, in amounts that vary considerably for different species.

**3.2.5 - Others products** : various quantities of lipids, volatile substances, vitamins, sex factors, growth inhibitors or stimulators are frequently released by microorganisms.

In the case of a two chamber bioreactor, separated by a single limiting semi-permeable membrane allowing gas exchanges, liquids, molecules and minerals would also be exchanged. It would therefore be necessary to characterise and to measure the possible reciprocal effects of extracellular metabolites excreted at different growth phases in each compartment.

[18] Hellbust, JA, 1974, Extracellular products, in Stewart, WDP (ed.), Algal physiology and biochemistry, Blackwell Scientific Publication, pp.838-863.

# PART III - THE WORLD OF MICROORGANISMS

Among microorganisms, eukaryotic microbes (*Eucarya*) comprise fungi (including yeasts), algae and protozoa (Flagella, Rhizopodia, Cilia, Sporozoa...), while prokaryotic microbes (*Bacteria)* comprise bacteria and cyanobacteria (formerly called blue-green algae). Recently it has become evident that a third group of microbes, the prokaryotic *Archaea* (archaebacteria), exists[19].

## 1- MAJOR GROUPS OF PROKARYOTES

The prokaryotes consist of millions of genetically-distinct unicellular organisms. What they lack in structural diversity, they make up for in their physiological diversity. In Bergey's Manual[20], the groups of prokaryotes are formed based on easily-observed characteristics such as Gram stain, morphology (rods, cocci, etc.), motility, structural features (e.g. spores, filaments, sheaths, appendages, etc.), and on distinguishing physiological features (e.g. anoxygenic photosynthesis, methanogenesis, lithotrophy, etc.). Nowadays, the way to group organisms, especially prokaryotes, is on a genetic basis, i.e., by comparison of the nucleotide sequences of the small subunit ribosomal RNA that is contained in all cellular organisms.

In the ensuing description, prokaryotes are placed under trivial headings based on common structural, biochemical or ecological properties. This does not imply close genetic relatedness among all members in a group. Also herein, some prokaryotes are in more than one group, and some groups consist of both Archaea and Bacteria.

**Archae** : The constraints of similar growth temperature, salt composition of culture mediums and of oxygenic photosynthesis or of obligate aerobic metabolism eliminate archae as possible candidates.

**Bacteria** : Among the eleven distinct groups of Bacteria defined by phylogenetic analysis, only those containing oxygenic photosynthetic and aerobic bacteria will be discussed.

### 1.1 - Photosynthetic prokaryotes

Main physiological types of phototroph bacteria are given in the following table :

| | Prochloro bacteria | Cyanobacteria | Purple sulphur bacteria | Green sulphur bacteria | Heliobacteria |
|---|---|---|---|---|---|
| Photosynthesis | Oxygenic | Oxygenic | Anoxygenic | Anoxygenic | Anoxygenic |
| Energy conversion | ATP+NADH | ATP + NADH | ATP | ATP+NADH, ATP | ATP + NADH |
| Electron donor | H2O | H2O | H2, S | H2, S | Organic |
| Carbon source | CO2 | CO2 | CO2 / Organic | CO2 / Organic | Organic |

[19]Woese, CR, Kandler, O, Wheelis, ML, 1990, Towards a natural system of organisms : proposal for the domains *Archaea, Bacteria, Eucarya. Proc. Natl. Acad. Sci. USA*, 87 :4576-4579.

[20] Holt, J.G. (editor-in-chief). Bergey's Manual of Systematic Bacteriology. Volume 1, 1982. Gram-negative bacteria of medical or industrial importance. Volume 2, 1986. Gram-positive bacteria of medical or industrial importance. Volume 3, 1988. Other Gram-negative bacteria, cyanobacteria, Archaea. Volume 4, 1988. Other Gram- positive bacteria. This is the standard authoritative guide to bacterial taxonomy and identification. This the usual place to begin a literature survey or an identification process of a specific bacterial group.

The technical constraints lead to select an oxygenic photolithotroph strain in the photosynthetic compartment, therefore among cyanobacteria or prochlorobacteria.

### 1.1.1 - Cyanobacteria

Cyanobacteria are one of the most diversified group of bacteria on the basis of their morphology, metabolism, differentiation, and ecology. They deserve special emphasis because of their great ecological importance in the global carbon, oxygen and nitrogen cycles, as well as their evolutionary significance in relationship to plants. Photosynthetic cyanobacteria have chlorophyll a, carotenoids, and addition phycobilins. The planktonic cyanobacteria fix an enormous amount of $CO_2$ during photosynthesis, and as "primary producers" they are the basis of the food chain in marine environments. Their oxygenic photosynthesis, which utilises photosystem II, generates a substantial amount of oxygen present in the earth's atmosphere.

In the provisional classification of Rippka *et al.*[21] based on the axenic representatives in the Pasteur Culture Collection, cyanobacteria are subdivided into five major groups (sections), whose general characteristics are summarised in table below.

| | | | |
|---|---|---|---|
| Unicellular | | Binary fission, budding | Section I |
| | | Multiple fission, baeocytes | Section II |
| Filamentous | vegetative cells | Division in one plan | Section III |
| | vegetative cells and heterocysts | Division in one plan | Section IV |
| | | Division in more than one plan | Section V |

**Section I** (Order Chroococcales, Rippka et al. 1979[22])

Members of Section I are unicellular cyanobacteria whose cells may be spherical, cylindrical or oval ; most reproduce by binary fission, which may occur in one, two or three planes ; some reproduce by asymmetric binary fission (budding) ; colonial forms are held together by mucilage or sheath. Structurally, these are the simplest cyanobacteria, but they nevertheless show an extremely wide range of physiological and genetic diversity.

Genera of interest of this section :

| | *Chamaesiphon* | *Chroococcus* | *Cyanothece* | *Dactylococcopsis* | *Gloeobacter* | *Gloeocapsa* | *Gloeothece* | *Microcystis* | *Synechococcus group* | *Synechocystis group* |
|---|---|---|---|---|---|---|---|---|---|---|
| **Number of strains at PCC** | 3 | 3 | 10 | 1 | 2 | 7 | 9 | 5 | 53 | 45 |
| **Preservation modes** | L, D, C | L, D, C | L, D, C | L, D, C | L, D, C | L, D, C | L, D, C | L, D, C | L, D, C | L, D, C |
| **Resting cells, or spores** | - | - | - | - | - | - | - | - | - | - |
| **Nutritional type** | PH/OP/? | PH | OP | ? | OP/? | OP/PH/CH/? | OP/? | ? | OP (PH, ?) | PH/OP/? |
| **Toxin** | ? | ? | ? | ? | ? | ? | ? | +, some - | ? | ? |

Preservation mode : L: freeze-dried ; D : Desiccation ; C: cryopreservation in liquid nitrogen.
Nutritional type : OP : obligate photoautotroph ; PH : photoheterotroph ; CH : chemoheterotroph ; FPC : facultative photo and chemoheterotroph ; ? : unknown
Toxin production : + : yes ; -: no ; ?: not tested

[21] Rippka, R., 1988, Recognition and identification of cyanobacteria, *Meth. enz.*, 167:28-67.
[22] Rippka, R., Deruelles, J., Waterbury, J. B., Herdman, M. & Stanier, R. Y. (1979). Generic assignments, strain histories and properties of pure cultures of cyanobacteria. Journal of General Microbiology 111:1-61.

Among cyanobacteria of section I, the synechococcus and synechocystis groups are particularly interesting for our project because they contain numerous species and therefore offer a large choice these unicellular photosynthetic prokaryotes, which have been extensively studied and are already well known, and which, in addition, may be easily stocked and preserved under different forms.

**Summary of characteristics of strains of different clusters in *Synechococcus* group**

| | ***Cyanobacterium* cluster** | ***Cyanobium* cluster** | ***Synechococcus* cluster** | **marine cluster A** | **marine cluster B** | **marine cluster C** |
|---|---|---|---|---|---|---|
| **Number of strains** | 2 | 8 | 10 | 15 | 4 | 5 |
| **Mol% G+C of DNA** | 39-40 | 65.7-71.4 | 47.5-56 | 55-62 | 63-69 | 47.4-49.5 |
| **Morphology** | | | | | | |
| Cell diameter (μm) | 1.7-2.3 | 0.8-1.4 | 1-1.5(9), 3(1) | 0.6-1.7 | 0.8-1.4 | 1.2-2.1(5 |
| Defined sheath | - | - | - | - | - | - |
| **Vegetative multiplication** | | | | | | |
| Budding (asymmetric fission) | - | - | - | - | - | - |
| Bipartition | + | + | + | + | + | + |
| **Motility** | | | | | | |
| Gliding | - | - (?) | +(1), variable | - (?) | - (?) | - (?) |
| Swimming | - | - | - | + (4) | - | - |
| **Nutrition** | | | | | | |
| Obligate photoautotroph | + | + | + | + | + | - |
| Facultative heterotroph in the dark | - | - | - | - | - | - |
| Facultative photoheterotrophy | - | - | - | - | - | +(4) |
| **Nitrogen fixation** | | | | | | |
| Aerobic | not detected | not detected | not detected | not detected | not detected | not detected |
| Anaerobic | - | | - (5) | - | - | + (1) |
| **Pigments** | | | | | | |
| Chromatic adaptation | NA | NA | NA | - | NA | +(1) |
| **Polyunsaturated fatty acid content** | Low (1) | Low(4) | High(1) Low(6) | nd nd | Low (1) | High(3) |
| **Growth conditions** | | | | | | |
| Max. growth temperature. | not determ. | 35-37(6) | 37-43(5), >53(3) | ~30(15) | 41(1) | 39-43(2) |
| Salt tolerance | Low | Low | Low | High | High (1) | High (2) |
| **Ecology :** Fresh water | + | + | + | | | |
| Marine | | | | + | + | + |

**Summary of characteristics of strains of different clusters in *Synechocystis* group**

| | Low GC cluster | High GC cluster | Marine cluster |
|---|---|---|---|
| Number of strains | 5 | 11 | 2 |
| Mol% G+C of DNA | 35-37 (5) | 41.1-48.0 (11) | 30.5.- 31.7 (2) |
| **Morphology** | | | |
| Cell diameter (µm) | 3 -7 ( 5) | 2 - 3 ( 5) | 2.5 - 4 (2) |
| Defined sheath | - | - | - |
| **Vegetative multiplication** | | | |
| Bipartition | + | + | + |
| **Motility** | | | |
| Gliding | + (3) | + (4) | - |
| **Nutrition** | | | |
| Obligate photoautotroph | +, ? | - | n.d. |
| Facultative heterotroph | | | |
| in the dark | - | + (9) | - |
| Facultative photoheterotrophy | n.d. | n.d. | n.d. |
| **Nitrogen fixation** | | | |
| Aerobic | - | - | + |
| Anaerobic | - | - | + (2) |
| **Pigments** | | | |
| Chromatic adaptation | + (2) | NA | - |
| **Polyunsaturated fatty acid content** | Low (5) | High (5) | n.d.) |
| **Growth conditions** | | | |
| Max. growth temperature. | 37 -39 (5) | n.d. | 32 (2) |
| Salt tolerance | Low | Low | Low |
| **Ecology** : Fresh water | + | + | |
| Marine | + | + | + |

**Section II** (Order Pleurocapsales, , Waterbury & Stanier 1978[23])

Main genera of cyanobacteria assigned to Section II (see Waterbury, 1989[24], for a recent review) are *Chroococcidiopsis, Dermocarpella, Myxosarcina, Pleurocapsa group, Stanieria* and *Xenococcus*. They are unicellular cyanobacteria are characterised by a unique form of reproduction, not observed in any other group of prokaryotes : multiple fission (a series of rapid successive cell divisions without concomitant cell growth) yielding small daughter cells (baeocytes ["nannocytes" or "endospores" in the botanical literature]). Some members reproduce only by multiple fission, whereas others exhibit a combination of both binary and multiple fission. The number of baeocytes released from the vegetative cell ranges from as few as 2 to over 1000, depending on the strain and the growth conditions. The baeocytes of some members are motile and are "naked" at the time of their release, since the outer fibrous wall material is not synthesised during the process of multiple fission ; in others, synthesis of the outer fibrous layer continues and the baeocytes, surrounded by the wall layer, are not motile. Due to this mode of reproduction, cyanobacteria of this section are not good candidates and will not be retained.

NOTE: The orders Chroococcales and Pleurocapsales were combined into a single order (Chroococcales) in the revision of Komárek & Anagnostidis (1986)[25].

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[23] Waterbury, J. B. & Stanier, R. Y. (1978). Patterns of growth and development in pleurocapsalean cyanobacteria. Bacteriological Reviews 42, 2-44.

[24] Waterbury, J. B. (1989). Subsection II. Order Pleurocapsales Geitler 1925, emend. Waterbury and Stanier 1978. In Bergey's Manual of Systematic Bacteriology, Vol. 3, pp. 1746-1770. Edited by J. T. Staley, M. P. Bryant, N. Pfennig & J. G. Holt. Baltimore, Hong Kong, London, Sydney: Williams & Wilkins.

[25] Komárek, J. & Anagnostidis, K. (1986). Modern approach to the classification ststem of cyanophytes 2 - Chroococcales. Archiv für Hydrobiologie Suppl. 73, 157-226.

**Section III** (Order Oscillatoriales, Elenkin 1934[26])

Main genera are *Arthrospira, Geitlerinema, Leptolyngbya, Lyngbya, Microcoleus, Oscillatoria, Pseudonabaena, Spirulina, Symploca*. Members of Section III are filamentous cyanobacteria whose trichomes reproduce by binary fission in a single plane at right angles to the long axis and are consequently uniseriate. The trichome is composed only of vegetative cells, since differentiation of heterocysts or akinetes (see comments to Sections IV and V, below) does not occur. Some unsheathed members exhibit false branching; the latter results from breakage of the trichome within the sheath followed by protrusion of the ends through the sheath wall, after further growth.

These species, which are filamentous and not easily storable (particularly *Arthrospira platensis* PCC 8005) will not be retained.

**Section IV** (Order Nostocales,. Castenholz 1989[27])

Main genera are *Anabaena, Anabaenopsis, Aphanizomenon, Calothrix, Cylindrospermum, Microchaete, Nodularia, Nostoc, Scytonema, Tolypothrix*. Cyanobacteria assigned to Section IV are filamentous organisms that reproduce by binary fission in a single plane to produce uniseriate trichomes. Like the members of Section III, sheathed members may produce false branches. In the absence of combined nitrogen, one or more cells of each trichome differentiate into heterocysts, which may occupy terminal or interpolated positions; these specialised cells are the sites of nitrogen fixation under aerobic conditions. Some members may produce akinetes, which are usually resistant to cold and to desiccation and thus permit survival of the organism under extreme environmental conditions. Many produce hormogonia, filaments which differ from the mature trichome in possessing smaller cells (produced by rapid successive cell divisions in the absence of growth) and which may be gas-vacuolate, motile, or both; their function is one of dispersal from a sessile mature filament or colony, and they eventually develop into a mature trichome.

**Section V** (Order Stigonematales, Geitler 1925[28])

Main genera are *Chlorogloeopsis, Fischerella*. Members of Section V resemble those of Section IV in being filamentous cyanobacteria that differentiate heterocysts in the absence of combined nitrogen. They are distinguished, however, by their mode of cell division: reproduction of young primary trichomes occurs by binary fission in one plane, but some or all cells of older primary trichomes may divide in more than one plane, giving rise to multiseriate trichomes or trichomes with true lateral branches, or both ; heterocysts develop in terminal, intercalate and lateral positions. Hormogonia differentiate from the ends of both primary trichomes and lateral branches. Some strains produce akinetes. In some members the filamentous mode of growth is lost early in development, giving rise to groups of cells within a common sheath which resemble aggregates of unicellular cyanobacteria. Under $N_2$-fixing conditions, one to two cells in such unsheathed cell clusters differentiate into heterocysts. Short filamentous hormogonia are produced within the cellular aggregates by binary fission in one plane, under conditions which favour rapid growth.

Cyanobacteria of sections IV and V are filamentous, produce akinetes and exopolysaccharides and therefore do not satisfy the researched criteria.

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[26] Elenkin, A. A. (1934). Ob osnovnych principach sistemy Cyanophyceae. Sov. Bot. 1934, 51-83.

[27] Castenholz, R. W. (1989). Subsection IV. Order Nostocales. In Bergey's Manual of Systematic Bacteriology, Vol. 3, pp. 1780-1793. Edited by J. T. Staley, M. P. Bryant, N. Pfennig & J. G. Holt. Baltimore, Hong Kong, London, Sydney: Williams & Wilkins.

[28] Geitler, L. (1925). Synoptische Darstellung der Cyanophyceen in morphologischer und systematischer Hinsicht. Beihefte zum Botanischen Centralblatt 41:163-294.

In conclusion, cyanobacteria of section I seem the best adapted to the project because they are unicellular, they survive desiccation, storage in liquid nitrogen and lyophilisation. Moreover, the genera *Synechococcus* and *Synechocystis* are among the most studied and the best known photosynthetic microorganisms, and therefore would be good candidates

### 1.1.2 - Prochlorobacteria

Prochlorophytes were recently discovered and only 3 species are known for the moment. They are chlorophyll a and b containing phototrophic prokaryotes which resemble both cyanobacteria (because they are prokaryotic and contain chlorophyll a) and the plant chloroplast (because they contain chlorophyll b instead of phycobilins).

| Genera - species | *Prochloron didemni* | *Prochlorococcus* | *Prochlorotrix holandica* |
|---|---|---|---|
| Cell organisation | Unicellular | Unicellular | Filamentous |
| Size | 8-14µm | 0.4-0.8µm | Diam < 2µm |
| Symbiosis | Ascidians | | |
| Multiplication | binary fission | binary fission | binary fission |
| Ecology | marine, benthic | marine, planktonic | fresh water, planktonic |
| Temperature | >25 | | 20-30 |

The products of metabolism are principally aminoacids and short chain polysaccharides. , They do not produce free disaccharides[29] (sucrose for example), contrary to chlorobionts, nor poly-b-hydroxybutyric acid, contrary to cyanobacteria ; however, phenolic acids are present[30].

## 1.2- Chemo-organo-heterotrophic and aerobic prokaryotes

Main groups and their principal characteristics are presented in the following table

| **Groups** | **Growth** | **Morphology** | **Spores** | **wall** | **Parasitism** |
|---|---|---|---|---|---|
| Sheathed bacteria | A/M | Pluricellular | | + | |
| Budding or appendaged bacteria | A/ANF | Appendaged | | +/- | |
| Gliding bacteria | A/ANF | Pluri/Unicellular | | + | |
| Spirocheta | AN/ANF | Unicellular | | + | |
| Actinomyceta | A/AN | Pluricellular | | + | |
| Chimiolithotrophic bacteria | A/AN | | | + | |
| Gram negative aerobic rods and cocci | A | Unicellular | | + | +/- |
| Gram negative rods | ANF | Unicellular | | + | |
| Gram negative anaerobic rods | AN | | | + | |
| Gram positive bacteria | A/ANF | | + or - | + | |
| Mycoplasma | ANF | Unicellular | | - | |
| Rickettsia | | Pluricellular | | + | + |
| Chlamydia | | | | +/- | + |
| Archaebacteria | | | | + | |

A = Aerobic ; AN = Anaerobic ; ANF = Anaerobic facultative ; M = Microaerophilic

Taking into account the criteria imposed by the technical ant biological constraints for the selection of the heterotrophic partner, we will focus attention on unicellular, non spore forming,

[29]Kremer, B.P., Pardy, R., Lewin, R.A., 1982, Carbon fixation and photosynthetates of *Prochloron*, a green alga symbiotic with an ascidian, *Lissoclinum patella*. *Phycologia* U.K., 21(3):258-263.

[30]Whatley, F.R., 1989, Biochemical features of *Prochloron*. In Lewin R.A. and Cheng L. (eds), Prochloron, a microbial enigma, Chapman and Hall publ., New York, USA, pp. 53-64.

aerobic and chemoorganotroph bacteria, and therefore on gliding bacteria, Gram negatives aerobic rods and cocci, Gram positive aerobic cocci (the constraints imposed by the nephelometric measurement of biomass eliminates the irregular aerobic rods).

### 1.2.1 - Gliding bacteria

Gliding bacteria do not have flagella, but can propel themselves when in contact with surfaces. The precise mechanisms of gliding motility have not been defined, but may involve rotating structures near the cell surface or the secretion of chemical surfactants. They contain four main groups : the *Lysobacterales*, *Myxococcales*, *Cytophagales* and *Beggiatoales*.

The *Lysobacteria* are rod-like cells which produce brown, water soluble pigments. *Myxobacteria* have a complex life cycle, which culminates in the production of fruiting bodies containing resting structures called myxospores. These two groups, that excrete hydrolytic enzymes and exert strong lytic activities of neighbouring bacteria, yeasts, fungi, algae and even of nematodes, should therefore be avoided in the case of a two compartment ecosystem.

*Cytophaga* form an heterogeneous group of microorganisms which probably do not belong to a single taxonomic genera, and which probably present the higher potential for aerobic degradation of organic matter. Numerous species (20) have been described in the 1989 Bergey's manual. They are strict aerobes or falcutative anaerobes chemoorganotrophs, and some may use $NO_3^-$ as terminal electron acceptor. In the case of fermentative metabolism, acetate, propionate and succinate may be produced. However, strictly aerobic strains may produce organic acids during growth, particularly on sugar-containing media. All are able to degrade one or several kinds of macromolecules, mainly proteins and polysaccharides, including cellulose, agar, chitin, pectin and starch. Optimum temperature is 20-35°C, optimum pH is around 7. They are common in soil, decomposing organic matter, freshwater and marine habitats. The type species is *Cytophaga hutchinsonii*.

*Beggiatoales* are in majority multicellular

Among gliding bacteria, Cytophagales therefore could be retained as potential candidates for the heterotrophic compartment.

### 1.2.2 - Gram negative aerobic rods and cocci

This very heterogeneous group contains several species pathogenic to humans (*Legionella*, *Neisseria*, *Brucella*) which will be eliminated for security reasons. Nitrogen fixing bacteria, methylotrophs and methanotrophs will not be considered either. This group contains however some candidates for the project.

**Pseudomonads** is a term for bacteria which morphologically and physiologically resemble members of the genus *Pseudomonas*, a very diverse group of Gram-negative rods with a strictly-respiratory mode of metabolism. Usually the term is reserved for members of the genera *Pseudomonas*, *Xanthomonas*, *Frateuria* and *Zooglea*, but many other related bacteria share the definitive characteristics of pseudomonads, i.e., Gram-negative bacteria which typically live by aerobic (as opposed to facultative) means.

. In Bergey's Manual, these bacteria are unified as Gram-negative aerobic rods and cocci. In Woese's Universal Phylogenetic tree the genera are scattered about among the Purple Bacteria, with some being close relatives of the *Enterobacteriaceae*.

Most pseudomonads are free-living organisms in soil and water ; they play an important role in decomposition, biodegradation, and the C and N cycles.

*Xanthomonas* and *Zooglea*, which are known to produce large amounts of flocculating polysaccharides and to form biofilms, will not be retained for the project.

***Pseudomonas*** is an important genus of Gram-negative rods with polar flagella widely distributed in nature. Straight or slightly curved rods, but not helical, 0.5-1.0 x 1.5-5.0 µm. Many species accumulate poly-β-hydroxybutyrate as carbon reserve material. They are not surrounded by sheaths. No resting stages are known. These aerobic organotrophs, having a strictly respiratory type of metabolism with oxygen as the terminal electron acceptor, do not carry out fermentative metabolism. In some cases nitrate can be used as an alternate electron acceptor, allowing growth to occur anaerobically. They are known for their ability to degrade a very large variety of organic compounds, but they are usually unable to degrade biopolymers such as cellulose and lignin. The temperature range is 4-43°C, depending on the species. Most, if not all, species fail to grow under acidic conditions (pH 4.5). Most species do not require organic growth factors.

There are about 150 species of *Pseudomonas*. Some species are pathogenic for humans, animals or plants. The type species is *Pseudomonas aeruginosa*. Some species are isolated from fresh and distilled water : *P. huttiensis*, *P. lanceolata*, *P. spinosa*. Some species are isolated from sea water : *P. doudoroffii*, *P. nautica*, *P. perfectomarina*, *P. stanieri*, *P. elongata*, *P. gelidicola*, sodium ions are required for growth. Some species are isolated from hypersaline lakes : *P. halophila*. For characteristics of these strains see *Bergey's Manual of Systematic Bacteriology, vol.1* and *Bergey's Manual of Determinative Bacteriology, 9th edition*.

**Others genera of interest** : The genera *Alcaligenes* and *Deleya* use a large variety of organic acids and aminoacids as carbon sources may be candidates for the project

The strict respiratory type of metabolism with oxygen as terminal electron acceptor and the absence of fermentative processes by bacteria of the genus *pseudomonas* make them possible candidates for the project.

### 1.2.3 - Aerobic gram positive cocci

**Gram-positive cocci** consist of quite diverse genera that are placed together for convenience because of the spherical shape of their Gram positive staining. They do not form endospores. Motility is uncommon. The genera fall into reasonably distinct groupings of aerobic, facultative anaerobic and strictly anaerobic genera.
The major aerobic genera are presented in table below :

| | Deinobacter | Deinococcus | Marinococcus | Micrococcus | Planococcus | Salinicoccus |
|---|---|---|---|---|---|---|
| Motility | - | - | + | - | + | - |
| Halophily (7.5% NaCl for growth) | - | - | + | - | - | + |
| Acidification of the culture medium | - | weak or - | - | often + | - | - |

*Deinobacter* are non motile, rod-shaped cells of 0.6-1.2 x 1.5-4.0 µm. They are aerobic . chemoorganotrophs, with a respiratory metabolism. They are usually able to hydrolyse gelatine and do not use carbohydrates[31] ; they do not reduce nitrate. The optimum temperature is 30-35°C. Highly resistant to γ-radiation, this organism closely resembles, except for cell shape, the radiation resistant genus *Deinococcus*, to which it seems close on ribosomal RNA evidence.

[31] Martineau, B, 1997, Systématique bactérienne, guide d'identification des bactéries aérobies. Décarie, Montréal, 159 p.

## 2- MAJORS GROUPS OF MICRO-EUKARYOTES

There are about 80 major types of eukaryotes[32]. This number will be reduced as relationships emerge and will increase as new types of organisms are discovered. The earliest diverging eukaryotes are protists which lack mitochondria (amitochondriate taxa). The divergence of amitochondriate taxa is followed by a series of independent protist lineages, many are amoebae or flagellates. Late in evolution, five complex assemblages (plants, animals, fungi, stramenopiles and alveolates) as well as a number of smaller groups diverge, but the relationships among these are still unclear. These late-evolving groups define the "crown" of the eukaryotic subtree[33].

### 2.1- Photosynthetic micro-eukaryotes

Algae, photosynthetic eukaryote microorganisms, contain chlorophyll and carry out oxygenic photosynthesis with water serving as the electron donor. Seven independent specific groups (at least) are distinguished by the type of accessory pigment they contain, the chemical composition of carbon reserve compounds and cell walls, and motility. Algae can be either green, containing only chlorophyll, or brown and red. The later contain not only chlorophyll but pigments such as carotenoids that hide the green colour. The chlorophyll is contained thylakoïd membranes. Algae can occur as either single cells or as aggregates of cells. Algae are found primarily in aquatic habitats and at the soil surface. Algae should not be confused with cyanobacteria, which are bacteria.

The world of photosynthetic microorganisms is very large and interesting, but their utilisation is limited by the constraints imposed by the project. Most may be conserved in preference in liquid nitrogen. In addition, most genera of each group form resting cells and therefore stop growth under unfavourable conditions that may not be controlled during the projected experiment in the moon environment (magnetic field, cosmic radiations, ...). Finally, these microorganisms often require vitamins and may adapt to heterotrophic conditions.

We therefore give here some examples of photosynthetic microorganisms that may be used for the project, keeping in mind that complementary bibliographic and experimental studies

| | Chlorobionts | Chromobionts | Rhodobionts | Euglenobionts | Cryptobionts | Dinobionts | Rhaphidobionts |
|---|---|---|---|---|---|---|---|
| Unicellular | + | + | + | + | + | + | + |
| Pluricellular | + | + | + | | | | |
| Resting cell | + | + | - | + | - | + | ? |
| terrestrial | + | + | | | | | |
| aquatic | + | + | + | + | + | + | + |

The genera of each group of interest (Chlorobionts, Chromobionts, Rhodobionts, Euglenobionts and Cryptobionts) are described below:

#### 2.1.1 -Unicellular chlorobionts

Chlorobiont is the most diverse group of algae, with more than 7000 species growing in a variety of habitats (about 800 of which are marine). Like the plants, the green algae contain two forms of chlorophyll a and b, ß-carotene; various xanthophylls, starch, some fats or oils. The major groups of "green algae" are distinguishable on the basis of their flagellar insertion

They may be unicellular, multicellular, colonial or coenocytic (composed of one large cell without cross-walls that may be uninucleate or multinucleate). They have membrane-bound

[32]Handbook of protoctista, 1989, Margulis, L., Corliss, J.O., Melkonian, M., Chapman, D.J. (Eds). Jones and bartlett Publishers, Boston.

[33] Knoll, A.H. (1992): The early evolution of eukaryotes: a geological perspective. *Science* 256:622-627.

chloroplasts and nuclei. Most species are aquatic and are found in both freshwater or marine habitats; some are terrestrial, growing on soil, trees, or rocks. Some are symbiotic with fungi giving lichens. Some are symbiotic with animals, e.g. *Hydra* has a symbiotic species of *Chlorella*.

We give below two examples of unicellular species.

***Chlorella :*** Its diameter is between 2-8 microns. Under favourable growth conditions, the process of reproduction can generally be divided into several steps ; growth-ripening-maturity-division. At the division stage, a "mother" cell divides into 4 'daughter' cells. This complete reproduction cycle can take less than 24 hours.

***Chlamydomonas :*** Very large genus of unicellular, motile flagellates occurring in stagnant water and on damp soil. The cells have a glycoprotein wall (not cellulose) and two equal flagella. The nucleus is enclosed in a cup-shaped chloroplast, which has a single large pyrenoid where starch is formed from photosynthetic products. Two small contractile vacuoles, which have an excretory function, are located near the flagella. There is also a red pigment spot which is light-sensitive, allowing the cell to swim towards light.

They have two types of cycle. Asexual reproduction is by fission, the protoplast dividing to form 4-8 zoospores similar to the parent. Nutrient deprivation induces cells to form isogametes, which fuse in pairs, forming a zygote; the zygote loses its flagella and forms a thick wall which is resistant to adverse conditions; the zygote undergoes meiosis to form 4 haploid zoospores.

Resting spores. *Polytomella citri, Haematococcus pluvialis, Chlamydomonas spp.*

### 2.1.2 - Unicellular Chromobionts

Even though most of them are photosynthetic, Chromobionts are not at all closely related to plants, or even to other algae. Unlike plants, the Chromobionts have chlorophyll c, and do not store their energy in the form of starch. They are some of the most important organisms in aquatic ecosystems. The cool and temperate coasts of continents are lined with kelp forests, where many commercially important fish and shellfish feed and reproduce, and diatoms are frequently the primary source of food for both marine and fresh-water organisms. Chromobionts are almost all aquatic organisms. These may occur, however, in both freshwater and marine environments. The Phaeophyta, for example, are primarily known for their large marine kelps, but the group also includes microscopic freshwater species. Likewise the diatoms have many marine and freshwater groups.

Some of their chracteristics and their ecology[34] are presented in the table below.

| | **Primnesiophyceae** | **Dictyochophyceae** | **Eustigmatophyceae** | **Bacillariophyceae** | **Tribophyceae** | **Chrysophyceae** | **Fucophyceae** |
|---|---|---|---|---|---|---|---|
| Unicellular | + | + | + | + | + | + | - |
| Colonial | - | - | - | + | - | - | - |
| Filamentous | - | - | - | - | + | + | + |
| Multicellular | - | - | - | - | - | - | + |
| Terrestrial | none | none | frequent | rare | rare | none | none |
| Fresh waters | rare | none | frequent | frequent | frequent | frequent | rare |
| Marine | frequent | frequent | none | frequent | rare | not frequent | frequent |
| Benthic | rare | none | rare | frequent | rare | rare | frequent |
| Planktonic | frequent | frequent | frequent | frequent | frequent | frequent | none |
| resting cells | | | | + | | + | - |

[34] Boudouresque C.-F., Gomez, A., Ribera, M. A., 1993, *Une approche moderne du monde végétal, première partie*. Version provisoire, GIS Posidonie Publishers, Marseille.

**Diatoms** are in the Division Chrysophycota, Class Bacillariophyceae. They are all unicellular, although chain like colonies are also found. They may vary in size from 2-200 µm in length and as already noted may be solitary or form chains up to 2 mm in total length. This class of algae contains over 100,000 species distributed in over 250 genera. They are abundant in the marine environment, but are also found in freshwater and in damp habitats (e.g. on rocks or soil). Many of the genera contain a large number of species, such as *Navicula*. This group is probably, on a world scale, the most productive of algae, since it is estimated that they contribute 20-25% of the global annual production of dry mass. McQuoid and Hobson provide an excellent review of diatom ecology[35].

### 2.1.3 - Euglenobionts

Euglenobionts are green or colourless, unicellular and motile organisms (two genera are know to be sessile). Most genera and many species are world-wide distributed and are found in a variety of freshwater and marine habitats, especially those rich in organic matter. Many exhibit wide tolerance to salinity, pH, light/dark, temperature, even radioactivity and others forms of pollution. They require vitamins B1 and B12.

There are six orders of euglenobionts ; the three orders of interest : *Eutreptiales*, *Euglenales* and *Rhabdomonadales* are non-phagotrophic or non endozoic. They are described below:

| Order | Eutreptiales | Euglenales | Rhabdomonadales |
|---|---|---|---|
| Genera | *Eutreptia Eutreptiella* | *Euglena Astasia* | *Rhabdomonas* |
| Characteristics | Green or colourless | Green or colourless | Colourless ,Osmotrophs |

Genera and species of Euglenales (*Euglena, Astasia, Phacus, Trachelomonas...*) are most studied[36]. The species *Euglena gracilis*[37] is described below:

| Genera - Species | *Euglena gracilis*(Z) |
|---|---|
| Strain origin | ATCC 12716 |
| Morphology | cigar-shaped |
| Dimensions | 10 x 50 µm |
| Multiplication | Vegetative |
| Ecology | cosmopolitan |
| Storage | Cryopreservation |
| Culture medium | Cramer and Myers |
| Temperature (°C) | 25 (20-30) |
| Salinity (%°) | variable |
| pH | 6,8 (3 to 9) |
| Light | 3500-7000 lux[38] |
| Generation time (h) | 10.5-12.9 |

### 2.1.4 - Unicellular Rhodobionts

*Rhodosorus marinus, Porphyridium cruentum* and *Rhodella violacea* are the most studied species among micro-Rhodobionts growing in marine culture mediums. Their main characteristics are presented in the table below :

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[35]McQuoid, M. R. and L. A. Hobson. 1996. Diatom resting stages. *J. Phycology* 32:889-902.

[36] Handbook of protoctista, 1989, Margulis, L., Corliss, J.O., Melkonian, M., Chapman, D.J. (Eds). Jones and bartlett Publishers, Boston.

[37] The biology of Euglena, vol. 3, physiology, DE, Buetow (ed.), Academic Press, 1982, p. 363.

[38]Edmunds, LN, Jr., 1965, Studies on synchronously dividing cultures of *Euglena gracilis* Klebs (strain Z). 1- Attainment and characterisation of rhythmic cell division. *J. Cell. Comp.Physiol.*, 66 : 147-158.

| Genera - Species | *Rhodosorus marinus* | *Porphyridium cruentum* | *Rhodella violacea* |
|---|---|---|---|
| Length | 4-8 µm | <10 µm | 8-14 µm |
| Vegetative multiplication | Bipartition | Bipartition | Bipartition |
| Ecology | Marine | Ubiquitous | Marine |
| Culture media | Conway | ASW, Conway, f, f/2 | Conway |
| Temperature (°C) | 20 (18-25) | 21-25 (10-35) | 25 |
| Salinity (‰) | >17 | 4,5-35 | |
| pH | | 7,5-7,8 (>7) | |
| Light | 5000-10000 lux | 5000-10000 lux | |
| Generation time (h) | 20-28 | 23 | 38 |

### 2.1.5 - Cryptobionts

Cryptobionts are marine or freshwater planktonic organisms. They are generally more important in cold water habitats. Temperature optimum is about 20°C with a moderate range of temperature. Most species are photosynthetic and motile (*Chilomonas* are colourless), non motile palmelloid forms are also know. Cryptobionts are relatively slow growing : maximum growth rates seldom exceed one cell division per day.

## 2.2- Heterotrophic micro-eukaryotes

**Protozoa** are unicellular microorganisms that lack cell walls and obtain nutrients by ingesting other microbes, or by ingesting macromolecules in solution in a process of pinocytosis[39]. They lack pigments and may be motile. There are four major groups, which are distinguished by their mechanism of motility (flagella vs. pseudopodia vs. cilia vs. non-motile), as well as the characteristics of their life cycles. *Mastigophora* (flagellates) are motile through the use of flagella, *Sarcodina* (amoebas) are motile with amoeboid movement, *Ciliophora* use cilia for movement and *Sporozoa* are non-motile. Each group contains representatives that cause important human diseases. These organisms will be discarded because, as phagotrophs, they are not adapted to axenic culture mediums and because they are pathogen to humans.

**Fungi** are chemoorganotrophs, lack chlorophyll, and have simple nutritional requirements as compared to bacteria. The three important groups of fungi are moulds, yeasts, and mushrooms. Fungi are particularly important in the decomposition of wood or wood products such as paper. Unlike the Bacteria and Archaea, there is great diversity in both fungal morphology and sexual life cycles.

Yeasts are true fungi and are characterised by a wide dispersion of natural habitats. Yeasts multiply as single cells that divide by budding or direct division (fission), or they may grow as simple irregular filaments (mycelium). Yeasts have the ability to ferment individual sugars, and for this reason, they will not be retained for the project, in spite of their other characteristics.

[39]Patterson, D.J. (1994): Protozoa: Evolution and Systematics. In: Hausmann, K. and Hulsmann, N. (eds.): Progress in Protozoology, (Proceedings of the IX International Congress of Protozoology, Berlin 1993). Gustav Fischer Verlag, Stuttgart, Jena, New York USA, pp 1-14.

# CONCLUSION : CHOICE OF THE PARTNERS

## Analysis of technical constraints

The microorganisms selected to colonise the compartments of the projected ecosystem should be unicellular in order to simplify biomass formation by optical methods. In addition, they will have to be maintained in resting conditions and to survive during the ~ 35 days long waiting period in orbit before landing. The preservation and storage procedure are therefore of critical importance for the success of the experiment. We selected three methods to temporarily block any metabolic activity for cells storage during this waiting phase, one based on natural spore formation, and two others using cold or desiccation.

These methods of preservation provide varying degrees of success, depending on the different species of microorganisms, and never results in 100% recovery of the preserved cells. When it is vitally important that cultures are not lost, it is advisable to use two methods in parallel. If desiccation may be used without important risks for the conservation of prokaryotic cells, storage in liquid nitrogen and freeze-drying techniques have become standard methods for the long-term maintenance of many micro-organism cultures because they are the best documented and they provide the best chances of success.

The viability of cells varies from 10 to 90% depending on the used method and on the susceptibility of the strains. It is therefore necessary to determine for each of the selected strains the proportion of cells surviving the selected preservation method, in order to define the minimal cell concentration of the stored inoculum that would allow a successful experiment.

Moreover, one should take care to the dead or lysed biomass which could be oxidised and therefore consume and divert the oxygen required for respiration of the heterotrophic partner ; in that case, the addition of antioxidants in the culture medium should be considered. The chemical oxygen demand (COD) of the culture medium containing this dead biomass, and the biological oxygen demand (BOD) of cells for respiration should therefore be determined in order to evaluate the amount of antioxidant to be added to the culture medium (for example, vitamins A and E for lipids, vitamin C for hydrosoluble compounds).

In addition, the selected microorganisms should not form biofilms nor excrete important amounts of polysaccharides which both could disturb gas (and eventually nutrient) transfers in and between the two compartments.

Finally, the design of the ecosystem introduces other technical constraints. In its simplest form, it may consist of two compartment separated by a single membrane permeable to gases, but also to minerals and small molecules that may exert undesirable effects ; it may also consist of two compartment separated by an intercalating gas chamber, in which case only gases exchanges would be allowed.

## The biological constraints

In the projected ecosystem consisting in the coupled growth of two partners on the basis of gas (eventually nutrients) exchanges, one of the partner should be a strict photoautotroph whose growth would be supported by light, CO2 produced by the heterotrophic partner and minerals. Growth of its heterotrophic partner on organic nutrients provided in the culture medium should occur only in the presence oxygen produced by the photosynthetic partner, that implies that the selected heterotrophic micro-organism would be strictly aerobic, and would be unable to grow by fermentative processes. Moreover, attention should be paid to possible exchanges of secreted metabolites which may exert undesirable inhibitory effects.

The selection these microorganisms therefore requires knowledge of nutritional and growth characteristics of putative candidates. Choices will be therefore largely based on nutrient

requirements, on specific growth rate or generation time, and on some metabolic specificities, particularly excretion of secondary metabolites. For the probable case where the simplest design of bioreactor would be retained, the selected microorganisms should be able to grow under similar physicochemical conditions, i.e. at similar temperature and pH, on a common basic mineral culture medium for the two selected partners.

Finally, microorganisms which form resting cells should be avoided because they prefer stop growth as soon as environmental conditions become unfavourable. Unless very reliable technical controls of the bioreactor, fluctuations in the microorganisms environment could occur, and the formation of resting cells would then compromise the experiment.

**The choice of the partners**

Putative unicellular candidates may be found among eukaryotic or prokaryotic microorganisms. Eukaryotic microbes (*Eucarya*) comprise fungi (including yeasts), algae and protozoa (Flagella, Rhizopodia, Cilia, Sporozoa...), while prokaryotic microbes (*Bacteria)* comprise bacteria and cyanobacteria (formerly called blue-green algae). Recently it has become evident that a third group of microbes, the prokaryotic *Archaea* (archaebacteria), exists.

The two partners in the projected ecosystem may *a priori* be prokaryotic or eukaryotic or a combination of both types of microorganisms.

**For the oxygen producing compartment,** the prokaryotic cyanobacteria may be used. Some eukaryotic photosynthetic micro algae among Chlorobionts, Chromobionts, Rhodobionts, Euglenobionts, Cryptobionts could be candidates, on condition that they are obligate phototrophs and that they do not form resting cells. However, cyanobacteria and particularly species belonging to the genera *Synechococcus* and *Synechocystis* appear to be the best adapted to the project because these photosynthetic unicellular microorganisms may be stored using different methods (desiccation, lyophilisation and storage in liquid nitrogen) ; they do not excrete large amounts of polysaccharides or other secondary metabolic, they do not form resting cells and they are resistant to radiation ; moreover, they have been extensively studied and are well known.

**For the oxygen consuming compartment,** the heterotrophic, obligate aerobic prokaryotes belonging to Cytophaga-like bacteria, Pseudomonads, Aerobic Gram positive cocci may be used. If it is decided to use as nutrients the unavoidable dead biomass produced by any of the preservation method chosen, cytophaga like bacteria may be selected.

Among obligate aerobic heterotrophic micro-eukaryotes available in culture collections under the required conservation form, the best characterised are generally to humans and should be rejected. However, one can consider the possibility to use mutants of micro algae which have lost photosynthetic pigments or activity. These are necessarily heterotrophic, as none are known to be able to derive their energy from oxidation of inorganic compounds. In the case of the simplest two compartment ecosystem exchanging both gasses and liquids with minerals and organic molecules in solution, *Pseudomonas* like bacteria would be good candidates since they are able to adapt to numerous substrates. If environmental constraints such as radiations have priority, gram positive aerobic cocci, *Deinobacter grandis*, *Deinococcus radiodurans*, *D. radiopugnans* should be considered.

It becomes then possible to design an ecosystem which could be constituted of exclusively prokaryotic or eukaryotic microorganisms, or of a combination of types ; in that latter case, the photosynthetic, oxygen producing partner should be a cyanobacteria while the heterotrophic, oxygen consumer should be one of the described micro-eukaryote.

The final selection of possible candidate, on the basis of the preceding theoretical analysis will become possible when the experimental conditions of the experiment and the subsequent

constraints will be well defined. A complementary bibliographic study, specific to the selected micro-organisms will then be required to precise their requirements for and/or tolerance to different nutrients, physico-chemical parameters (pH, temperature, salinity and ionic strength of the culture medium...), their metabolic specificities (excretion of secondary metabolites, ...), ....

In the perspective of the subsequent experimental phase that may point out unexpected problems or incompatibilities, several species of oxygen producers and consumers should be selected. The experimental phase would then consist in the characterisation of the resistance of each of the selected strains to the different procedures of conservation procedures, in the measurements of growth kinetics parameters (biomass production, gas exchanges) and in the characterisation of possible secondary interactions between the two partners, determined by minerals, nutrients, or secondary metabolites exchanges through the delimiting semipermeable membrane. Finally, the coupled growth of the two partners should succeed on earth before landing on moon.

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# ANNEX

## STORAGE OF CYANOBACTERIA IN LIQUID NITROGEN

**Sample preparation** [40]: Transfer cyanobacterial liquid culture into a cryovial[1] in preparation for cryopreservation. Alternatively the culture can be grown directly as a lawn on a tiny agar slant prepared within the cryovial[41]. If the cryovial contains liquid culture, pellet the cells by centrifugation in a clinical centrifuge and discard the supernatant [2]. Add cryoprotective solution containing 1.0 ml of half-strength growth medium (BG-11 works well for virtually all fresh-water cyanobacteria) containing 5% methanol or 8% DMSO to the pelleted cells [3]. Alternatively, if the culture is growing on an agar slant, transfer 1 ml of cryoprotective solution above the slant[4,5].

[1] Two-ml or 1.8-ml polyethylene or polypropylene cryovials are especially convenient for handling and storage efficiency, although 1-ml and 5-ml cryovials also work well.

[2] We have constructed acrylic sleeves that fit into the tube holders of the rotor, positioning the cryovials securely in place within a clinical centrifuge rotor, flush with the top of the tube holders. The cryovials can also be inserted into unmodified tube holders for centrifugation in a clinical centrifuge.

[3] Although glycerol is an effective cryoprotective agent for many bacteria, it is not effective for most cyanobacteria. Methanol at approximately 5% (v/v) is suitable for most strains. However, we have been successful with concentrations of methanol ranging from 2% to 12.5%, and DMSO ranging from 4 to 15 %, depending on the culture. A small fraction of some cultures survive with no added cryoprotective agent.

[4] When the cryoprotective agent is added directly above the culture on an agar slant, the tube is shaken gently prior to freezing, to dislodge some of the cells and ensure that the liquid penetrates through the culture. Cells pelleted from liquid suspension are fully suspended in the cryoprotective solution.

[5] Cells are killed by exposure to bright light when in cryoprotective solution. Keep the culture in subdued room light while handling, and in complete darkness at other times.

**Freezing** : The cryovial containing the culture in cryoprotective agent at room temperature is inserted into a special "freezing container"[6] which has been pre-chilled to refrigerator temperature. The freezing container is then placed into a -70 C freezer for 2 hours. Then the cryovial is quickly removed from the freezing container, placed into a storage container, and plunged into liquid nitrogen for indefinite storage.[7]

[6] The "Mr. Frosty" freezing container (Nalgene) is satisfactory for nearly all cyanobacteria. It is inexpensive to purchase and holds eighteen 2-ml cryovials simultaneously. Its contents cool at slightly less than 1°C per minute when it is placed into a -70 C freezer.

[7] Sterility is a problem when storing plastic cryovials in liquid nitrogen. Vials equipped with gaskets and those with inside threads seal most tightly, but liquid nitrogen always creeps into

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[40] Brand J. J., 1996, Cryopreservation of cyanobacteria, *Cyanonews*, 12 (2)
[41] Bodas, K., Diller, K.R., Brand, J.J., 1995, *Cryo-Letters*, 16:267-274.

some cryovials. This provides a conduit for entry of bacteria, some of which remain viable in bulk liquid nitrogen. Several manufacturers sell heat-shrink tubing that serves as a tight-fitting sleeve around the entire cryovial and lid, thereby eliminating liquid nitrogen leakage. Bacterial contamination can be eliminated also by storage in sealed glass ampoules or by storing plastic cryovials in the nitrogen vapour just above the liquid, although these procedures introduce additional safety and convenience considerations.

**Thawing and recovery** : Cultures to be revived are removed from liquid nitrogen storage and warmed rapidly to room temperature[8]. Cells are immediately pelleted by centrifugation of the cryovial[9,10], and the supernatant is discarded. One ml of fresh growth medium is placed into the vial to suspend the pellet. The cryovial lid is slightly loosened to allow gas exchange, and the contents of the vial are kept in complete darkness for 1-2 days. The culture can then be placed on agar or in liquid growth media under normal growth conditions. The viable cells should begin normal growth within 1 - 2 days in light, although they are especially susceptible to damage by excessive light intensity for the first day or two of illumination.

[8] Warm rapidly by plunging the tightly sealed, still-frozen cryovials into a dish of water at 35°C. An appropriately selected volume of water will cool to approximately 25 °C as the cryovial contents are warmed to that same temperature.

[9] Centrifugation of a thawed culture in a cryovial containing an agar slant is best done in an angle rotor that pellets the cells on the agar surface without appreciably altering the position of the agar in the tube.

[10] Cultures of eukaryotic algae especially, and cyanobacteria to some extent, are susceptible to mechanical damage during recovery from storage at low temperature. Cells should be pelleted at the minimum R.C.F. that facilitates pelleting. Excessive agitation should be avoided when suspending the pellet.