



## **ECOMAWARU**

### **ECO-sustainable MAnagement of WAter and wastewater in RUral communities**

#### **D4.2 – A4.2 Technical Report on microalgae selection**

31<sup>st</sup> December 2010

## **Technical report on microalgae selection**

### **Action 4.2: Microalgae selection**

## **IMPLEMENTATION OF THE DATA BASE**

Some manual activity are conducted in order to select the autochthonous algal species presents in the wastewater of Varese Ligure:

- ⇒ to take a different samples of water from the territory of Varese Ligure
- ⇒ to prepare nearby our laboratory the culture system e.g. the common method for cultivation of microalgal cells: batch culture.

### **Selection of the source of microalgae**

Wastewater and water collected from diverse points of municipal treatment plant such as effluent and influent (see **FIGURE 1 A**). Aquatic algae often appear in planktonic forms, but in this case, they are not visible any algal cell, collecting a representative volume of water and observed directly using the optical microscope Laica DMLS. So it is necessary to prepare a culture system in the laboratory for the growth and re production of the algae.

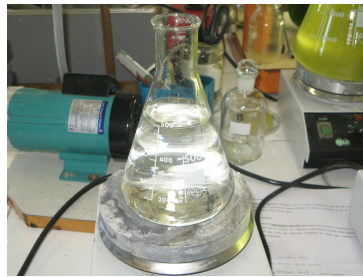
### **Microalgae in Screening Approach**

The laboratory procedure are:

- A** - Storage and deposit cultures
- B** - Production of biomass- start
- C** – Production of biomass- after 12 days
- D** – Production of biomass- after 20 days
- E** - Identification



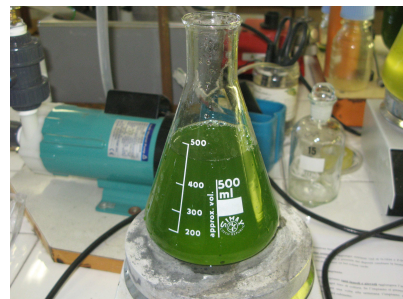
(A) Wastewater and water collected from diverse points such as (1) effluent and (2) influent of S. Pietro Vara plant.



(B) The start of batch culture a sample of wastewater has been put in the flask. The condition of the culture is optimal to growth the algal biomass.



(C) Batch cultures after 12days.



Batch culture after 20days.



(D) Growth and reproduction of algal biomass.



(E) Identification using by optical microscope.

**FIGURE 1:** These are the main steps of the microalgae from nature to stage of testing for identify.

### Culture system: batch culture

The batch cultures were kept in a culture room at  $22\pm 2^{\circ}\text{C}$ , with 12 h light: 12 h darkness photoperiod at  $30\ \mu\text{E m}^{-2}\ \text{s}^{-1}$  PAR (photosynthetically active radiation). The 250-ml glass flasks were used and the culture medium was composed of water (effluent) and wastewater (influent) enriched with 6.8 mg P / l and 50 mg N/l, final concentration (N/P = 7). This culture medium will be named "eutrophic medium". These were the physical conditions that the batch cultures were kept from March to December.

After 12 days it is observed the first forms of algal cells, and it is started the monitoring using microscope (Laica DMLS) the variations in the community structure. It is observed the presents of some algae, of the herbivores and some bacteria.

In particular the competition between species of algae is also studied, and the viability of the species as inocula after a senescence period.

Moreover the presence of herbivores (predation) and bacteria (competition) are the mechanisms affecting microalgae assemblages, originating different alternative states.

However, the effect of these mechanisms may depending on algal proprieties such as size, growth rate and variability as an inoculum.

In **TABLE 1** there are show some results of the analytic campaign: wastewater of S. Pietro Vara; this wastewater has been used to feed algal culture in batch culture.

DATA	pH	N-NH4 [mg/l]	P-PO4 [mg/l]	COD [mg/l]
27/05/2010	7,35	6,10	3,65	35
31/05/2010	6,68	24,51	4,79	95
01/06/2010	6,68	67,07	5,65	80
02/06/2010	6,87	53,66	4,77	100
03/06/2010	6,75	36,22	5,28	92,5
04/06/2010	6,93	29,02	4,95	75
05/06/2010	6,89	18,78	3,43	92,5
06/06/2010	6,88	13,05	4,45	62,5
08/06/2010	6,8	7,19	4,71	80
09/06/2010	6,9	7,32	3,78	70
10/06/2010	7,04	11,10	3,62	62,5
11/06/2010	7,06	9,15	3,86	
12/06/2010	7,05	5,00	5,65	
13/06/2010	7,09	5,37	1,18	
14/06/2010	7,13	6,46	3,78	
15+16/06	7,13	4,76	1,34	25
24/06/2010	6,58	7,44	2,52	

25/06/2010	7,22	2,32	0,64	
26/06/2010	7,18	1,83	0,88	
27/06/2010	7,25	3,29	0,43	0
28/06/2010	7,2	1,10	0,37	
29/06/2010	7,3	5,12	0,29	15
09/07/2010	7,3	1,71	0,35	35
10/07/2010	7,38	6,34	0,62	
11/07/2010	7,55	6,22	0,88	55
12/07/2010	7,59	5,49	0,43	
13/07/2010	7,54	2,07	0,24	67,5
14/07/2010	7,48	2,93	0,2	
15/07/2010	7,85	0,49		62,5
16/11/2010	4,982	0,732	1,205	17,5
17/11/2010	5,124	2,805	0,080	10
18/11/2010	7,34	10,609	2,009	102,5
19/11/2010	7,51	4,146	0,777	
20/11/2010	7,59	3,171	0,000	17,5
21/11/2010	7,6	3,780	0,080	
22/11/2010	7,59	4,024	0,643	
23/11/2010	7,48	3,171	0,268	2,5
24/11/2010	7,43	0,000	0,348	
25/11/10	7,8	0,000	0,482	10
26/11/10	7,71	0,488	0,348	
27/11/10	7,72	3,049	1,098	12,5
28/11/10	7,64	0,000	0,402	
29/11/10	7,61	0,000	0,134	
30/11/10	7,52	1,098	0,402	2,5
01/12/10	7,57	0,000	0,054	
02/12/2010	7,74	6,951	0,187	80,982
03/12/2010	7,76	4,390	0,161	
04/12/2010	7,79	8,048	0,134	40,491
05/12/2010	7,8	6,585	0,214	
06/12/2010	7,8	5,731	0,402	
07/12/2010	7,72	3,049	2,946	32,393
08/12/2010	7,69	4,024	0,321	
09/12/2010	7,71	4,878	1,044	
10/12/2010	7,7	4,390	0,964	
11/12/2010	7,77	3,049	0,375	40,491
12/12/2010	7,75	4,268	3,963	
13/12/2010	7,72	10,121	0,536	
14/12/2010	8,85	7,926	1,098	16,196

TABLE 1: Data of the samples coming from plant of S. Pietro Vara (May- December 2010).

## Measurement of cell growth and culture productivity

Dry mass is measured as total biomass dry weight and the chlorophyll concentration is measured as total photosynthetic biomass.

The results of this analysis are shown following in **FIGURE 2**. The results show that the values are floating for the period of 30days, after the grown of the algal cells is stationary.

## Maintenance of mass culture

In order to maintain long-term algal cultures, it is necessary to provide a medium (wastewater of S. Pietro Vara) and maintain the algal culture in good and constant physical-chemical parameters.

The basic requisite for correct maintenance concerns consistent evaluation of some basic parameters (light, temperature, pH and nutrients) from which to infer the relative culture performance in reference to the maximal performance known by scientific literature experience and our experience.

In practical terms, the aim for management of mass cultures is to sustain the perfect condition. By the research in the scientific literature and by our experiences it is stand out that one of all basic parameters is essential to growth of algal biomass: it is the light. For this culture the optimal intensity light has been  $30 \mu\text{E m}^{-2} \text{s}^{-1}$  PAR and others parameters, describes in section "culture system", have been good for the development of algal culture.

**So when it has been obtained a good concentration in the batch culture of 0.5g/l in July, it is started the maintenance phase in order to have a inoculum ready. In this batch culture the algal species, that is prevalent, is *Chlorella sp.***

But it is probably that in natural ambient of Varese Ligure, **TABLE 2** shows the environmental condition of Varese Ligure ( it is taken by Deliverable 1 A1 ), the algal species probably change in relation of new environmental condition.

A	B	C	D
Parameter	Critical point and evidences	Possible solution	Varese Ligure
Temperature	Two seasonal periods have been identified.		Average annual temperature 9-14°C
	<b>1. November –March</b>	It is necessary to increase the temperature in the culture system until 10 °C: <u>it can be useful to have a greenhouse</u> , in which to place the algal culture system.	The average temperature in the winter are nearing 0°C÷ -1°C
	<b>2. April - October</b>	No solution is required because temperatures are in the optimal range for the algal process	Temperate climate
Light	Light greatly influences the algal growth: <b>the optimal range of incident irradiance is from 800 to 1400 µE/m²s.</b>		Latitude: <b>44°22'38"28 N</b>  Longitude: <b>09°35'42"72 E</b>
	Photo-inhibition troubles: <b>It is possible to have photo-inhibition effects about 1630 µE/m²s.</b> The peak light level may exceed 2000 µE/m²s , which is several times above the saturation irradiance level (Molina et al., 1999).	Summer period: it is necessary to screen the light in the central hours of the day.	
Nutrients	The algal cultures can have several problems with high concentration of nutrients (nitrogen and/or phosphorus), while with low nutrient concentration the algal culture is able to grow	<b><u>Optimum relation</u></b> <b>1:10</b> Phosphorus: Nitrogen (from scientific literature).  <b><u>Classical Redfield ratio</u></b> <b>1:16</b> P:N (from Voltolina et al., 1998).  <b><u>Low concentration of P</u></b>	The wastewater quality coming from the primary settler of the plant of <b>San Pietro Vara</b> of Varese Ligure is in these ranges :  N-NH <sub>4</sub> = 9.8-68mg/l N-NO <sub>3</sub> = 0.4-0.5 mg/l P-PO <sub>4</sub> = 0.30-2.70mg/l  Relation: 1:30 P:N

		<p>1:15 P:N 1:30 P:N (from others scientific works)</p> <p><u>Low concentration of N</u> 1:5 P:N (from others scientific works)</p>	
O <sub>2</sub> and CO <sub>2</sub> supply	It is necessary to supply CO <sub>2</sub> to the algal system	To supply CO <sub>2</sub>	Probably the plants: photobioreactor and pond will build open system.
	<b>In the open algal system (pond) CO<sub>2</sub> comes from the air;</b>	It is possible to increase the gas exchange moving the algal culture.	
	<b>In the closed system (photobioreactor) the CO<sub>2</sub> is artificially supplied</b>	It is possible to build the capture and sequestration systems of CO <sub>2</sub>	
pH	<p>pH is continuously monitored.</p> <p>It is maintained in the optimal range. pH range is dissimilar to different algal species.</p> <p>But at pH &gt;8 phosphorous precipitates and ammonium nitrogen gasifies, so the nutrients are not available to the algal biomass.</p>		

**TABLE 2:** Environmental parameters, critical points and solutions that can influence the algal phytodepuration in the territory of Varese Figure.

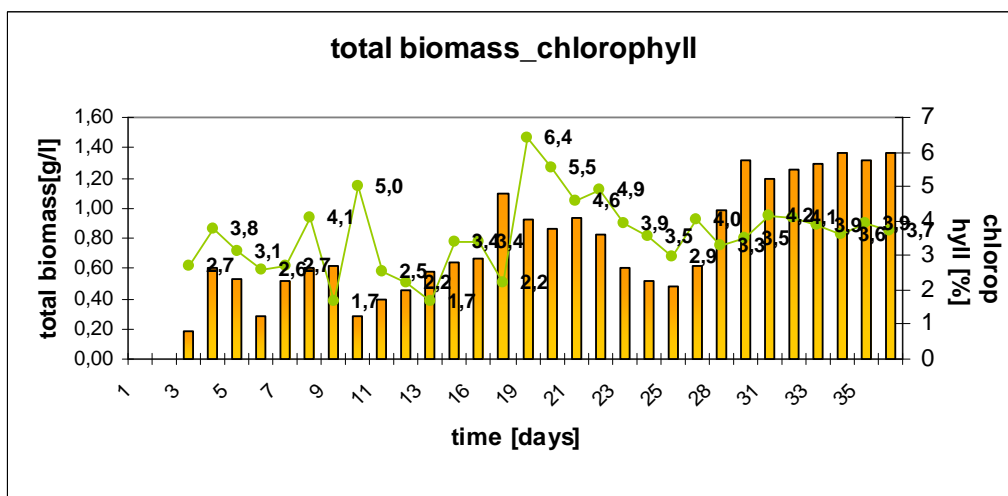
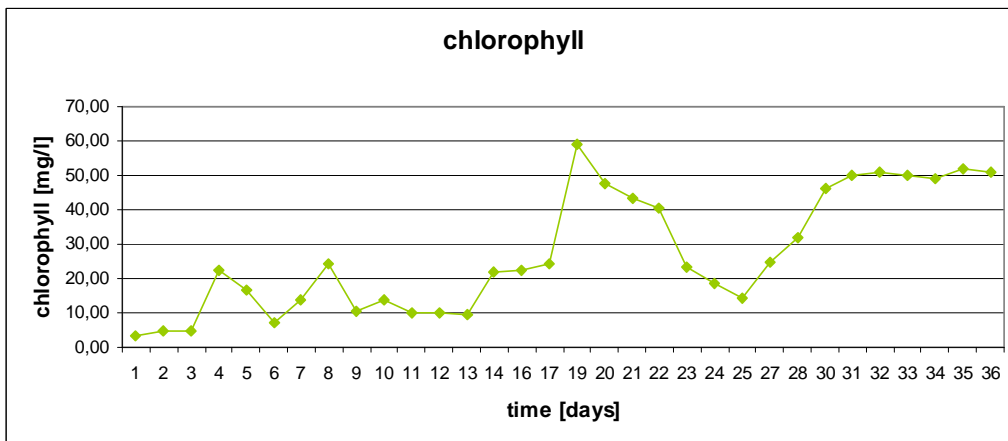
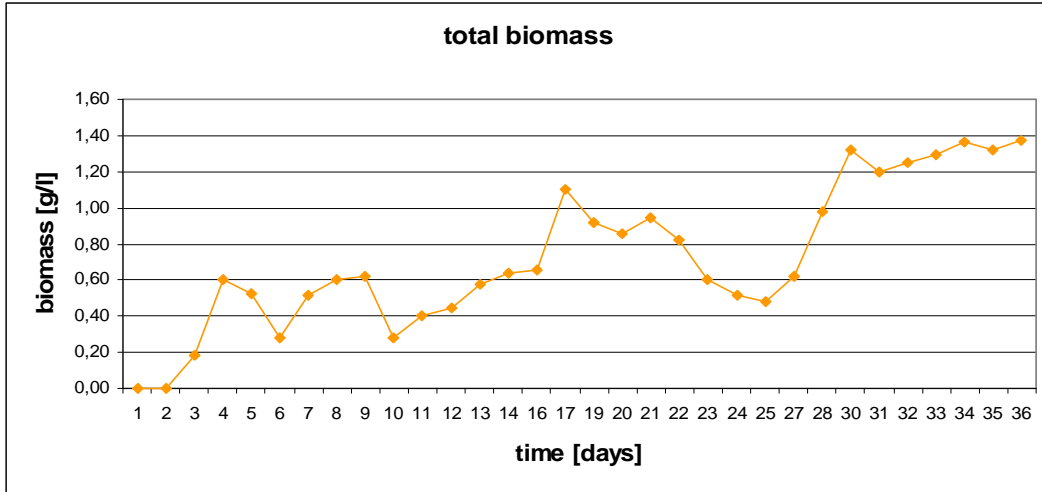


FIGURE 2: Biomass production during this action: same results of the monitoring culture.

## Mass culture: identification

The microorganism presents in the wastewater of S. Pietro Vara are:

**Prokaryotes** (bacteria): organisms without internal organelles

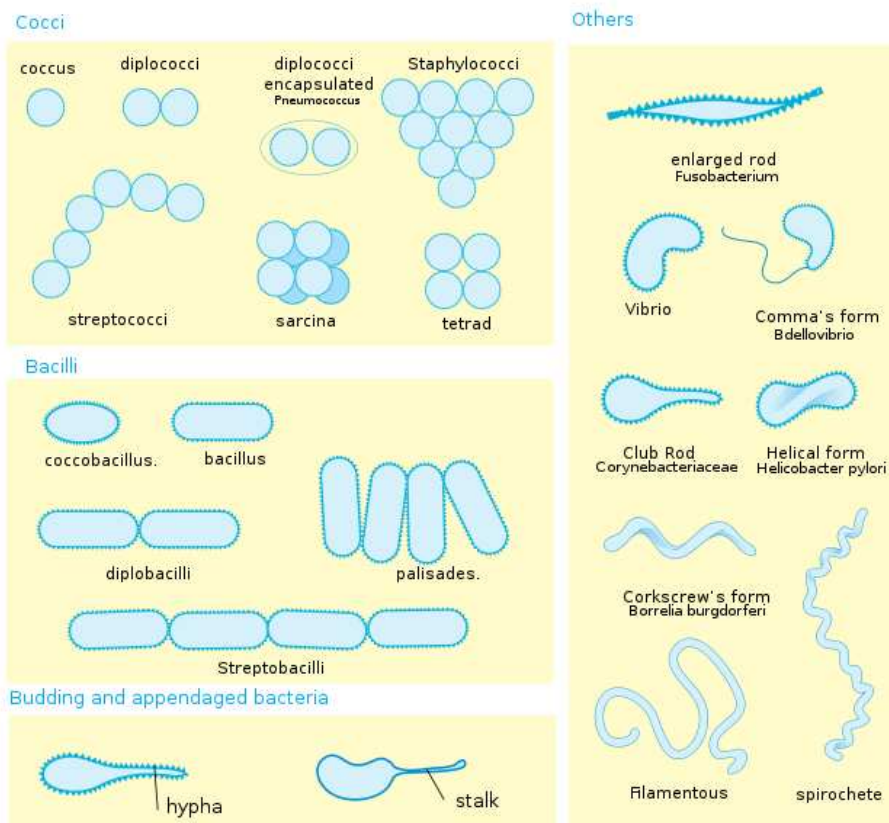
**Microalgae** (blue –green algae, diatoms and green algae): protists with chloroplast

**Protozoa** (ciliates, flagellates, amoebae): small organisms from 5 to 1000µm with/ without movements

**Metazoa** (gastrotrichs, rotifers, nematodes, flatworms, tardigrades): organisms with many cells which are arranged in epithelia.

Prokaryotes are organism typically a few micrometres in length. In this wastewater we found some different types of bacteria: single cells and others associate in characteristic patterns as in "bunch of grapes" clusters. In **FIGURE 3** they are shown some shapes of bacteria.

Species, that are probably presented in this wastewater of S. Pietro Vara plant, are: bacteria filamentous, cluster bacteria.

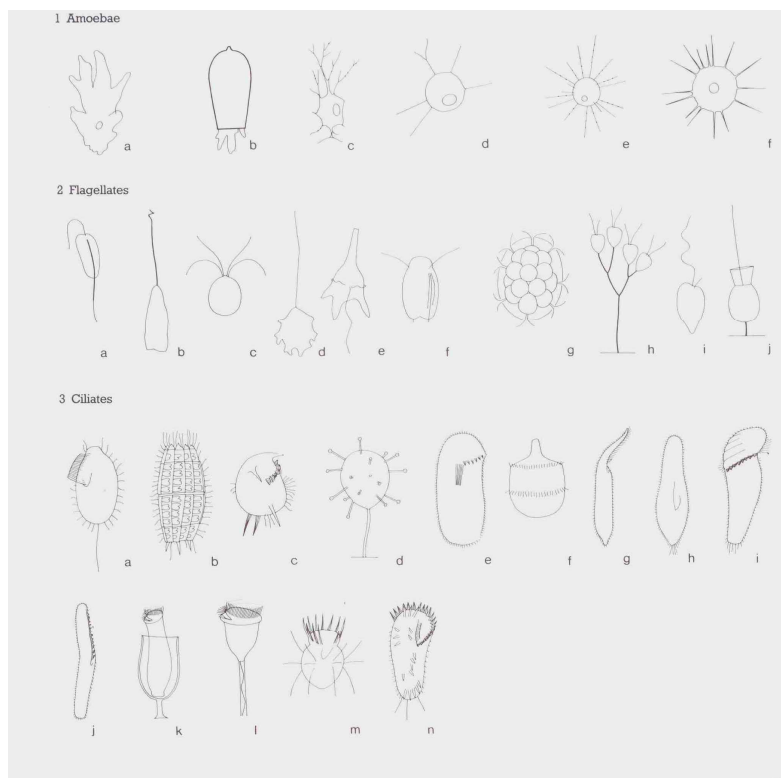


**FIGURE 3:** Some basic type of Prokaryotes.

Microalgae are microscopic photosynthetic organism. The species more common are diatoms, flagellated and chlorococcalean green algae, and filamentous blue-green algae, ranging in size from a few micrometer to more than 100  $\mu\text{m}$ . In wastewater of S. Pietro Vara plant we found:

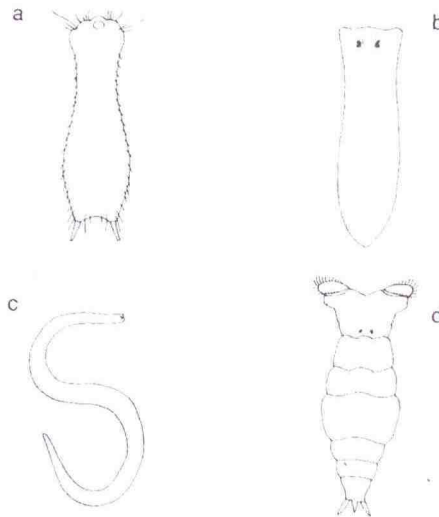
- the single and spherical cells as *Chlorella sp.*, green algae Chlorophyceae,
- some little spherical Cyanophyceae, a blue-green algae,
- some cells of Pirrophyta as probably *Gymnodinium sp.*
- and some pennate diatoms Diatomea;
- some filamentous algae as *Oscillatoria sp.* and a blue-green algae *Cianophyceae.*

Protozoa are unicellular organisms with nucleus, that move or feed using flagella (flagellates), cilia (ciliates) or pseudopodia (amoebae). Some basic types are illustrated in **FIGURE 4.** In wastewater of S. Pietro Vara plant, there are: *Vorticella sp.*, *Paramecium sp.*, *Amoebae sp.*,



**FIGURE 4:** Some basic type of Protozoa.

Metazoa are organisms of multicellular. In this wastewater have a little size (1mm) in the **FIGURE 5** they are shown some types of this organism presented in the wastewater of S. Pietro Vara: gastrotrich, flatworm, nematode and rotifer



**FIGURE 5:** Some basic type of little Metazoa: (a) gastrotrich, (b) flatworm, (c) nematode and (d) rotifer.

### Characteristic of algal biomass

The heterogenic algal culture have some parameters as photosynthetic rate (P) [ $\mu\text{mol mgchl}^{-1}\text{h}^{-1}$ ], chlorophyll concentration to biomass unit [% dry biomaas] and growth rate ( $\mu$ ) [ $\text{d}^{-1}$ ] that are very good. This values are present following in **FIGURE 6**.

	<b>light</b>	<b>chlorophyll rate</b>	<b>P net</b>	<b><math>\mu</math></b>
	$\mu\text{mol fotoni m}^{-2}\text{s}^{-1}$	% dry biomass	$\text{Mmolmgchl}^{-1}\text{h}^{-1}$	$\text{d}^{-1}$
Scientific literature	<b>30</b>	<b>1÷6</b>	<b>57</b>	<b>1</b>
our algal biomass	<b>24</b>	<b>5</b>	<b>81,82</b>	<b>0,1</b>

**FIGURE 6:** The comparison between the values of our algal biomass and values of the scientific literature: photosynthetic rate (P net), chlorophyll concentration and growth rate ( $\mu$ ).

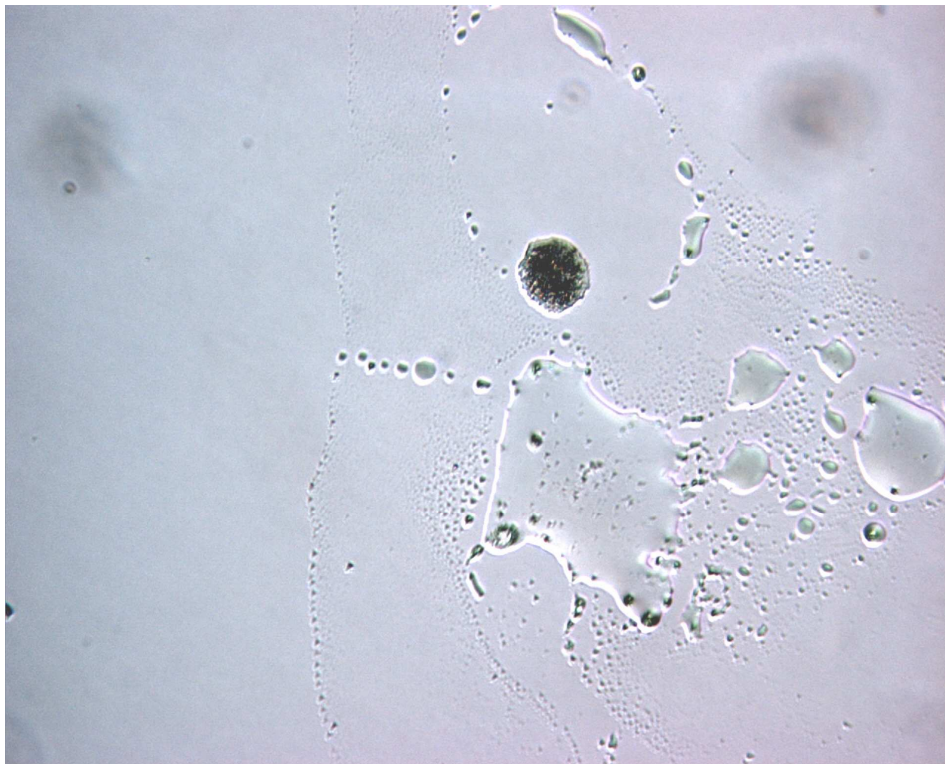
## MICRO PHYTODEPURATION ALGAE: PHOTOGRAPHIC GALLERY

In these pages have been reported only the imagines that the we considered relevant for each month.

Photo n° 1

Month of the algal culture: **April 2010**

Organisms: **Protozoa** Amoebae (probably)

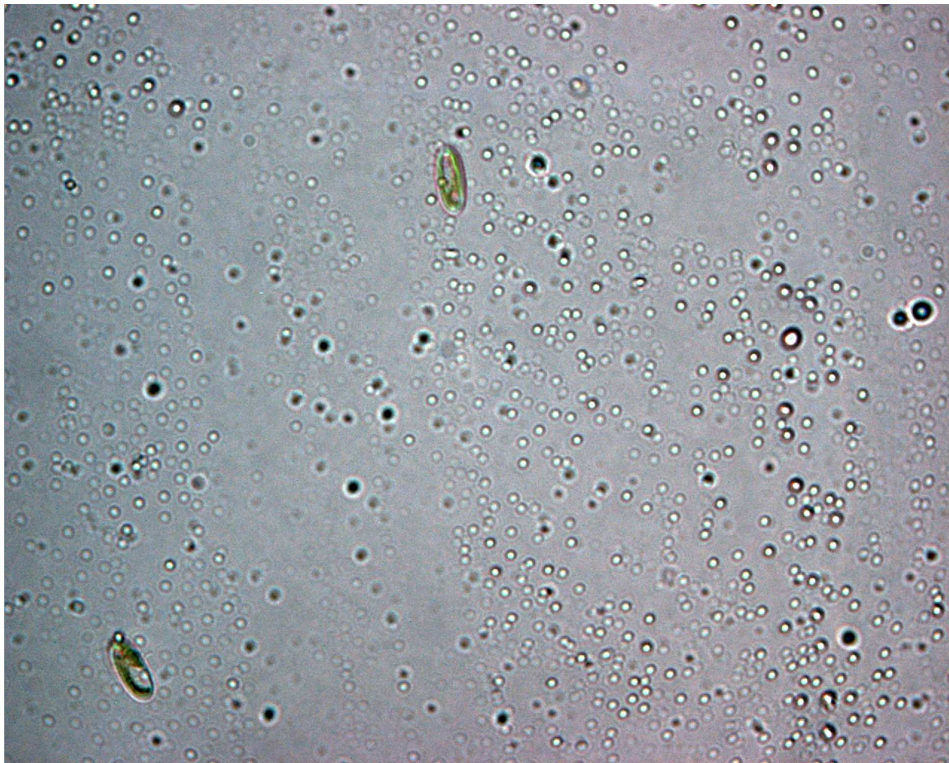


**Additional information:** in the batch culture some of this organisms are always present, a common genus of amoebae, observation to 1000x.

Photo n° 2

Month of the algal culture: **April 2010**

Organisms: **Micro Algae** Diatomea, a pinnate diatoms (probably)

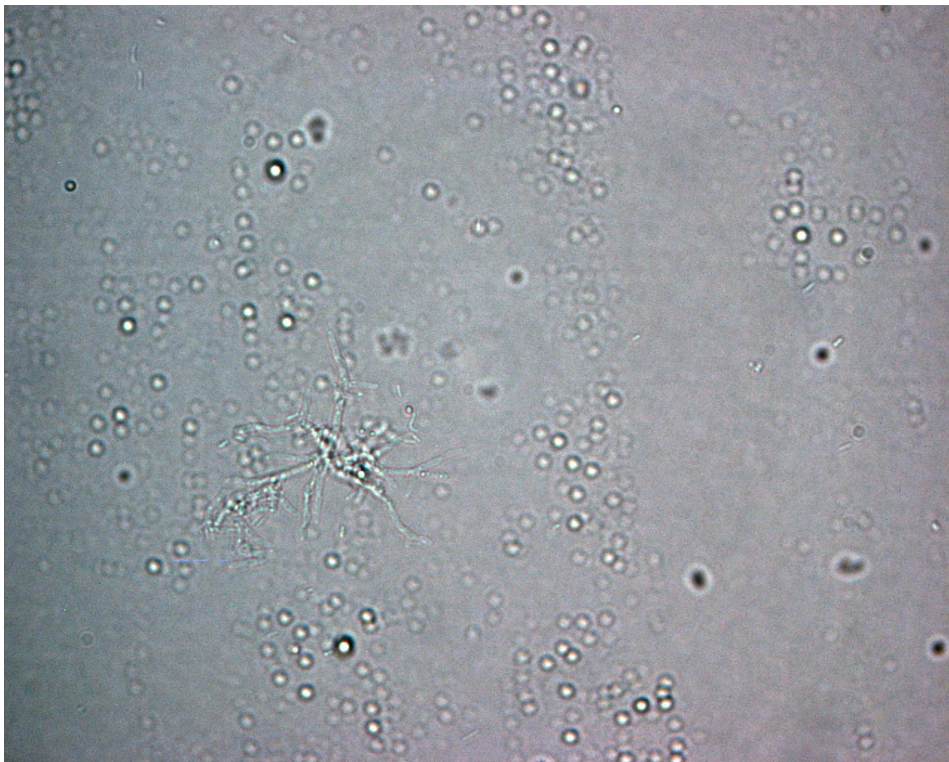


**Additional information:** observation to 1000x

Photo n° 3

Month of the algal culture: **April 2010**

Organisms: **Prokaryotes**-cluster and branched mycelia (probably)

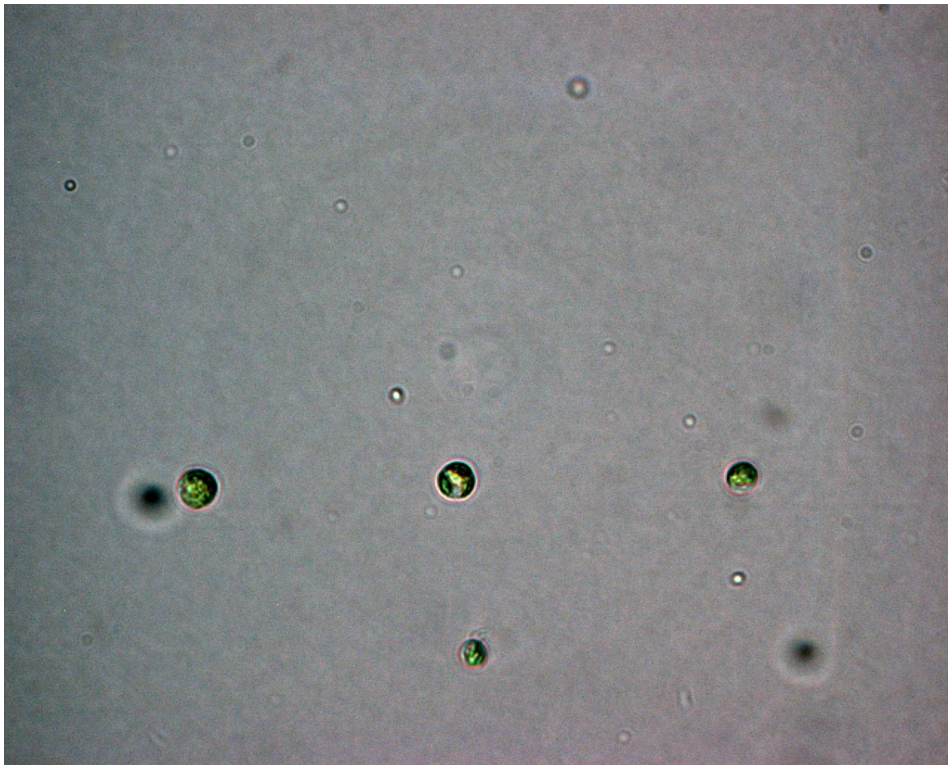


**Additional information:** observation to 1000x

Photo n° 4

Month of the algal culture: **April 2010**

Organisms: **Micro Algae** Chlorophyceae, green algae *Chlorella sp.*

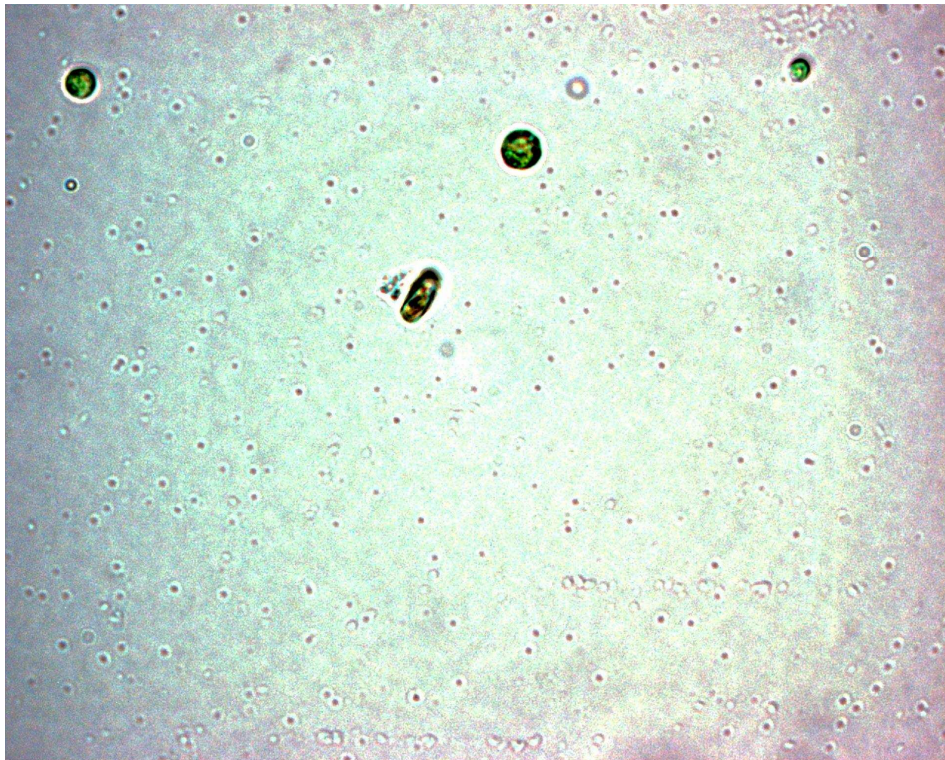


**Additional information:** in the batch culture these organisms are always present so many, observation to 1000x

Photo n° 5

Month of the algal culture: **May 2010**

Organisms: **Micro Algae** Chlorophyceae -green algae *Chlorella sp* and **Protozoa** Paramecium

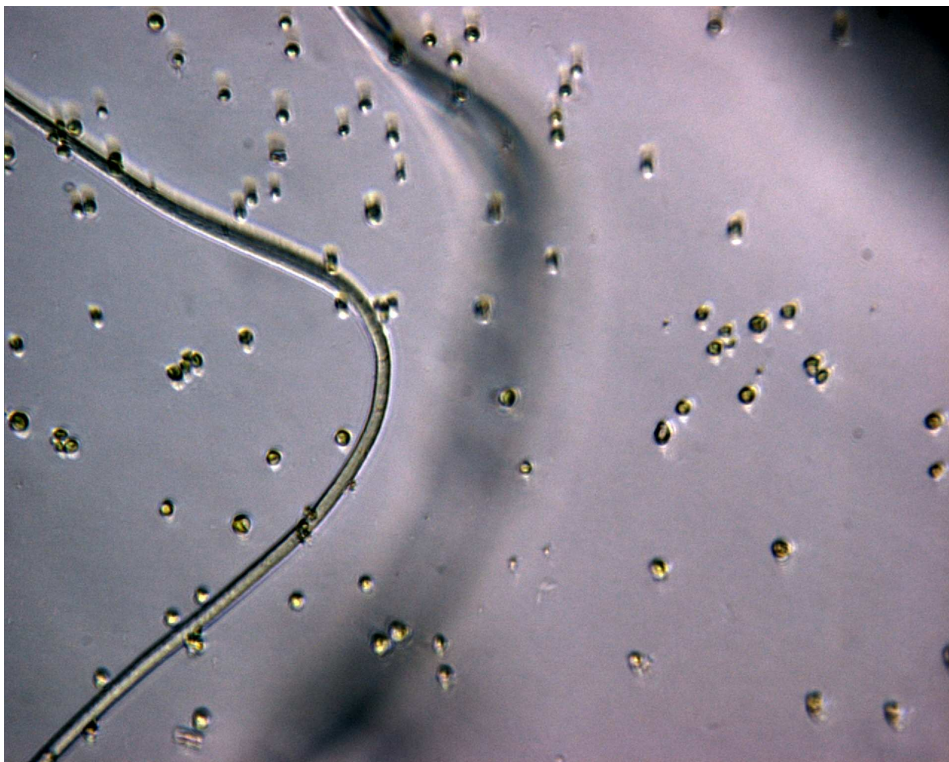


**Additional information:** the spherical organisms are the green algae *Chlorella sp.*, whereas the oblong cell with tuft of hair is Paramecium, observation to 1000x .

Photo n° 6

Month of the algal culture: **May 2010**

Organisms: **Micro Algae** Chlorophyceae -green algae *Chlorella sp* and filamentous of Cyanophyceae

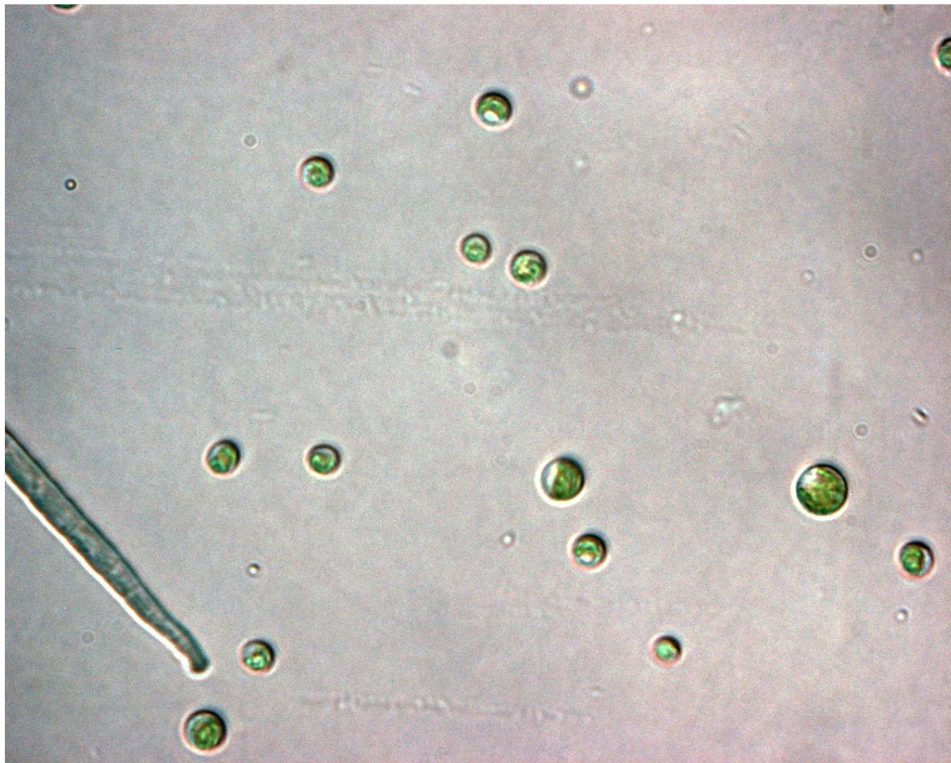


**Additional information:** the spherical organisms are the green algae *Chlorella sp.*, and the filamentous of Cyanophyceae , observation to 400x .

Photo n° 7

Month of the algal culture: **May 2010**

Organisms: **Micro Algae** Chlorophyceae -green algae *Chlorella sp* and filamentous of Cyanophyceae

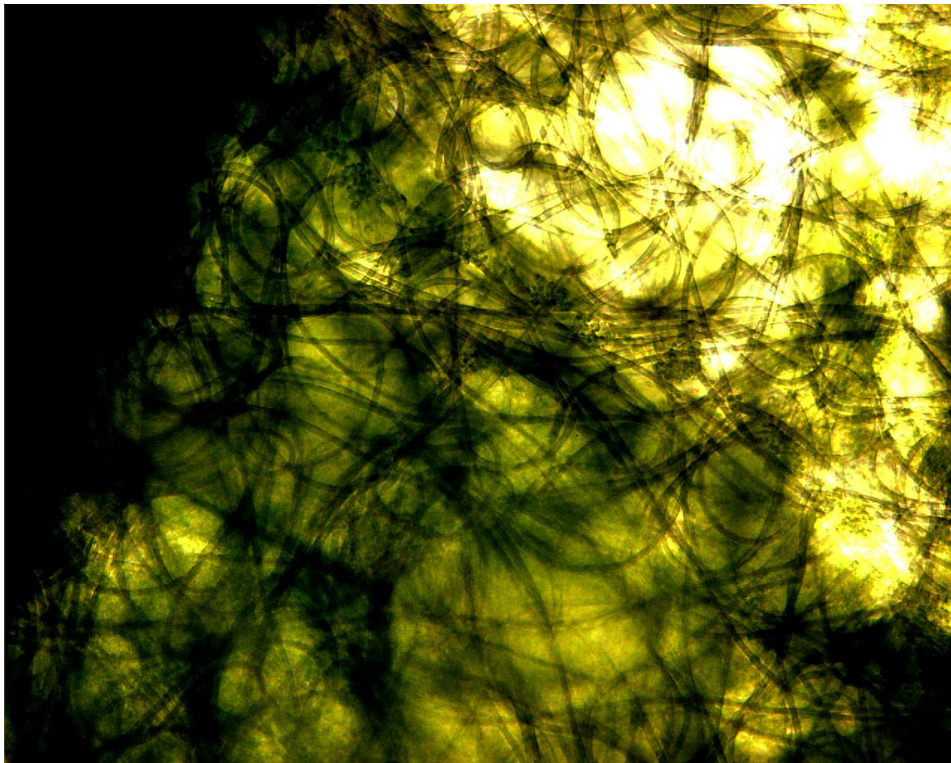


**Additional information:** the spherical organisms are the green algae *Chlorella sp.*, and the filamentous of Cyanophyceae , it is *Oscillatoria sp.*, observation to 1000x .

Photo n° 8

Month of the algal culture: **June 2010**

Organisms: **Micro Algae**- filamentous of Cyanophyceae



**Additional information:** filamentous algae, their genus are probably *Oscillatoria* (Cyanophyceae), observation to 200x.

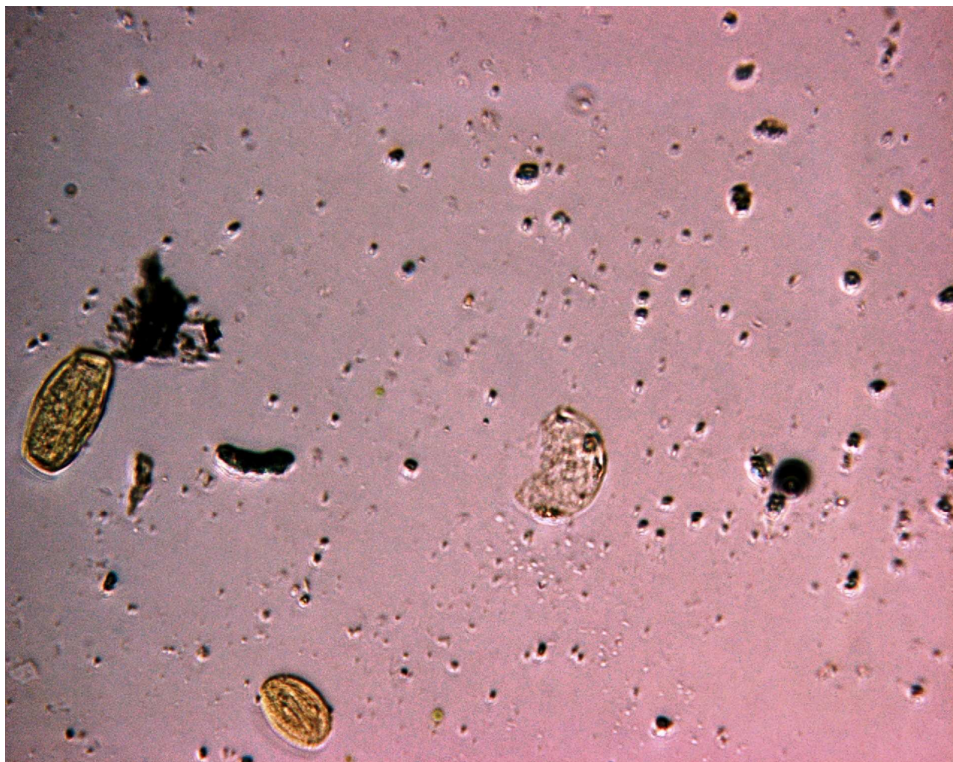
In the month of June the temperature is increase ( $T > 25^{\circ}\text{C}$ ) and pH in the algal batch culture is decrease ( $\text{pH} < 6$ ), in this condition the filamentous of Cyanophyceae are growth up more than Chlorophyceae. These changes must be to possible present of acidophilus bacteria as Lactobacillus.

Lactobacillus is bacteria it is present in the working of the yogurt and the wastewater of S. Pietro Vara contained wastewater coming from industrial yogurt manufactures.

Photo n° 9

Month of the algal culture: **June 2010**

Organisms: **Protozoa ciliates**

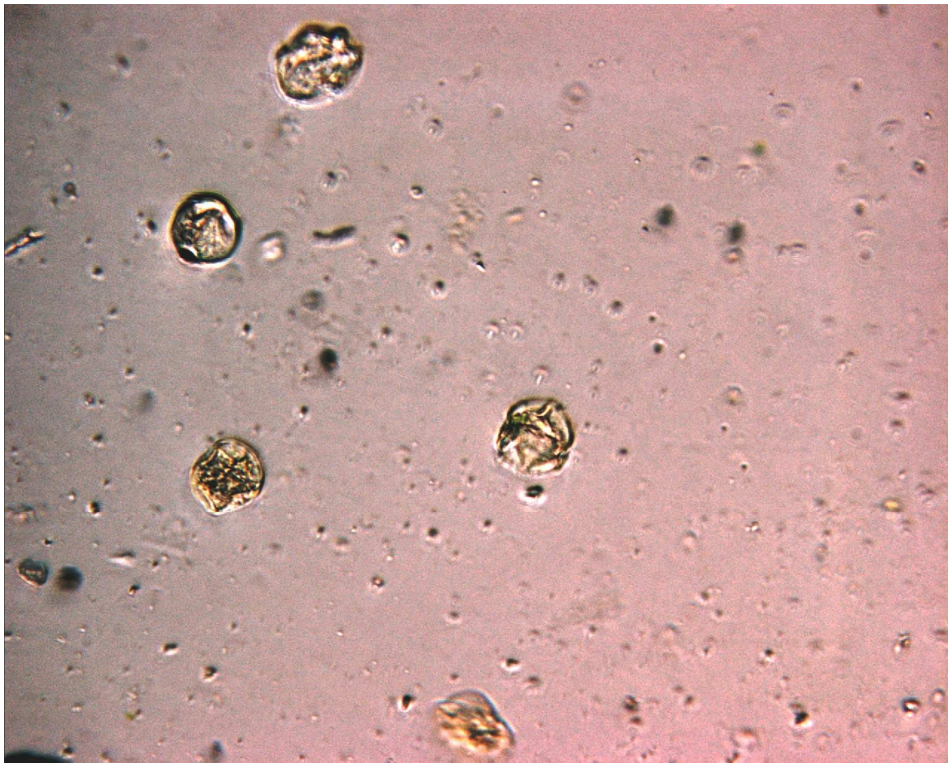


**Additional information:** observation to 400x.

Photo n° 10

Month of the algal culture: **June 2010**

Organisms: **Micro-algae** Pyrrophyta

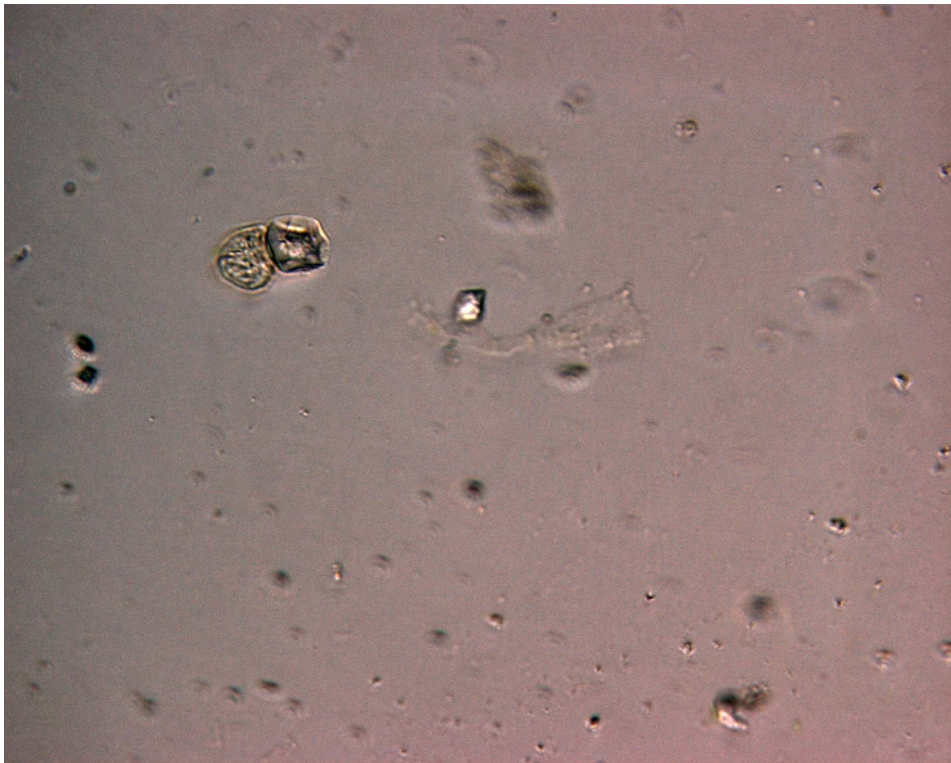


**Additional information:** the probably genus of these algae are *Gymnodinium* sp. observation to 400x.

Photo n° 11

Month of the algal culture: **June 2010**

Organisms: **Protozoa** Vorticella

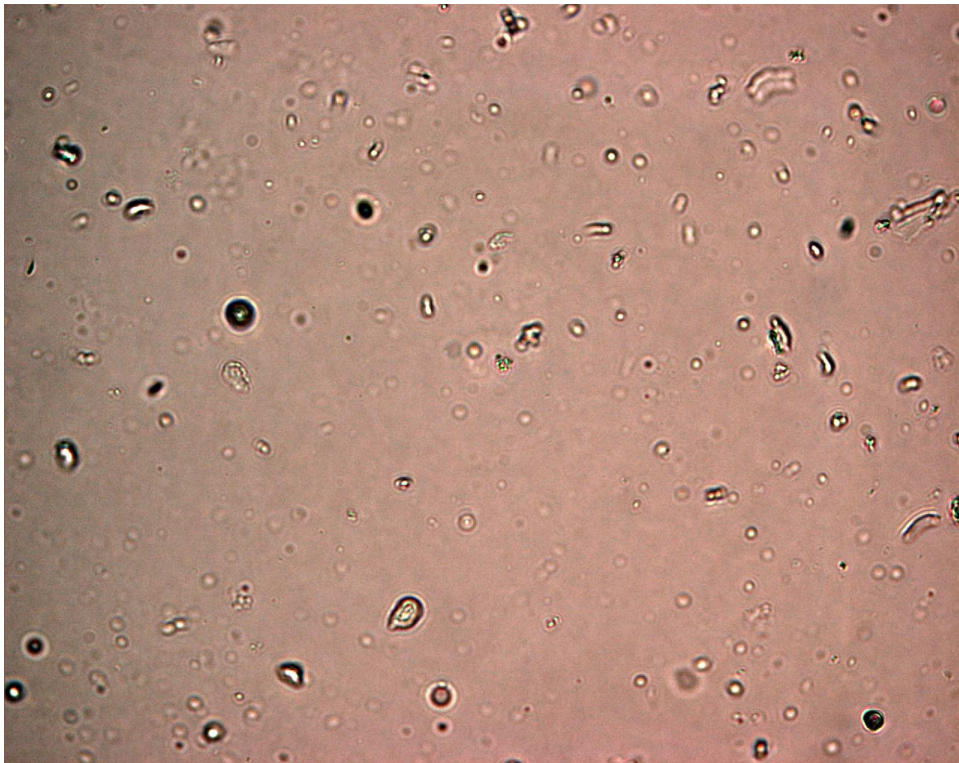


**Additional information:** observation to 400x.

Photo n° 12

Month of the algal culture: **June 2010**

Organisms: **Prokaryotes** (bacteria) bacillus and vibrio

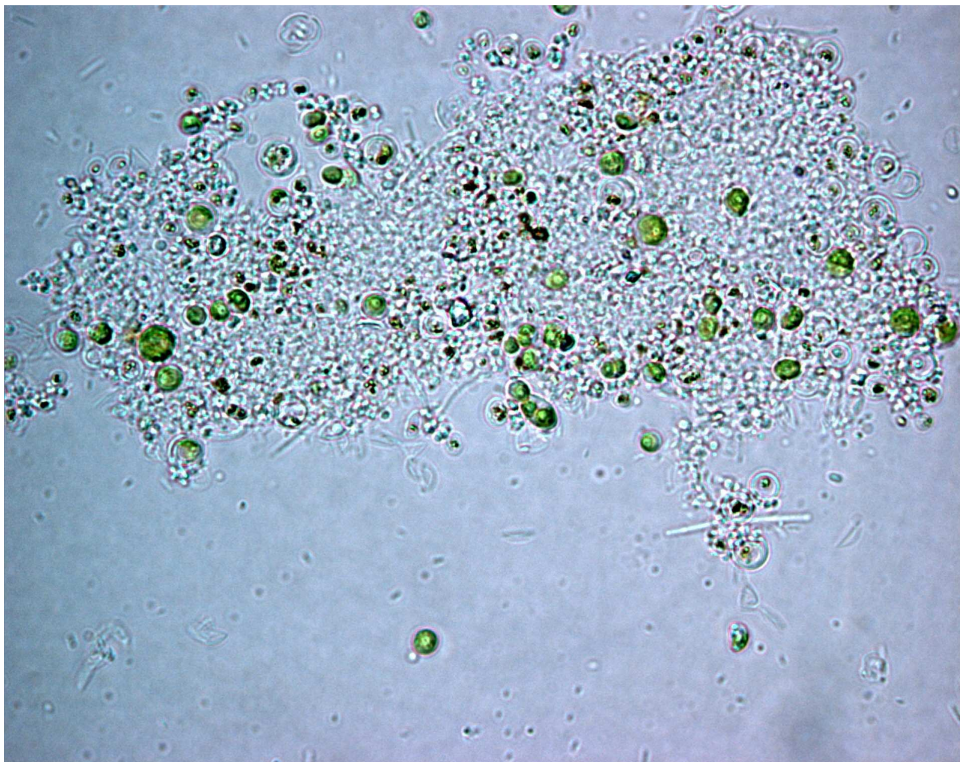


**Additional information:** observation to 1000x.

Photo n° 13

Month of the algal culture: **June 2010**

Organisms: **Micro Algae** Chlorophyceae - green algae *Chlorella* sp. and **Prokaryotes** cluster of bacteria

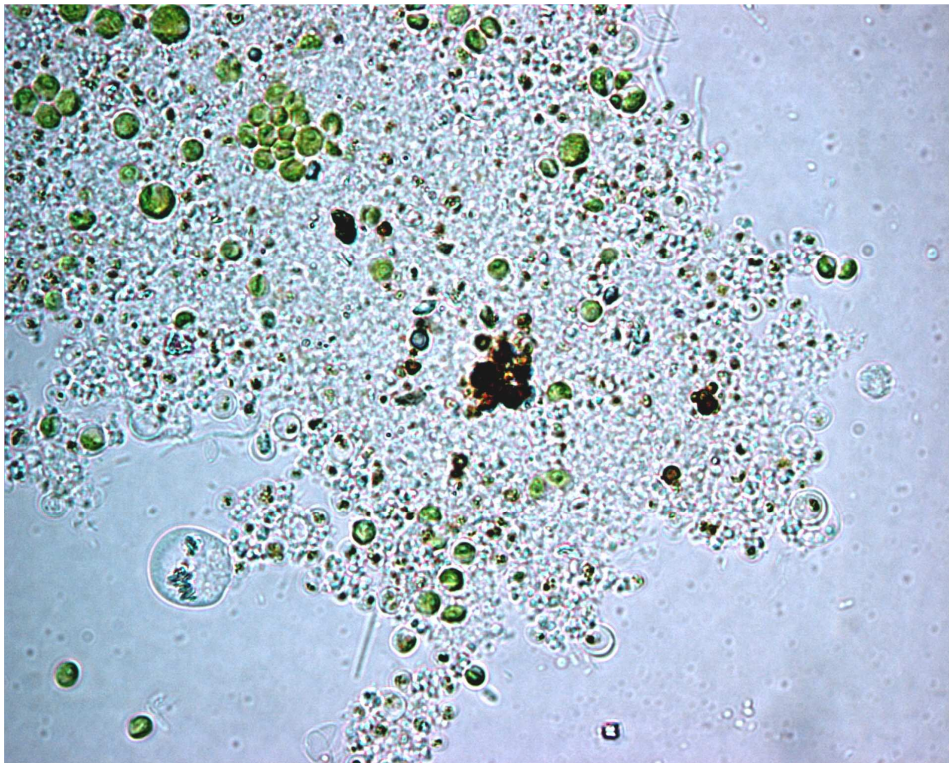


**Additional information:** the condition culture is no good, there are more bacteria clusters than green algae , observation to 1000x.

Photo n° 14

Month of the algal culture: **June 2010**

Organisms: **Micro Algae** Chlorophyceae - green algae *Chlorella* sp. and **Prokaryotes** cluster of bacteria

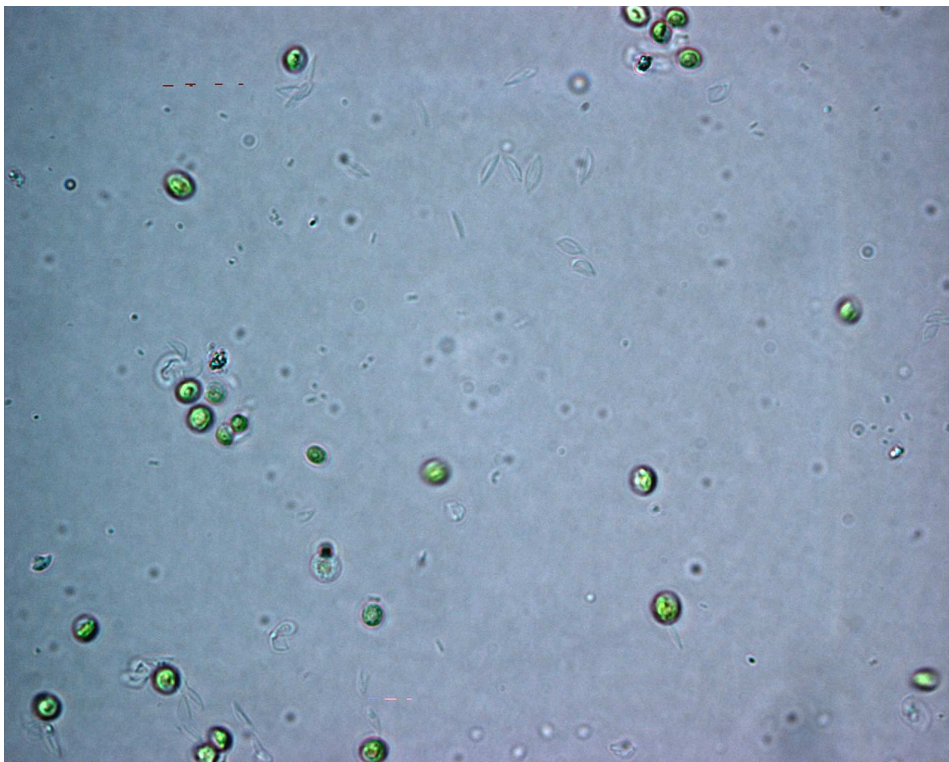


**Additional information:** the condition culture is no good, there are more bacteria clusters than green algae, observation to 1000x.

Photo n° 15

Month of the algal culture: **July 2010**

Organisms: **Micro Algae** Chlorophyceae - green algae *Chlorella* sp.

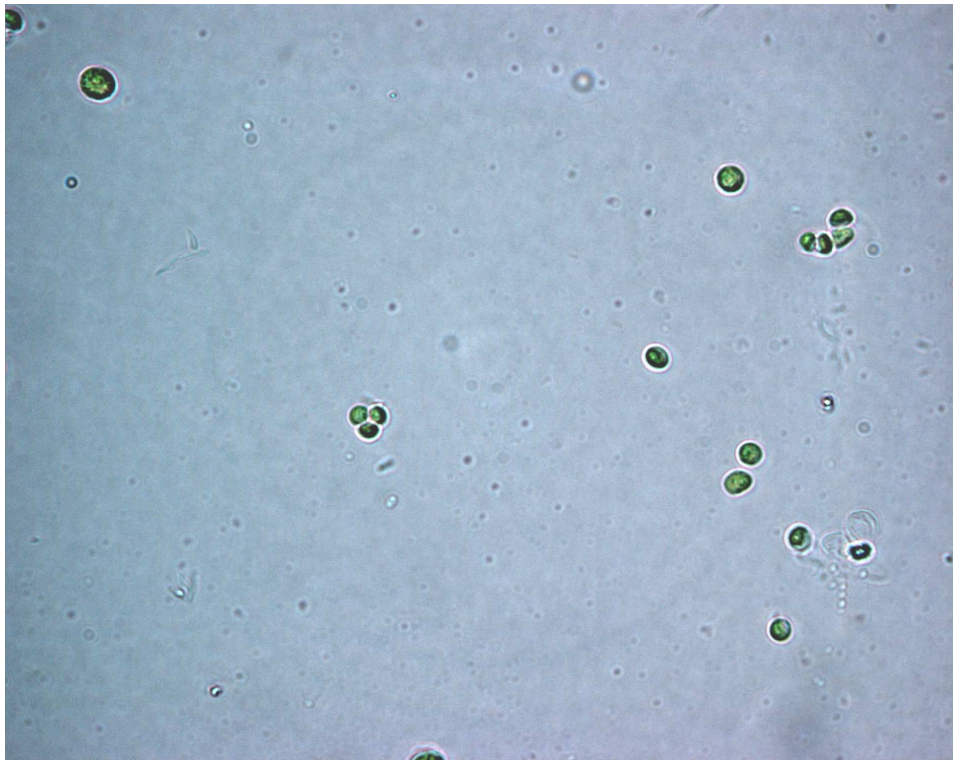


**Additional information:** In July the algae culture changes again, in this period the species predominant are *Chlorella* sp., the temperature of batch culture are  $20 \pm 2$  (in ambient  $T = 25^\circ\text{C}$ ) and pH are value from 5 to 2 in this condition also acidophilic algal species live as the *Chlorella* sp. (datum confirmed by scientific literature). In this culture condition the competitions (bacteria) and predators (protozoa and metazoa) are decrease in the batch culture, because the pH is acid (observation to 1000x).

Photo n° 16

Month of the algal culture: **July 2010**

Organisms: **Micro Algae** Chlorophyceae - green algae *Chlorella* sp.

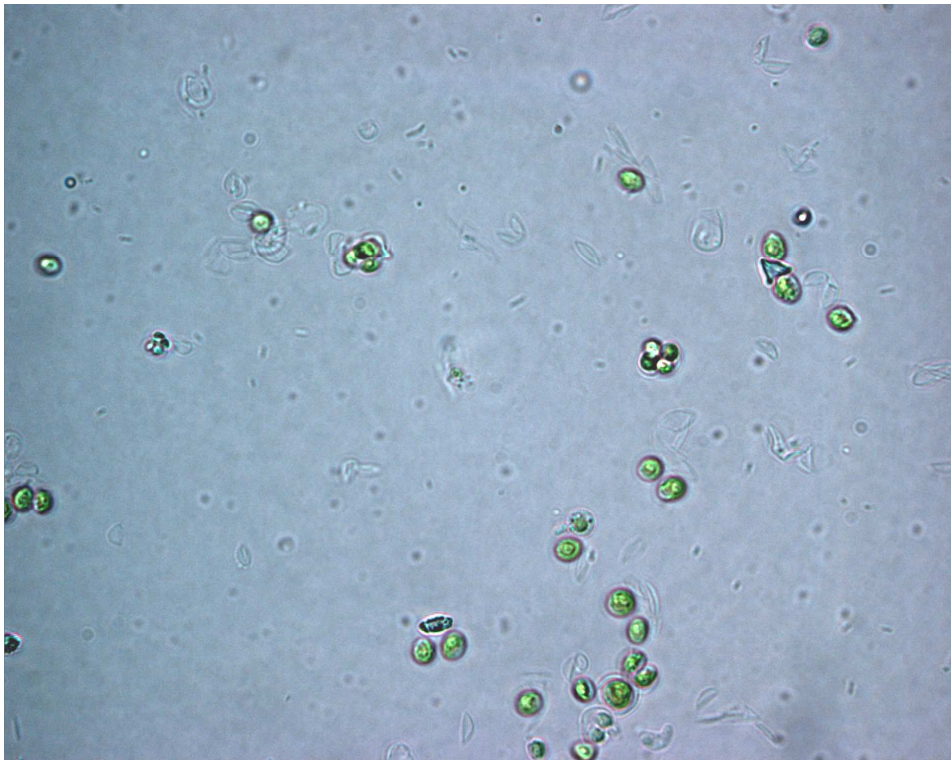


**Additional information:** the condition culture is good, there are more microalgae *Chlorella* sp. than other competitor organisms (other algae, bacteria, metazoa), observation to 1000x.

Photo n° 17

Month of the algal culture: **July 2010**

Organisms: **Micro Algae** Chlorophyceae - green algae *Chlorella* sp.

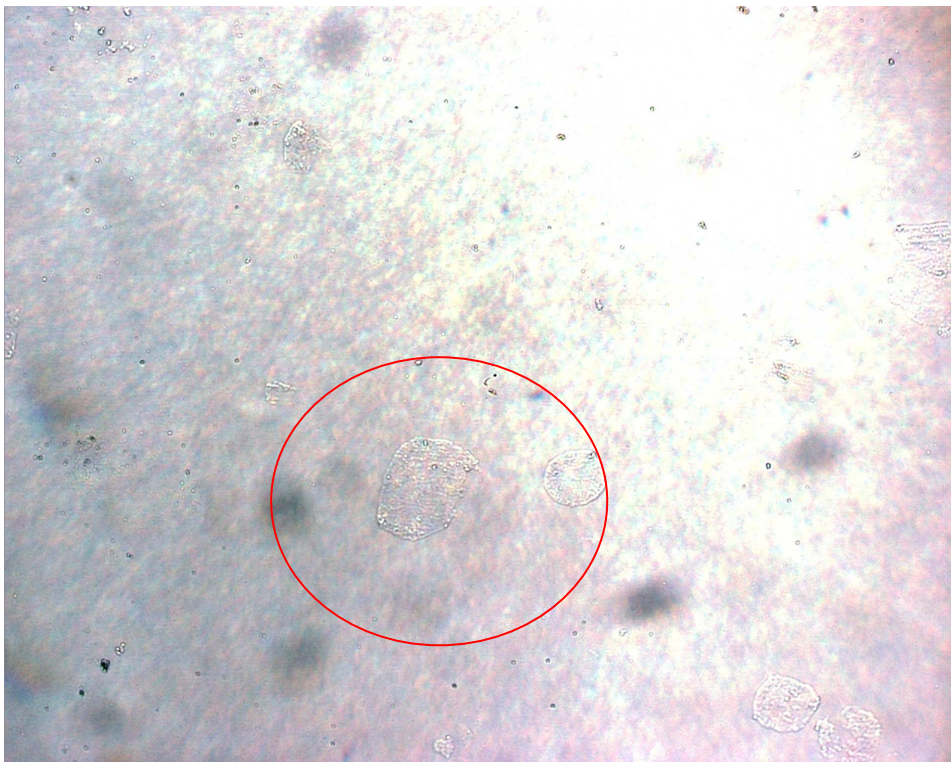


**Additional information:** the condition culture is good, there are more microalgae *Chlorella* sp than competitor organisms, observation to 1000x.

Photo n° 18

Month of the algal culture: **August 2010**

Organisms: **Protozoa** Amoebae without teca

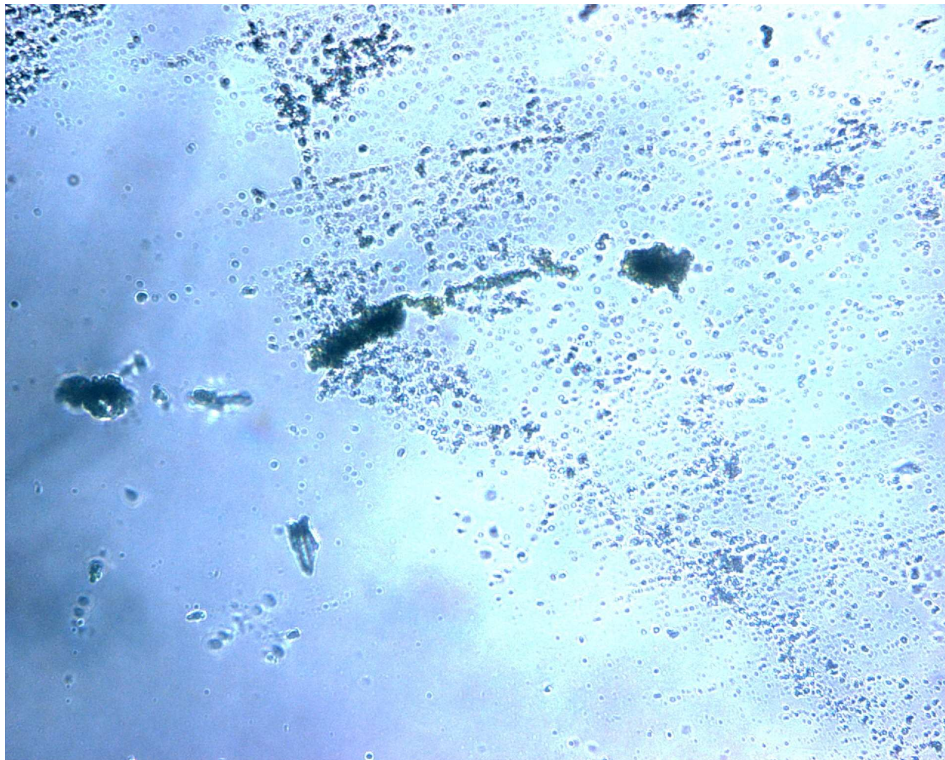


**Additional information:** observation to 100x.

Photo n° 19

Month of the algal culture: **August 2010**

Organisms: //

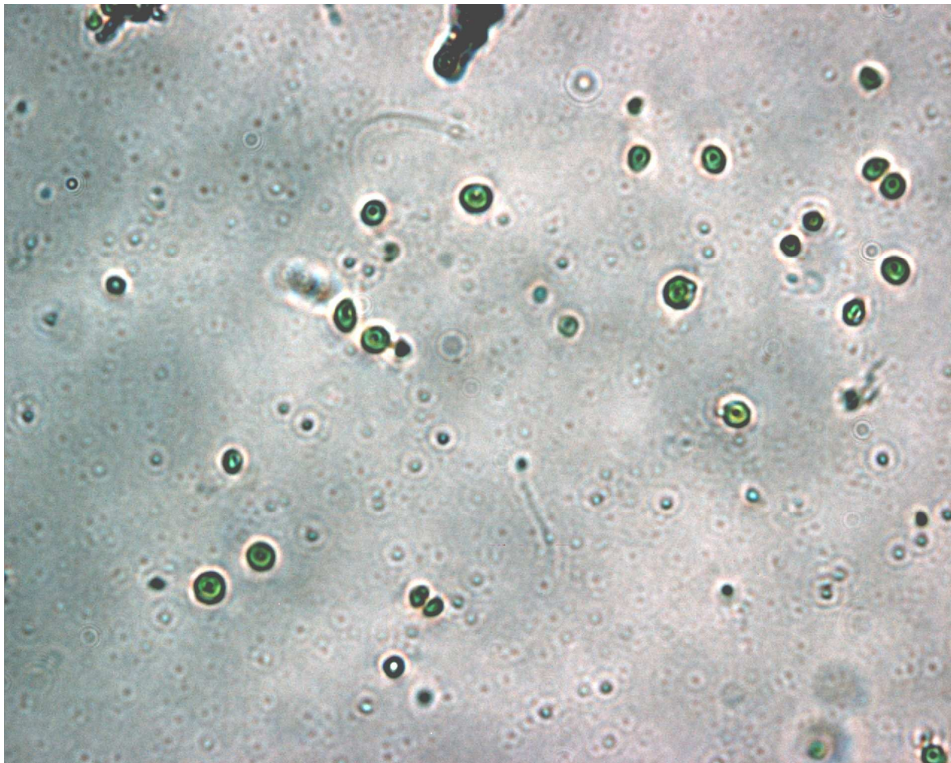


**Additional information:** the condition of the batch culture are changed, however there are both microalgae *Chlorella* sp. and the filamentous of Cyanophyceae, observation to 100x.

Photo n° 20

Month of the algal culture: **August 2010**

Organisms: **Micro Algae** Chlorophyceae - green algae *Chlorella sp.* and Cyanophyceae

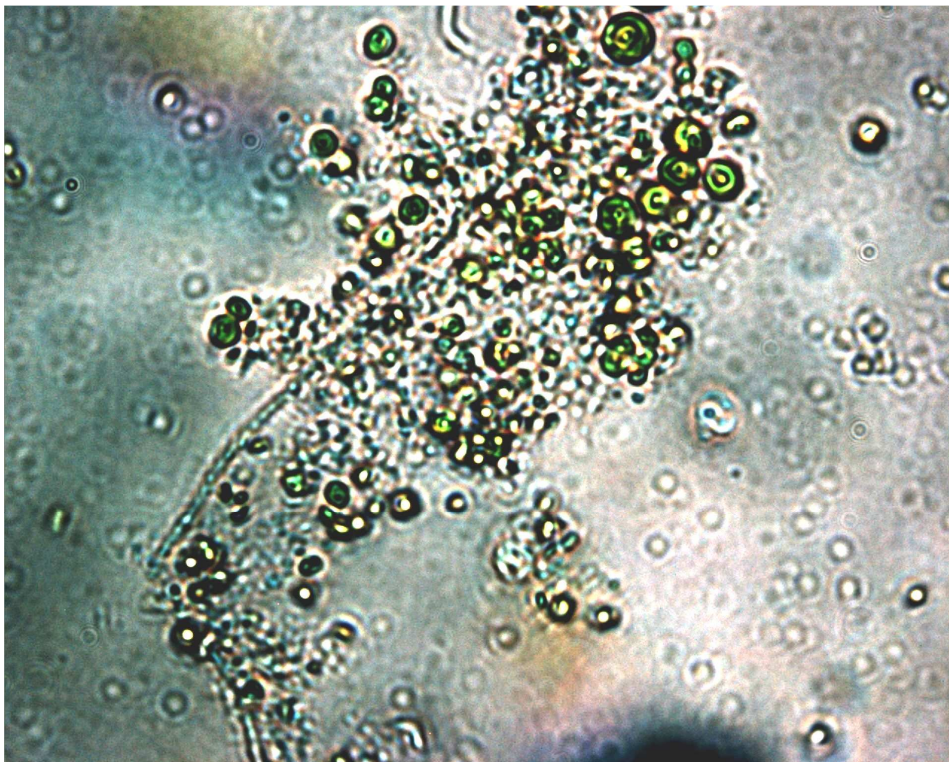


**Additional information:** in the algal culture there are both microalgae *Chlorella sp.* and the filamentous of Cyanophyceae, observation to 1000x.

Photo n° 21

Month of the algal culture: **August 2010**

Organisms: **Micro Algae** Chlorophyceae – green algae *Chlorella* sp., Cyanophyceae and **Prokaryotes** (bacteria)

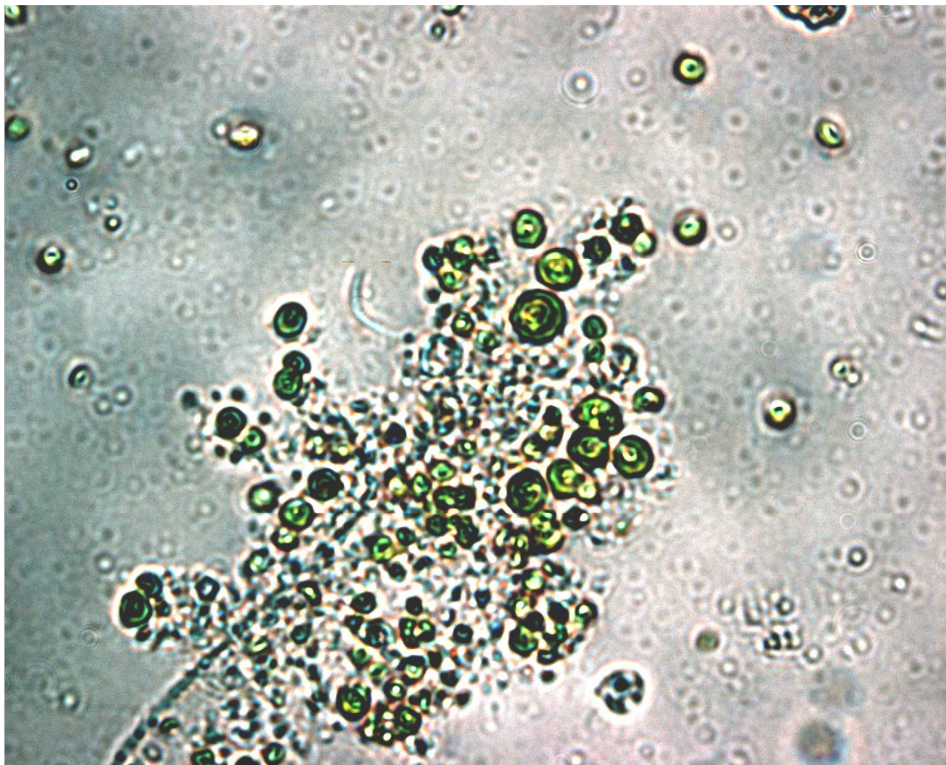


**Additional information:** Cyanophyta is probably to belong to genus Anabaene sp., observation to 1000x.

Photo n° 22

Month of the algal culture: **August 2010**

Organisms: **Micro Algae** Chlorophyceae - green algae *Chlorella* sp., Cyanophyceae and **Prokaryotes** (bacteria)

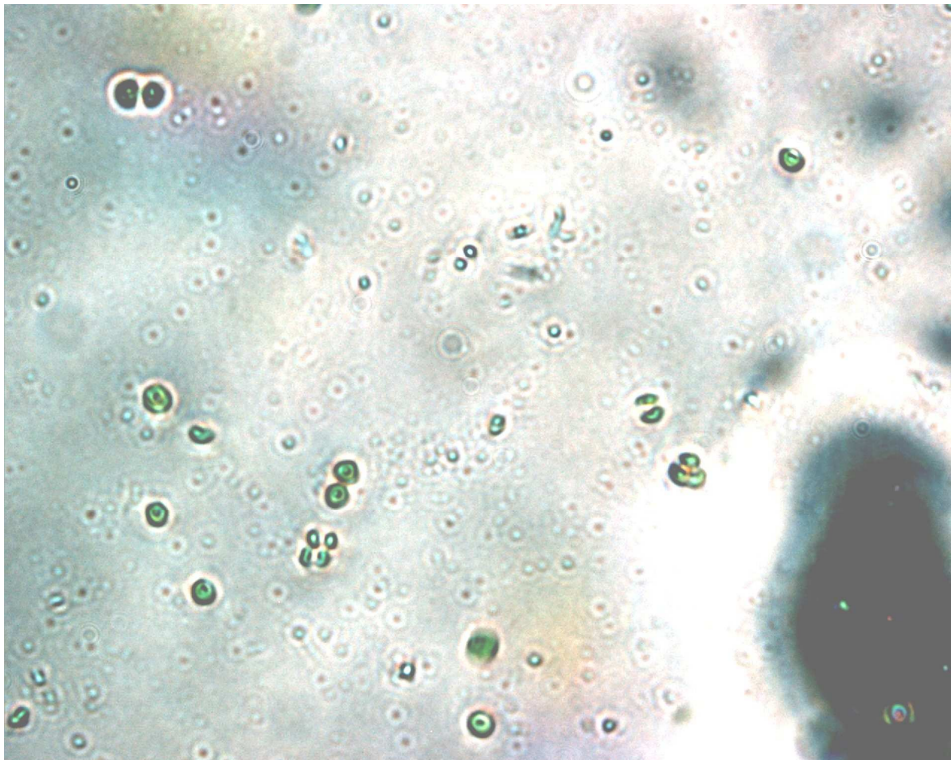


**Additional information:** Cyanophyta is probably to belong to genus *Anabaena* sp., observation to 1000x.

Photo n° 23

Month of the algal culture: **September 2010**

Organisms: **Micro Algae** Chlorophyceae - green algae *Chlorella* sp.



**Additional information:** observation to 1000x.

Photo n° 24

Month of the algal culture: **September 2010**

Organisms: **Metazoa** *Convoluta roscoffensis*

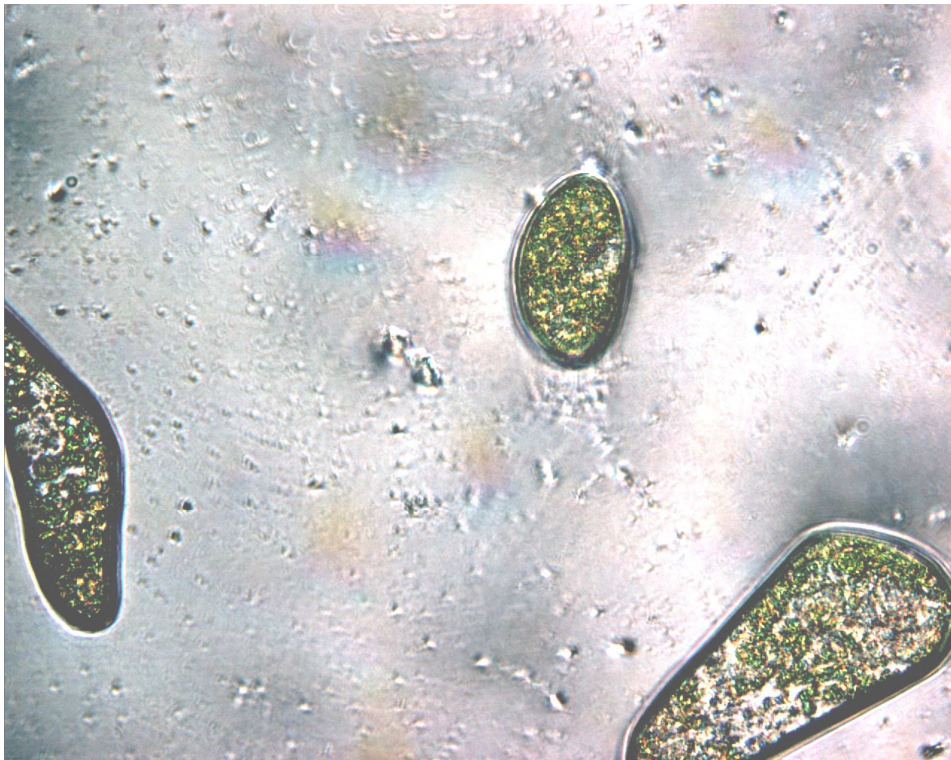


**Additional information:** A microscopic inspection of a translucent juvenile worm reveals that gut and parenchyma cells are filled with photosynthetic symbionts, microalgae of the genus *Clorella*. In the juvenile state those microalgae are ingested but not degraded and become endosymbionts. The algae remain photosynthetically active and thus, significantly contribute to the energy intake of the worms. After entering the adult phase, crucial anatomical changes such as loss of a functional pharynx and mouth, demonstrate that the worms now completely rely on their endosymbionts. They have become photoautotrophic organisms consuming sugars provided by the symbiotic algae (observation to 1000x).

Photo n° 25

Month of the algal culture: **September 2010**

Organisms: **Metazoa** *Convoluta roscoffensis*

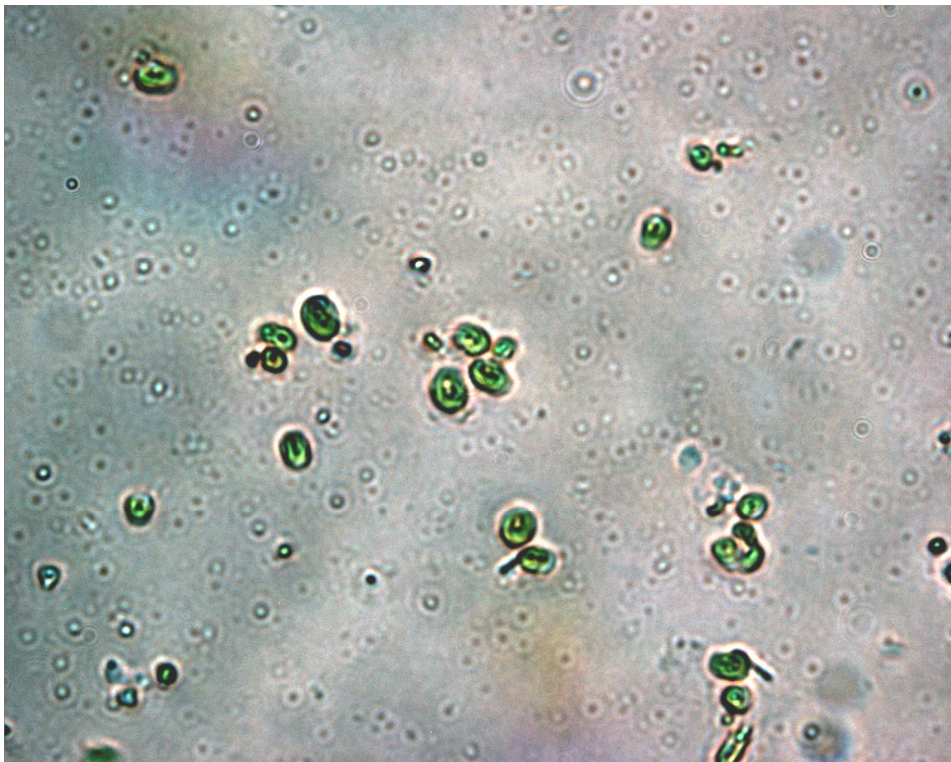


**Additional information:** observation to 1000x.

Photo n° 26

Month of the algal culture: **October 2010**

Organisms: **Micro Algae** Chlorophyceae - green algae *Chlorella saccharophylla* (probably)

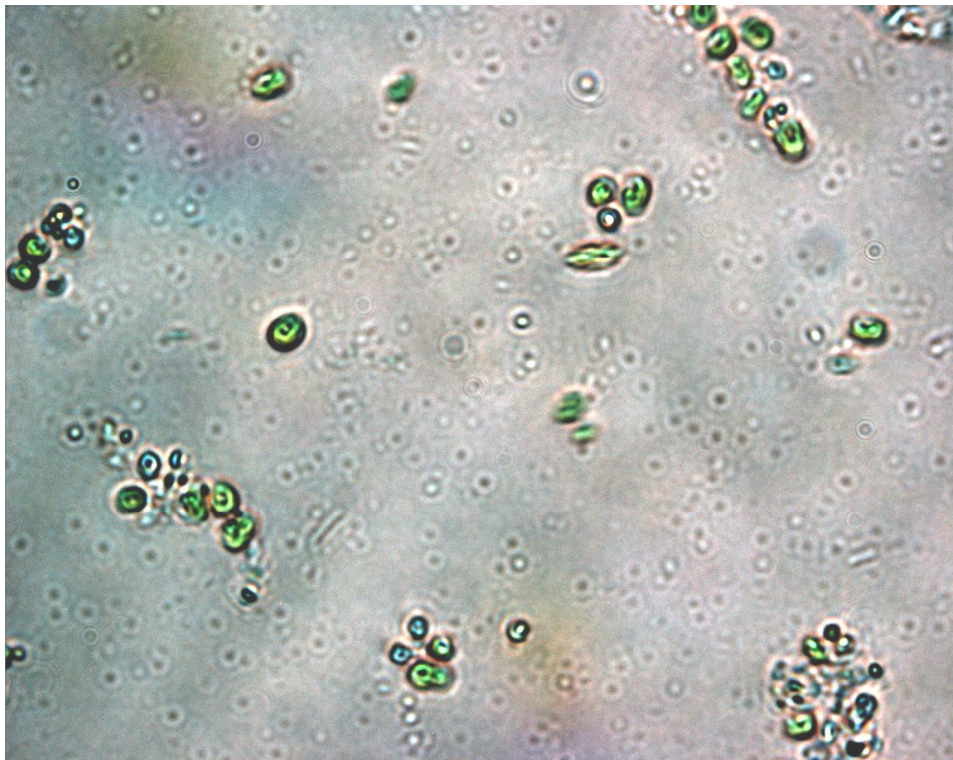


**Additional information:** in the batch culture there is always present the green algae, observation to 1000x.

Photo n° 27

Month of the algal culture: **November 2010**

Organisms: **Micro Algae** Chlorophyceae - green algae *saccarophylla* (probably)

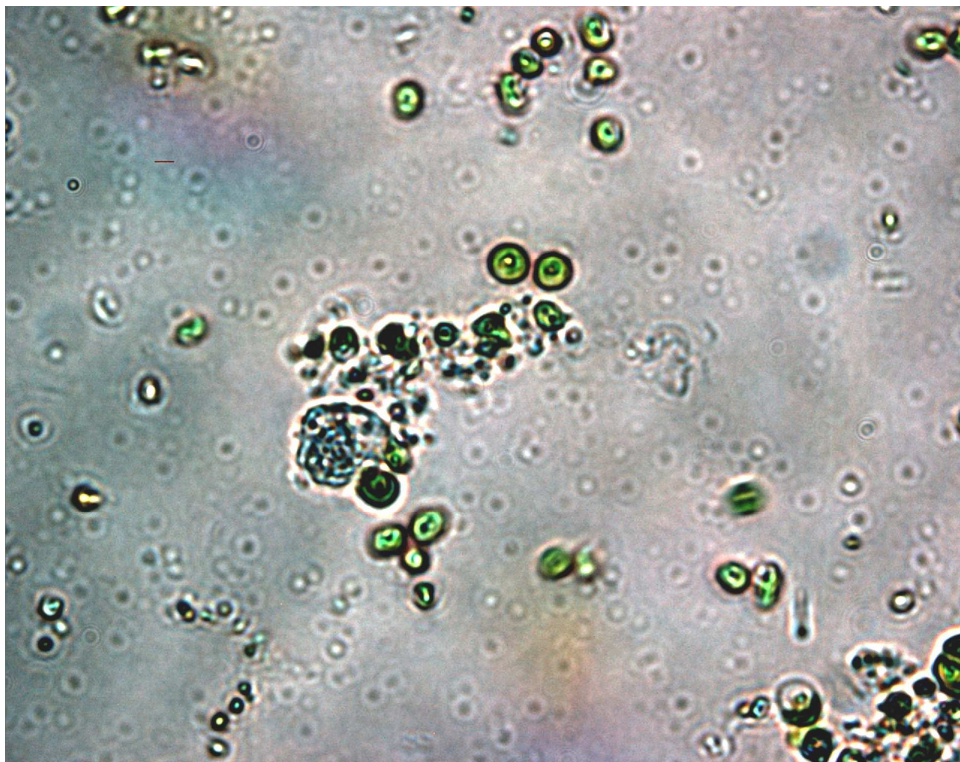


**Additional information:** in the batch culture there is always present the green algae, observation to 1000x.

Photo n° 28

Month of the algal culture: **December 2010**

Organisms: **Micro Algae** Chlorophyceae - green algae *Chlorella* sp.



**Additional information:** in the batch culture there is always present the green algae, observation to 1000x.

## Micro algae biomass

**ALGAL BIOMASS SELECTED : GOOD**

**PRODUCTION A SUFFICIENT QUANTITY OF MICRO ALGAL BIOMASS :  
YES**

**PRESENCE OF PREDATORS (HERBIVOROUS): YES**

**PRESENCE OF COMPETITIONS (BACTERIA) : YES**

### **PHOTOGRAPHIC MATERIAL:**

*Photographic material collected during the selection species is presented in pages 14 to 41*

### Summary of algal culture:

Months	Algae	
	<i>Chlorophyceae</i>	<i>Cyanophyceae</i>
April 2010	<b>so many *</b>	not many **
May 2010	<b>so many</b>	not many
June 2010	not many	<b>so many</b>
July 2010	not many	not many
August 2010	not many	<b>so many</b>
September 2010	<b>so many</b>	not many
November 2010	<b>so many</b>	not many
December 2010	<b>so many</b>	not many

\* it is the prevalent specie in the batch cultures.

\*\* it is not the prevalent specie in the batch cultures

**In light of the results of this action 4.2 the algal culture HAS BEEN SELECTED (i.e.quantity and quality very good), for the inoculum of the two phytodepuration systems.**