# STUDENT REPORT

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# *Algal production and Aquaculture*


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Finally, we would like to say many thanks to all the team of the Marine Biological Research Centre for their hospitality. They made our work placement such a pleasant stay and we exchanged a lot and shared good moments !

**Remember Kerteminde.** *(Diane KOCH, Théo BRUEY, Mélissa MIHOUB, Charles GOUILLARD).*

# ABBREVIATIONS

μ – growth rate

μm – micrometer

$\mu_{max}$ – maximal growth rate

ADP – adenosine-diphosphate

ATP – adenosine-triphosphate

$CO_2$ – carbon dioxide

D – Day

G – Gram

mL – milliliter

$N_2$ – atmospheric nitrogen

NADH – nicotinamide adenine dinucleotide

NADPH – nicotinamide adenine dinucleotide phosphate

$NH_4^+$ – ammoniac

$NO_2$ – nitrite

$NO_3$ – nitrate

$Q_n$ – nitrate specific uptake rate

$Q_p$ – phosphate specific uptake rate

$R^2$ – coefficient de determination

RuBisCO - Ribulose-1,5-Biphosphate Carboxylase Oxygenase

T – time

$T_d$ – doubling time

X – Biomass

$X_{max}$ – maximal biomass

$Y_{x/n}$ – yield of biomass in relation to the nitrate

$Y_{x/p}$ – yield of biomass in relation to the phosphate

# CONTENTS

# INTRODUCTION

Microalgae became an interesting resource in many fields of industry, particularly for food industry due to the increasing world population and the consequently increase in need of food. Biomass composition with high quantities of protein and 'healthy' polyunsaturated fatty acids gives algal cells a better nutritional value than rice or soybean (Spolaore et al., 2006). Microalgae are therefore not only used for human food supplement but also in animal nutrition (e. g. in aquaculture). Other applications are in cosmetics or in biofuel production (Spolaore et al., 2006).

The second part of this project deals with blue mussels. The blue mussel or *Mytilus edulis* is an edible bivalve used predominantly for diet but which can also be used for its shell and found in the recipe of some cosmetics.
In 2009, the mussel represented 1.6 tons on a total of 20.1 million tons of products produced worldwide in marine aquacultures. With an average annual growth rate of production of 3.7% (from 2000 to 2008) and a market's weight of 13.3 billion dollars (Food and Agriculture organisation, 2010), the cultivation of mussels represents therefore a very attractive and profitable area.
In Denmark, the production increased from 11 tons in 2003 to 2 643 tons (Danish Directorate of Fisheries) in 2009 (Food and Agriculture organisation, 2010) and more than 800 people were employed in production in 2008 while the number of licenses delivered was increasing with a worldwide request (Dewan and Roth, 2010).

Studies of bioenergetics and growth behavior of blue mussel are therefore interesting in a way to find new aquaculture farming sides and to increase the productivity. In Denmark the MarBioShell project deals with the question about the potential of line-mussel production in the Great Belt, an area what was not used for aquaculture so far. The main interest is therefore the growth rate of *Mytilus edulis* at different algal concentrations in surroundings. In order to answer that question growth studies in laboratory need to be performed over a couple of weeks supplied by the planktonic algal strain *Rhodomonas salina*. For this purpose, however, a high quantity of algal biomass with a constant biochemical composition is needed and hardly to produce without improvement of different cultivation techniques available.

The report deals with the question about production of sufficient algal biomass for growth studies of *M. edulis* and is separated in following tasks:

1. Investigation of *R. salina* in flask culture.
2. Investigation of batch cultures grown in a photo-bioreactor
3. Continuous production of *R. salina* in a photo-bioreactor
4. Growth and bioenergetic study of the blue mussel (*M. edulis*)

# THEORY

## I) Mytilus edulis

The Blue mussel (*Mytilus edulis)*, is an edible marine bivalve of the Mitilidae family. The mussel has a wide distribution pattern and can be found in temperate and polar waters from the Europe to the north Pacific coast of America. This adaptability is allowed by the capacity of mussels to withstand fluctuations in temperature, salinity, oxygen tension and desiccation (Food and Agriculture Organisation, 2010).
Living settled at rocks in water swept areas, the mussel is an animal with a soft body protected by a calcareous shell. Mussels are filter-feeders enable to grow on phyto- and partly zooplankton in diluted environments (Riisgård et al., 2011)
Easily cultivatable, mussels grow attached to suspended ropes. If not already placed, mussel larvae can naturally fix themselves at ropes in spring time. In autumn, mussels are transferred to socks and are harvested when they reach a commercially size of 45-55 mm (Food and Agriculture Organisation, 2010).

## II) Algae

### 1. Commercially use

Algal pigments are used in natural dyes, or as coloring agents of food and feed. Algae also contain essential polyunsaturated fatty acids used as food supplements. They are also appreciated for their nutritive values in aquaculture. Furthermore they synthesize essential amino acids for humans and animals, polysaccharides like starch, lipids, and vitamins. For example, *Chroomonas*, *Cryptomonas* and *Rhodomonas* have been grown for decades because of their ability to produce proteins, vitamins, antibiotics, and others (Spolaore et al.).

### 2. Morphology of the Cryptophyceae

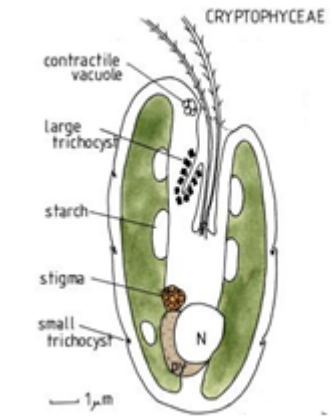


***Figure1.*** ***Morphology of Cryptophyceae (freshwaterlife.org).***

Cryptophyceae are a class of unicellular and mobile microorganism, which contain pigments. They are ovoid and own two flagella (Fig. 1). Crypthophyceae contain a contractile vacuole which

empties itself in a vestibular region. It enables the cell to remove waste and excess of water. The wall is invaginated in a belly furrow ending in a more or less deep canal; the cytopharynx which is used to ingest nutrients .The periplast contains thin plates made of proteins attached to the cell wall all around the cell. Trichocysts are cell organelles that release long filamentous proteins that capture predators in 'nets' to slow them down when the cell is disturbed. These proteins are nontoxic. They are located at the angles of periplast plates. The periplast compartment is separated by four walls, two belonging to the chloroplast and two belonging to the endoplasmic reticulum. There are also two chloroplasts, several pyrenoids, and a nucleomorphe. Pyrenoids can fix the carbon dioxide and form starch grains and the nucleomorph contains the cell's genom. Starch is a carbohydrate consisting of glucose units linked by a ß-1.4 glycosidic bound, and used as an energy source if carbon is limited. (Manguin) (Prescott et al., 2003)

The pigments are located in the chloroplasts. There are chlorophyll a, chlorophyll c2, one type of phycobilisome (depending on the species), and carotenoids like alloxantin, beta-caroten, monadoxanthin, or crocoxanthin.

## 3. Species studied

According to the taxonomic classification of the living beings, the algae studied have been identified as *Rhodomonas salina* by Hill and Wetherbee in 1989:

***Table 1.* Classification of *Rhodomonas salina*.**

| | |
|---|---|
| domain | *Eucaryote* |
| kingdom | *Chromalveolata* |
| phylum | *Cryptophyte* |
| class | *Cryptophyceae* |
| order | *Pyrenomonadales* |
| family | *Pyrenomonodaceae* |
| genus | *Rhodomonas* |
| species | *salina* |

*Rhodomonas salina* is a species common in brackish and marine waters. Like the others *Cryptophyceae*, it is a photosynthetic unicellular algal strain (Fig. 2).

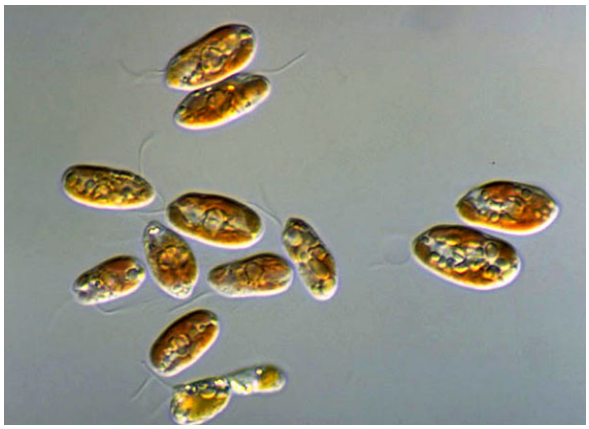

***Figure 1.* Photography of *Rhodomonas salina* observed under a microscope (cfb.unh.edu).**

## 4. Growth curve of Rhodomonas salina

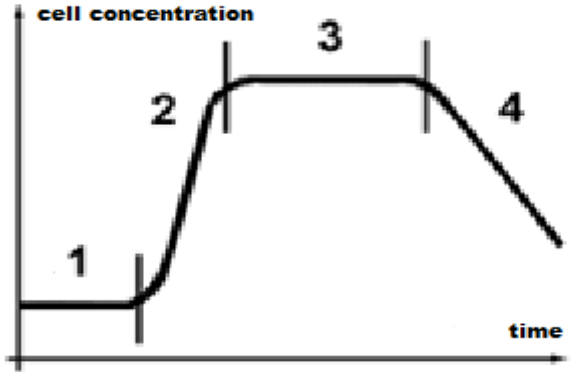


***Figure 3.*** ***General growth stages of a microorganism in a batch culture (phem.fr)***

**a) Lag phase (1):**

During lag phase cells adapt themselves to the physical and chemical conditions of the medium. They are like yellowish when previously nitrogen limited (Eriksen and Iversen, 1995a).

**b) Exponential phase (2):**

During exponential growth phase cells divide as fastest by asexual reproduction (Fig. 4). For the asexual reproduction *R. salina* encysts and losses its flagella and becomes non-motile. Cells appear red due to the phycoerythrin what dominates chlorophyll and takes up the blue-green light and emit red-orange light.

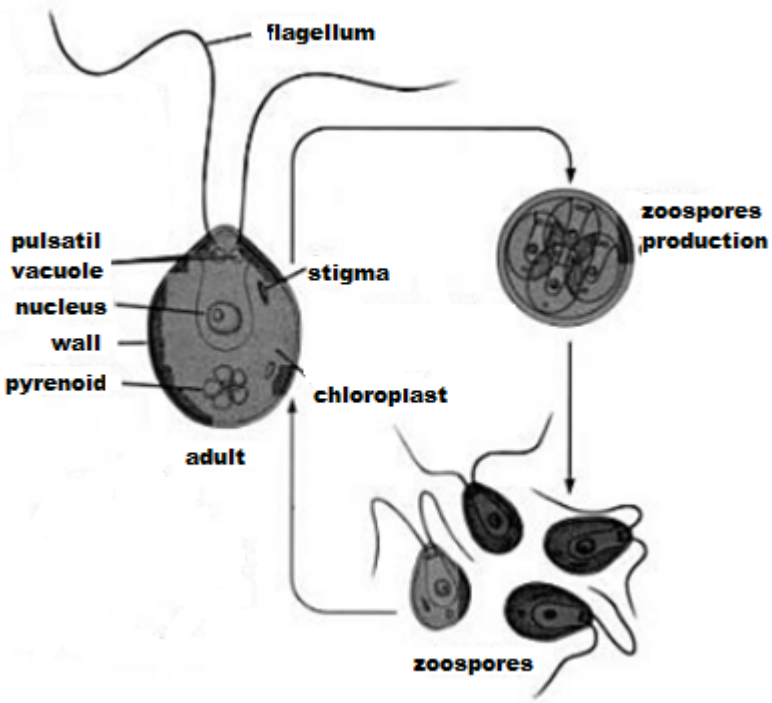


***Figure 4.*** ***Asexual reproduction (Prescott et al., 2003)***

**c) Stationary phase (3):**

The substrates like nitrate, phosphate, light and vitamins may become limiting and no further cell cycle can be performed. The cell concentration is constant. Moreover, *R. salina* may use phycoerythrin as nitrogen source and it changes the color to green, resulted by chlorophyll. (Eriksen and Iversen, 1995a)

**d) Death phase (4):**

With no addition of new substrates the cells dies along time and the population decrease. After the use of all the phycoerythrin (Eriksen and Iversen, 1995a), and chlorophyll as nitrogen sources, the carotenoids become visible and the cells appear yellowish.

## 5. Energy sources

### a) The photosynthesis

The photosynthesis is a process separated in two reactions (Mangin), the light and the dark reaction.

The light reaction (Fig. 5) based on photochemical reactions enabling the cell to absorb the light energy by photosynthetic pigments, and transform it into chemical energy in form of ATP. The photosynthetic pigments chlorophyll a and b, phycoerythrin and the carotenoids (e.g. beta-caroten), and alloxanthin are responsible for light absorption. The absorbed energy is used to excite electrons and to generate an electromotoric force (ATP synthase) for ATP synthesis (Fig. 5).

Two photosystems are present to transform the sun light into chemical energy. The photosystem I (PS I) absorbs light at high wavelengths and photosystem II (PS II) absorbs light at lower wavelengths and the absorbed energy used to excite electrons obtained from water.

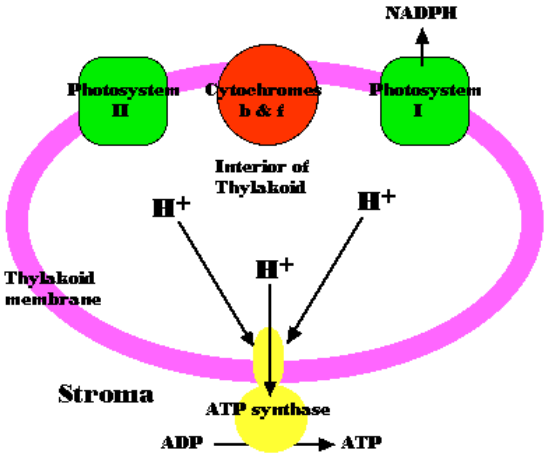


***Figure 5.* Photosynthesis process (Simmonet, 2001-2002)**

### b) The Calvin cycle

The Calvin cycle (Fig. 6) is a process, happening in chloroplast of photosynthetic organisms, using chemical energy (ATP and NADPH, nicotinamide adenine dinucleotide phosphate) obtained from light energy, to transform carbon dioxide into organic compounds. During the Calvin cycle (dark reaction) organic molecules are synthesized by

fixation of $CO_2$. RUBISCO (Ribulose-1,5-biphosphate carboxylase oxygenase) the most important enzyme on earth catalyses thereby the fixation of $CO_2$ (Curtis and Barnes, 1989). RUBISCO, however, can also bind to oxygen what inhibits the fixation of CO2. Therefore O2 has to be removed from the reactor by diffusion through a silicone tube.

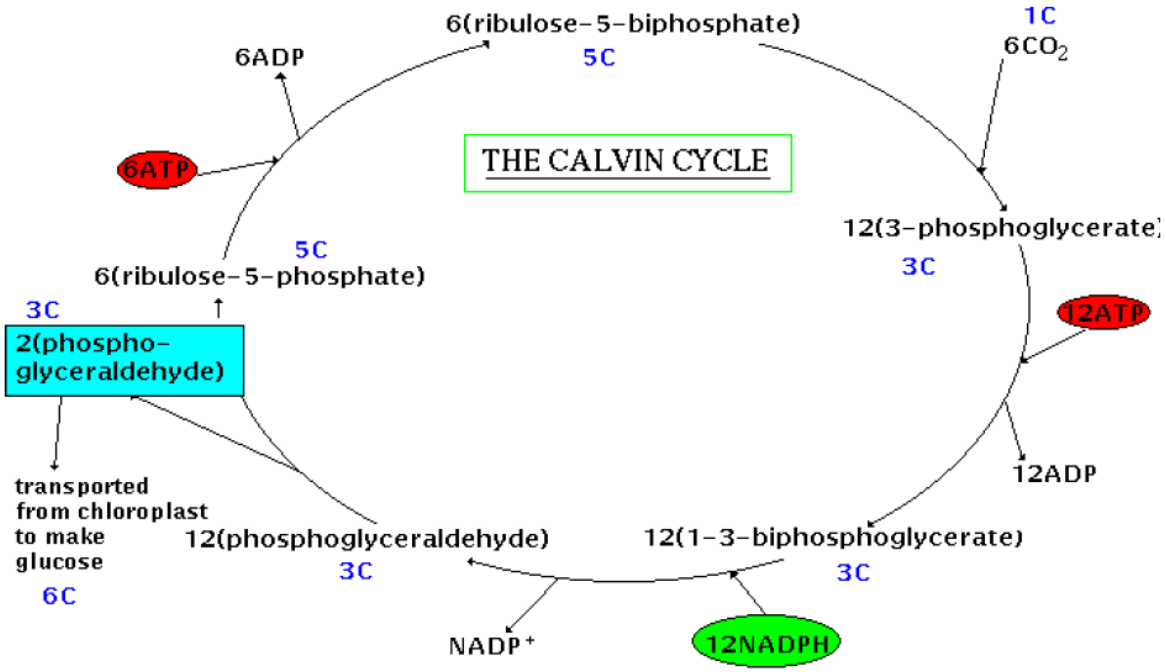


***Figure 6.*** ***Calvin cycle***

**c) Nitrogen metabolism**

Autotrophic organism incorporates nitrate, nitrite, or ammonium to cover the demand of nitrogen essential for amino acid synthesis and for fabrication of other molecules.

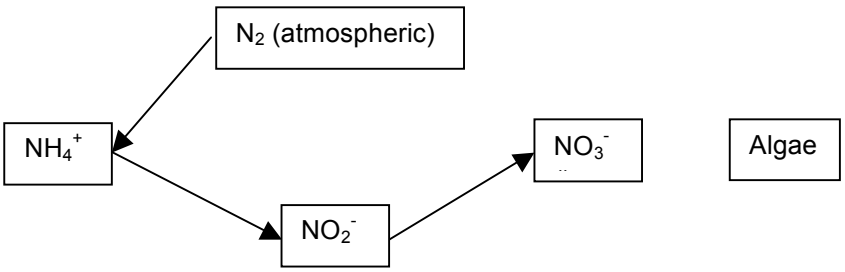


***Figure 2.*** ***Nitrogen cycle in sea water***

Aerobic bacteria are mainly responsible for the transformation of ammonium to the different nitrogen species (nitrate and nitrite) (Figs. 7 and 8). Ammonium can be released from organic nitrogen compounds and taken up by algae. Ammonium can also undergo nitrification (e.g by nitrobacter) and is subsequently oxidized to nitrite and nitrate. Both of which can be taken by algae and used as nitrogen sources (Darmangeat, 2005).

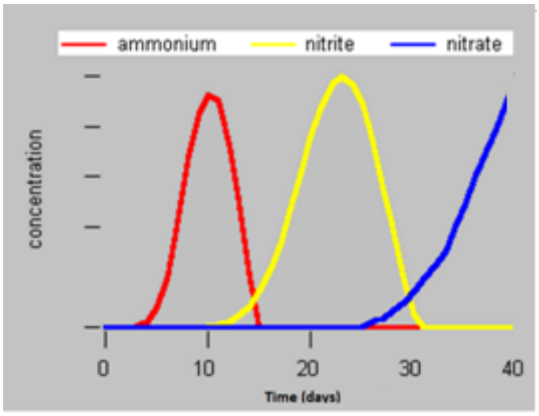


***Figure 8.*** ***Change of the different nitrogen sources in time (mars.reefkeepers.net)***

### III) Batch culture

A batch culture is a discontinuous culture, sterilized and inoculated by the microorganism wanted. It enables its growth until nutrients become limited. The medium has to contain at least one energy, carbon, nitrogen and mineral ions source (scribd.com).
The bioreactor need to be well mixed, the temperature and pH has to be well regulated because the metabolic reactions can change the pH of the medium or release heat (Eriksen et al., 2007).
In that kind of culture, growth, product formation, uptake of substrates may change the environment continuously.

**Advantages:**

- There is no microorganism lost during the culture
- Samples of biomass and products can be taken every time
- There are only few risks of contamination

**Disadvantages:**

- Limitation of cell growth by accumulation of waste products
- The presence of a lag phase lowers productivity
- The exponential phase is not maintained
- It need a long preparation time
- It is difficult to sterilize large volumes

### IV) Continuous flow culture

In order to start the bioenergetic experiment with *Mytilus edulis*, we need a significant amount of biomass with a stable biochemical composition over time. To reach this goal, a continuous production is the best solution. Therefore a batch culture will be changed to a continuous culture (Fogg and Thake, 1987).

The chemostat is carried out as well-mixed bioreactor where fresh medium containing nutrients is added continuously while the same amount of culture liquid is removed to keep a stable environment. The volume and the nutrients concentrations are approximately constant. (Fogg and Thake, 1987).

The continuous culture provides a better productivity, control of growth and substrate concentration by dilution rate, and avoids a stationary phase.

**Advantages:**

- Constant parameters make the continuous culture useful for research
- The culture conditions can be controlled by modifying the dilution rate
- High productivity

**Disadvantages:**

Contaminations are easier and require sterile conditions.

**Culture conditions of the *Rhodomonas salina*:**

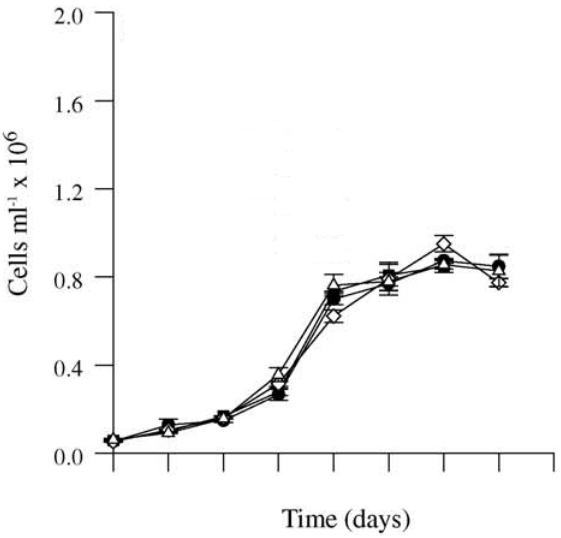


***Figure 9. Growth of R. salina with initial concentration of $NaNO_3$ and $NaH_2PO_4$ respectively of 621 and 29 uM in four different light conditions: 52 ( △), 68 (◇), 103 (■) and 142 ( ●) µmol photons $m^{-2}$ $s^{-1}$. (Lafarga-De la Cruz et al., 2006)***

According to several publications, the optimal growth conditions of *Rhodomonas salina* are at 20°C and pH 8 to 8.2, and continuous illumination (Hammer and al, 2002 ; Lafarga-De la Cruz and al, 2006).

Experiments performed by Hammer et al. (2002) showed that irradiances between 10 and 150 µmol photons $m^{-2}$ $s^{-1}$ didn´t affect the growth rate and maximal biomass of *R. salina*.
The $CO_2$ is an efficient acidifying agent and easy to use in order to regulate the pH. (Eriksen et al., 1998). Three different kinds of nitrogen sources nitrate, nitrite, and ammonium can be used.
According to Lafarga-De la Cruz et al. (2006), the optimal growth conditions to start a continuous culture were up to the fourth day of growth. From initial concentration of 5.64 $10^4$ cells $mL^{-1}$, 1.53 $10^6$ cells $mL^{-1}$ were obtained at a growth rate of 0.68 $day^{-1}$ at the best conditions of light and nutrients concentration reported.
Nitrate and phosphate were used as nutrients. Studies of their uptake by Lafarga-De la Cruz et al (2006) resulted in yields of biomass of 1.7 g $g^{-1}$ for nitrate and 26 g $g^{-1}$ for phosphate. Specific uptake rates were 0.37 g $g^{-1}$ $day^{-1}$ and 0.025 g $g^{-1}$ $day^{-1}$.

# MATERIELS AND METHODS

## I) Rhodomonas salina

The strain of *Rhodomonas salina* used was obtained from the University of Southern Denmark (SDU) and cultivated at the Marine Biological Research Centre Kerteminde (SDU) in illuminated flask cultures. Medium recipe listed in Appendix 1.

## II) Flasks cultures

In order to see the impact of different parameters on growth of *Rhodomonas salina*, experiments were performed in flask cultures, under different conditions of light, substrate concentrations, and pH's. Flasks were filled with 2L of artificial sea water (20 g $L^{-1}$, Marine Sea Salt, TETRA) and were autoclaved for 1 h at 80°C using a STRUERS autoclave. After autoclavation, two different volumes (2 or 6 mL) of two different main solution were added (one solution with precipitated salts called 'precipitated solution' while the other, non-precipitated, was called 'normal solution', both contained nutrients required for growth of *R. salina*) along with 1 mL of a vitamin solution (compositions are listed in appendix).

The first experiment was performed in duplicate. Four flasks with 2mL and 6mL of 'precipitated' and 'normal' solution, respectively, were placed under light limited condition (Fjord Laboratory, 19 $\mu mol.sec^{-1}.m^{-2}$) whereas the 4 others were placed under good light condition (Marine Station, 40 $\mu mol.sec^{-1}.m^{-2}$).

The second experiment was performed using five flasks adjusted to different pH's. The flasks contained 2L of artificial sea water (20 g $L^{-1}$), and 2mL of the 'normal' nutrient solution and 0,5mL of the vitamin solution were added to each flask after autoclavation. The pH's of the flasks were adjusted to 5, 6, 7, 8, 9 using 6M $H_2SO_4$ and 4M NaOH and were then placed under good light condition (Marine Station).

All flasks were inoculated with 10 mL of *R. salina*. $CO_2$ was used as carbon source, the temperature was approximately 20°C and a mechanical agitation was provided by a stirrer. Samples were taken and analyzed every day.

## III) Photo-bioreactor

The photo-bioreactor used for the experiments is a 3.0.1 Applikon BTS05 coming from Microlab Aarhus (Eriksen et al., 2007). The detailed scheme of the bioreactor is represented below. It consists of a glass cylinder (2L) placed between two plastic plates joined with silicon rubber. The bioreactor is placed in a box and illuminated by 6 fluorescence tubes placed on 3 of the 4 sides (Phillips TLD 18W/33). Since the lamps heat up the culture medium, a cooling system is necessary to maintain the temperature in the bioreactor at 20°C. Cold water is pumped (Sirai Bussero) (c.) from a cooling system (Reciprotor Haake K10 and C10) (d.) to a cooling finger (b.) placed inside the bioreactor. The cold water also cools a condenser (e.) fixed on the head-plate to prevent evaporation. The condenser is linked to an overflow bottle (f.) by a 10 meters silicon tube to eliminate the produced oxygen by diffusion. Since the gas circulates through a closed loop, it is important to eliminate the $O_2$ what is in competition with carbon dioxide for the fixation at RuBisCO. The overflow bottle saves the air pump (Hargraves) (g.) to what it is linked in case of a problem. $C0_2$, coming from a 50 liters bottle at 60 bars (l.), is mixed with recycled air before the pump (g.). $C0_2$is dosed by a controlled solenoid valve (Sirai)

(m.) whose opening is regulated by a pH and temperature controller Combicontrol PIC432B (k.). The controller is connected to a pH electrode (i.) and a thermometer (j.) immersed in the medium, and a computer for data analysis. In order to keep the pH between 8 and 8.2, the controller opens the solenoid valve when it is too high. The temperature inside the bioreactor is kept around 20°C. The air and the $CO_2$ are pumped into the asperging system (h.) which delivers big bubbles for mixing the culture medium (Eriksen et al., 1998). However, there is a small space between the asperging tube (h.) and the bottom of the bioreactor that is not mix efficiently, what makes it necessary to stir manually using a magnetic stirrer (o.).

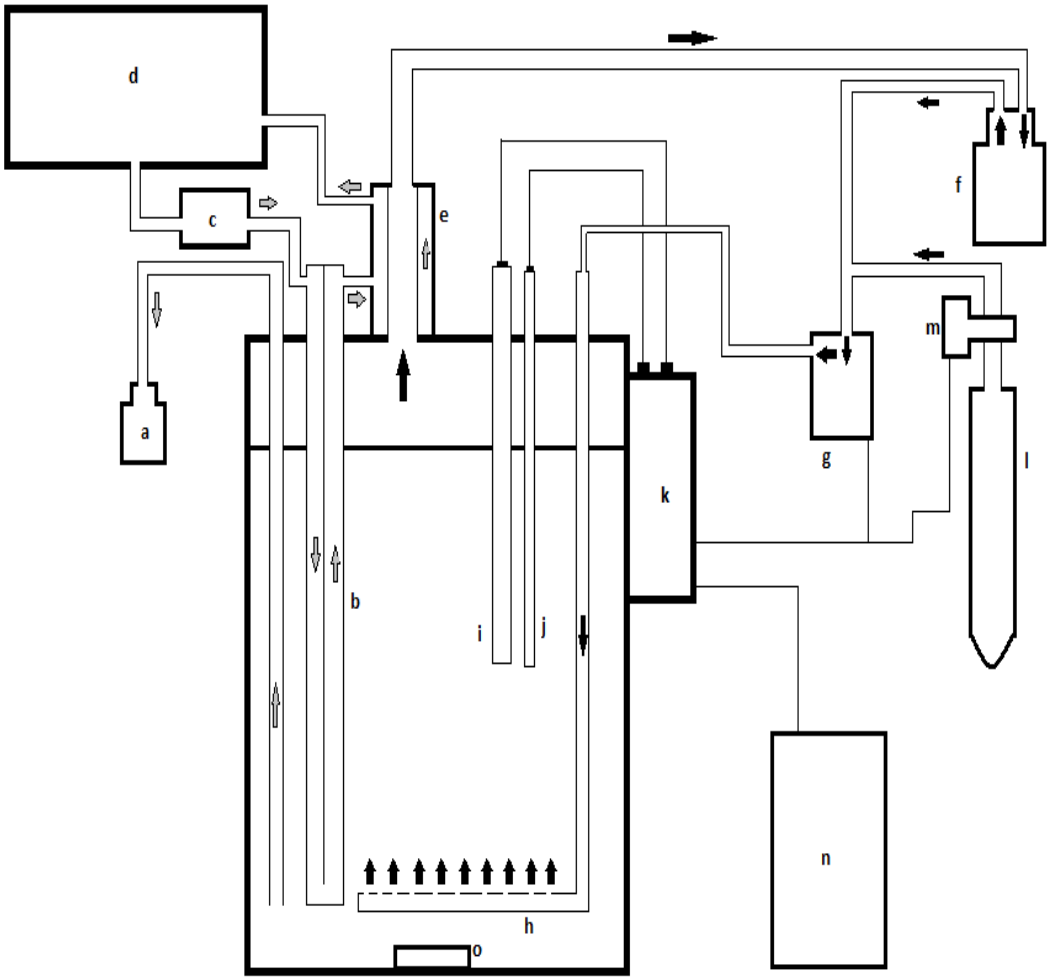


***Figure 10**. Scheme of the photo-bioreactor in batch culture*

**a.** sample bottle
**b.** cooling finger
**c.** water pump
**d.** cold water
**e.** condenser
**f.** overflow bottle
**g.** air pump
**h.** perforated sparger
**i.** pH electrode
**j.** thermometer
**k.** pH and temperature controller
**l.** $CO_2$ bottle
**m.** solenoid valve
**n.** computer
**o.** stirrer
gas flow
liquid flow

## IV) Photo-bioreactor cultures

The goal of this experiment is to obtain the maximal productivity of *R. salina* in a continuous flow culture in a photo-bioreactor. A batch culture, however, has to be performed prior to get all values needed for a continuous flow culture (the final concentration of biomass, growth rate, the doubling time and the yield of biomass).

### 1. Batch culture:

The first experiment was started by dilution of a previous batch culture of *R. salina*. To 0.5L of a previous batch, 1.5L of artificial sea water (20g $L^{-1}$) containing 2,5mL of 'normal solution' and 1mL of 'vitamin solution' was added. Growth and uptake of nutrients were followed over one week.

The second experiment was started by dilution of the batch culture used in the first experiment. The same method and volumes are used.

For the third experiment, the same method is applied except that the bioreactor is previously cleaned and autoclaved and then inoculated with about 2L of a *R. salina* solution previously prepared in a flask. The solution is the one prepared during the flask experiments on pH variations after a week of growth.

### *2.* Continuous culture:

After the cell concentration reached approximately $6.5x10^6$ cells $mL^{-1}$ the batch culture was changed to a continuous flow culture. Dilution was carried out using autoclaved seawater (20 g $L^{-1}$) containing sufficient nutrients to produce $1x10^7$ cells $mL^{-1}$ (approximately 87 mg of nitrate, 43 mg of phosphate, and 5 mL of vitamin solution).

The flow rate of fresh medium (Q) to the bioreactor represents the dilution rate D ($day^{-1}$) calculated using (Eq.1).

For steady state, D equals μ, what means that the quantity of biomass produced each day equals the quantity removed.

To avoid a wash out and because cells are limited in light, the dilution rate chosen (0.25 $day^{-1}$) was lower than the growth rate (0.27 $day^{-1}$) but was increased later because the growth rate was underestimated and the cell concentration was increasing.

Dilution rate was calculated by Eq. **(1)**:

$$D = \frac{Q}{V} \qquad \textbf{(1)}$$

Where D: dilution rate ($day^{-1}$)
Q: the flow rate (mL $day^{-1}$)
V: volume of the bioreactor (mL).

An initial flow rate of fresh medium of approximately 500 mL $day^{-1}$ corresponding to a dilution rate 0.26 $day^{-1}$ was used. The dilution rate was adjusted during cultivation to keep a steady state.

The specific uptake rate (q) of the culture was calculated with the following formula **(2)**:

$$q = [((C_R+(S_0-S_t))*D)/((X_0+X_t)/2) \qquad \textbf{(2)}$$

Where q = specific uptake rate for a given substrate ($g_{nutrient}\ g_{biomass}^{-1}\ day^{-1}$)
$C_R$ = substrate concentration of the flow ($mg\ L^{-1}$)
$S_X$ = substrate concentration at time x ($mg\ L^{-1}$)
D = dilution factor
$X_X$ = biomass concentration at time x ($cells\ mL^{-1}$)

The yield of biomass of substrate was then calculated with this formula **(3)**:

$$Y = D / q \qquad \textbf{(3)}$$

Where Y = biomass yield ($g_{biomass}\ g_{substrate}^{-1}$)
D = dilution factor
q = specific uptake rate ($g_{nutrient}\ g_{biomass}^{-1}\ day^{-1}$)

## V) Analysis of samples

**Photo-bioreactor:** Samples of approximately 10mL of the algae solution were taken twice a day, one in the morning and another in the afternoon for enumeration of cell number, pH measures, quantification of nitrate and phosphate.

**Flask cultures:** The same analyses were made once a day.

The pH was measured using a pH meter (PHM92, Radiometer Copenhagen) and the cell concentration enumerations were carried out by a Bürker-Türk counting chamber in duplicate and a microscope (Alphaphot YS, NIKON, zoom x400) according to the equation **(4)**:

$$N = \frac{number\ of\ cells * dilution\ factor}{number\ of\ square\ counted * volume\ of\ a\ square} \qquad \textbf{(4)}$$

With N: cell concentration (cells/µL).

First, to enumerate the non-moving cells, a fresh sample and second, to enumerate all cells a sample fixed by lugol, was counted. 5 µL of lugol were added per sample, lugol kills the cells without destroying it. Additionally, to follow the change in morphology, differences in cell color by counting the number of 'red' and 'green' cells were investigated and the percentage of red and green cells of each sample were calculated.

## VI) Phosphate and nitrate analysis

A sample of 5 mL was taken and centrifuged (Centrifuge 5416, EPPENDORF) for 10 min at 900 rcf to separate the medium from the biomass. The supernatant was stored frozen until analysis of nitrate and phosphate.

Nitrate was quantified by measures of the absorbance at 220 nm using a spectrophotometer (UVIKON 922, KONTRON instruments) and plastic micro-cuvettes and according to the method used by Collos et al. (1999). Correlation between absorbance and concentration was done by a calibration curve using $NaNO_3$ (0-500 $mg\ L^{-1}$, Fig. 1 Appendix 2).

Phosphate was quantified using the method described by Chen et al. (1956) based on the ammonium molybdate and the ascorbic acid method. Correlation between absorbance at 820 nm and concentration was done by a calibration curve using $NaH_2PO_4$ (0-400 $mg\ L^{-1}$, Fig. 2 Appendix 2).

## VII) Growth parameters

Several parameters were obtained from batch and the flasks cultures such as the growth rate and the doubling time. Growth rate was obtained by plotting the natural logarithm of cell numbers against time and using a linear trend line. The slope of the trend line corresponded to the growth rate ($\mu$) in days$^{-1}$.

The following formula **(5)** was used to determine the doubling time ($T_d$):

$$Td = \frac{\ln(2)}{\mu} \qquad \textbf{(5)}$$

Where μ: growth rate (days$^{-1}$)
$T_d$: doubling time (days).

The doubling time corresponds to the time needed to double the cell concentration.

Yields of biomass for substrates were obtained by using the equation **(6)** and **(7)**:

$$Yx/s = \frac{X - X0}{S0 - S} \qquad \textbf{(6)} \quad \text{with } Y_{x/s} \text{ in g g}^{-1}$$

$$Yx/s \text{ (g mol}^{-1}) = Yx/s \text{ (g g}^{-1}) * M(substrate) \qquad \textbf{(7)}$$

Where X: biomass concentration (mg L$^{-1}$),
$X_0$: initial biomass concentration (mg L$^{-1}$),
S: substrate concentration (mg L$^{-1}$),
$S_0$: initial substrate concentration (mg L$^{-1}$),
M: molar mass (g mol$^{-1}$),
$Y_{x/s}$: biomass yield (g g$^{-1}$)

The specific nutrient up-take was calculated with this formula **(8)**:

$$q = \frac{\mu}{Yx/s} \qquad \textbf{(8)} \quad \text{with } Y_{x/s} \text{ in g g}^{-1}$$

Where q: specific up-take rate (g.g$^{-1}$.day$^{-1}$).

## VIII) Mytilus edulis

The mussels used for the experiments were caught in the Kerteminde Bay and stored in aerated tanks at the Marine Biological Research Center Kerteminde. Approximately 45 mussels of 30.0 ± 2.0 mm shell length were used for the experiment and placed for 3 weeks prior the experiment in an aquarium under laboratory conditions. During this acclimatization period, the aquarium was supplied by fresh seawater.

To study the growth of *Mytilus edulis,* mussels were fed with a constant algal concentration of *Rhodomonas salina* obtained from a chemostat.

The cell concentration in the aquarium was adjusted to 8119 ± 3328 cells mL$^{-1}$ of *Rhodomonas salina* at the beginning of the experiment but we couldn't produce enough cells and the concentration dropped around 5000 cells mL$^{-1}$. Algal cell concentration was quantified continuously by measures of chlorophyll *a* using a fluorometer (TURNER DESIGNS, 10-AU Fluorometer). The aquarium used for the experiment was supplied with water obtained from other aquarium containing mussels, acting as a

bio-filter and removing all organic matter so that the experimental mussels only filtrate what was supplied in form of *Rhodomonas salina*. Temperature was maintained at 13,3±1,0□C by a cooling system.

Cell concentration was also measured in duplicate using a particle counter (Micromeritics, Elzone 5380). Additionally, twice a day, temperature, salinity, and clearance rate were measured.

In our case, the clearance rate (volume of water cleared of suspended particles per unit of time) (Riisgard, 2001) was representative of the filtration rate (pumping rate = volume flow rate) because the filtration efficiency was 100% (particles with a diameter superior to 4 µm are all filtrated by gills). The filtration rate was calculated by equation **(9)** from Kiørboe & Møhlenberg (1981).

$$F = 0.0012L^{2.14} \qquad \textbf{(9)}$$

Where F = filtration rate (L $h^{-1}$)
L = length of the shell (mm)

The equation where F is a function of the weight (Møhlenberg & Riisgård, 1979) was also used **(10)**.

$$F = FR = 7.45W^{0.66} \qquad \textbf{(10)}$$

Where FR = filtration rate (L $h^{-1}$)
W = weight (g)

The energy balance, used to determinate the estimated growth rate ($G_{est}$), is based on the energy ingested and the energy used for respiration, excretion and growth:

$$-I = -R - G - E$$

Where I = energy obtained from the ingested particles
R = energy used for respiration
G = energy used for growth
E = energy lost by excretion

The estimated growth rate ($G_{est}$) which represents the amount of biomass gained over time, was calculated from the energy balance using equation **(11)**:

$$G_{est} = [(F_{est} * AE * C_{Rho} * E_{Rho}) - Rm]/1.12 \qquad \textbf{(11)}$$

Where $G_{est}$= estimated growth rate (mg $day^{-1}$)
$F_{est}$= filtration rate (mL $min^{-1}$)
AE= 80%,
$C_{rho}$= *Rhodomonas* concentration in the aquarium (cells $mL^{-1}$)
$E_{rho}$= 1.75 µJ $cell^{-1}$, energy content of *Rhodomonas salina*
$R_m$= Maintenance respiratory rate (mL $O_2$ $h^{-1}$)
1.12 is the energy cost for growth (12% of the growth rate)

The maintenance respiratory rate was given by the equation **(12)** from Hamburger (1983):

$$R_m = 0.475W^{0.663} \qquad \textbf{(12)}$$

Where $R_m$ = maintenance respiratory rate (mL $O_2$ $h^{-1}$)
W = dry weight (g)

Where 1 mL $O_2$ $h^{-1}$ is equal to 5522 µW and µW = µJ $s^{-1}$.

The actual growth rate ($G_{act}$) and the actual weight specific growth rate (µ) were calculated with the formula **(13)** and **(14)** from Hamburger (1983), respectively:

$$G_{act} = \Delta W/\Delta t \quad \textbf{(13)}$$

Where $G_{act}$ = actual growth rate (mg $day^{-1}$)
$\Delta W$ = weight difference between $t_0$ and t (mg)
$\Delta t$ = duration of the experiment (days)

$$\mu = [\ln(W_t/W_0)/t]*100 \quad \textbf{(14)}$$

Where µ = actual weight specific growth rate, in (% $day^{-1}$)
$W_t$ = dry weight at time t (g)
$W_0$ = dry weight at time t (g)

The condition index (CI, mg $cm^{-3}$) was expressed as $CI = W/L^3$ (Riisgård, 2001) where W is the dry weight in mg and L the shell length in cm.

The dry weight was obtained after drying the mussels over night in an oven at 90°C until the weight was constant.

The same measurements of dry weight and condition index were performed on a group of mussels obtained from the MarBioShell research mussel farm in Kerteminde Bay so that the growth rate of mussels grown in Kerteminde Bay can be compared to those obtained from laboratory experiments. These mussels were separated in two different size groups (20 mm and 30 mm) and were hung up for 3 weeks in net bags in Kerteminde Bay to study the growth rate of different sized mussels.

The clearance experiments were performed on mussels from the mussel farm starved half a year in the Marine Biological Research Center of Kerteminde.

For each experiment, a control group of mussel was taken and stored in the freezer.

See Appendix 3 for details on the system.

# RESULTS AND DISCUSSION

## I) Investigation of *Rhodomonas salina* in flask cultures

### 1. Nutrient and light variation experiment.

### a) Growth study at Fjord Lab

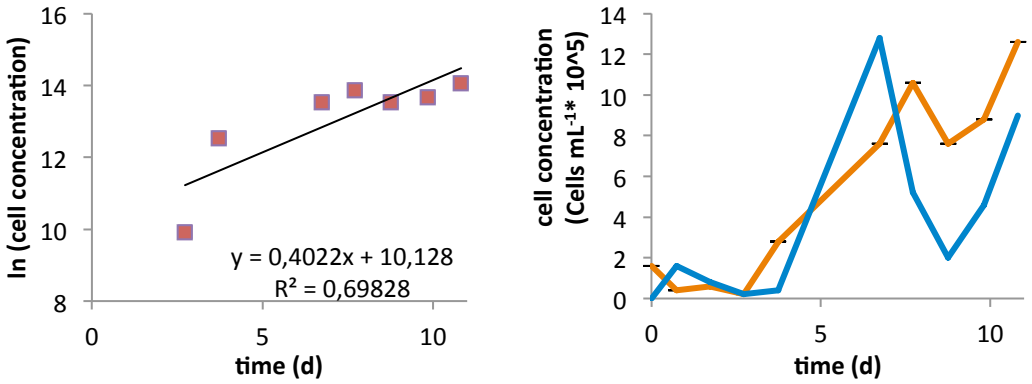


***Figure 11.*** *Flask with 6 mL of the precipitated nutrient solution. _ non moving cells with lugol, _ moving cells without lugol*

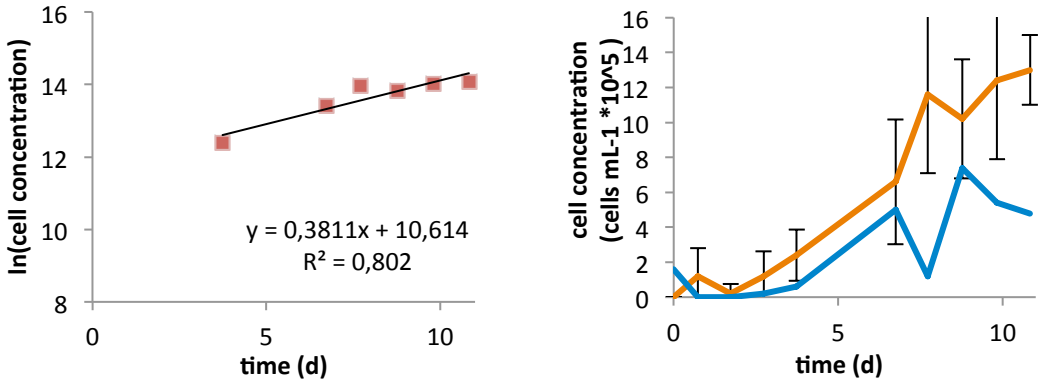


***Figure 12.*** *Flask with 2 mL of the precipitated nutrient solution. _ non moving cells with lugol, _ moving cells without lugol*

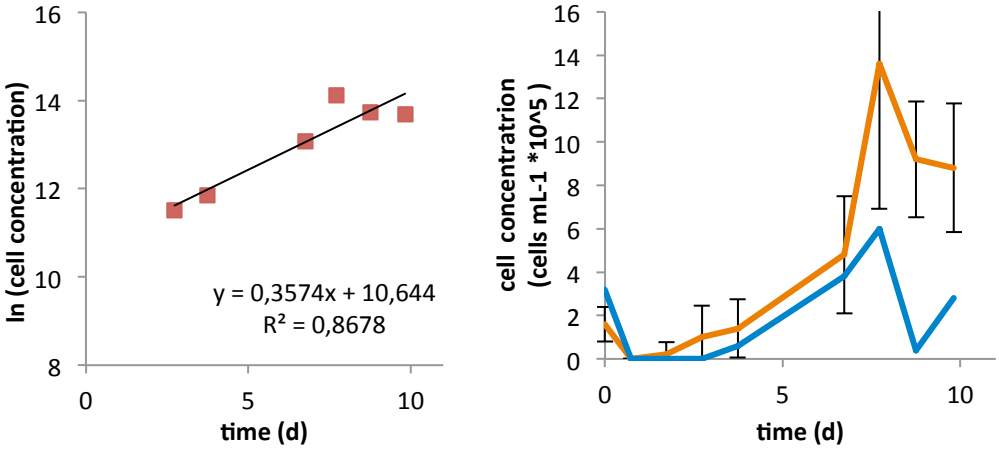


***Figure 13.*** *Flask with 6 mL of the clean nutrient solution. _ non moving cells with lugol, _ moving cells without lugol*

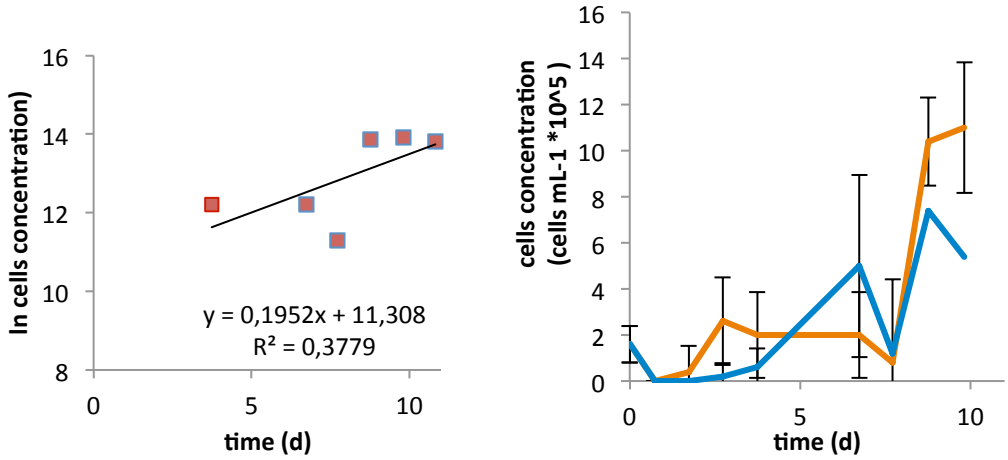


***Figure 14.*** *Flask with 2 mL of the clean nutrient solution. _ non moving cells with lugol, _ moving cells without lugol*

***Table 2.*** *Growth rate (μ), doubling time ($t_d$) , and maximal biomass concentration ($X_{max}$) of Rhodomonas salina grown in flask cultures at Fjord Lab.*

| | 6mL clean solution | 2mL clean solution | 2mL precipitated solution | 6 mL precipitated solution |
|---|---|---|---|---|
| **μ (day$^{-1}$)** | 0.36 | 0.19 | 0.38 | 0.40 |
| **$t_d$ (day)** | 1.9 | 3.6 | 1.8 | 1.7 |
| **Xmax (cell mL$^{-1}$)** | 1360000 | 1100000 | 1240000 | 1280000 |

The cultures with 2 mL nutrient solution (Figs. 12 and 14) started exponential growth earlier than the cultures performed with 6 mL of nutrient solution (Figs. 11 and 13). This may mean that the cells need certain time to adapt to higher nitrate and phosphate concentration and therefore the cultures grew faster in presence of 2 mL nutrient solution. We have also to take in consideration that at Fjord Lab the light conditions are less favorable than at the Marine Station. Except for the flask with 6 mL of the clean main solution the pH increased to 9 at the end of the exponential phase, what is too high for a good growth.

### b) Growth study in the Marine Station

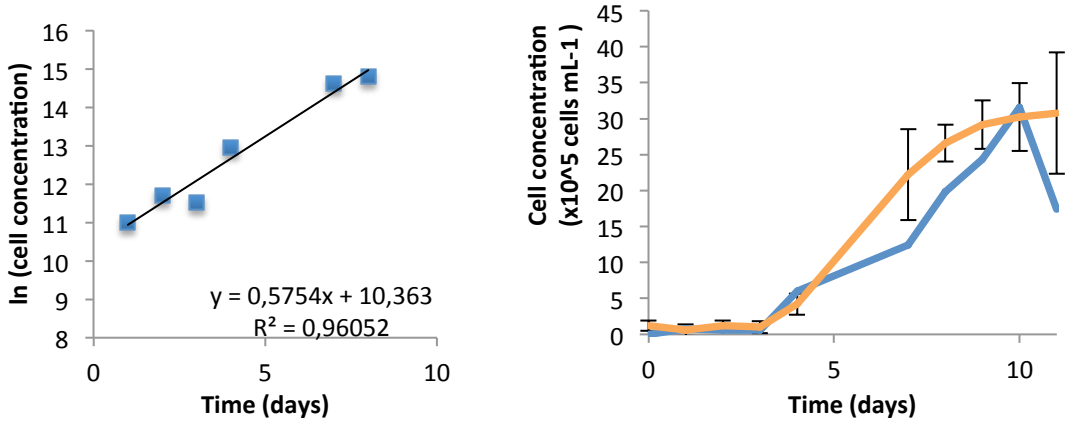


***Figure 15.*** *Flask with 6 mL of the precipitated nutrient solution. _ non moving cells with lugol, _ moving cells without lugol.*

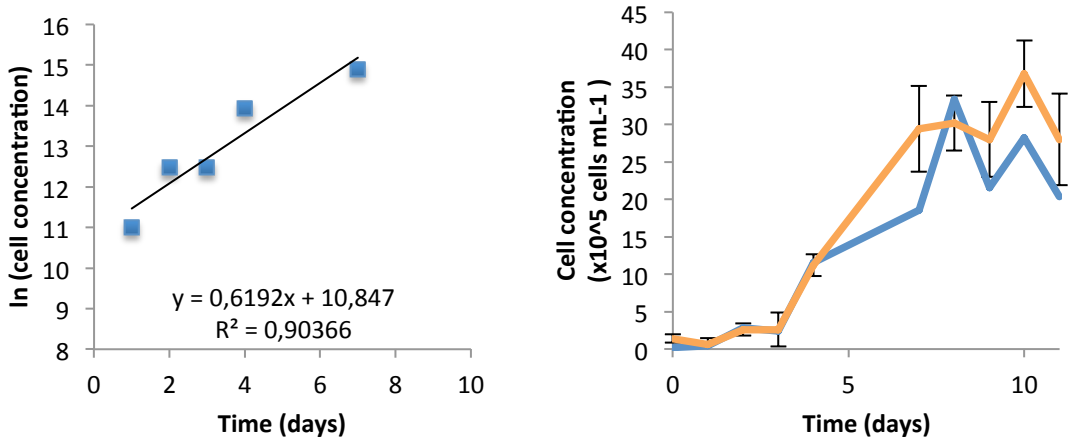


***Figure 16.*** *Flask with 2 mL of the precipitated nutrient solution _ non moving cells with lugol, _ moving cells without lugol.*

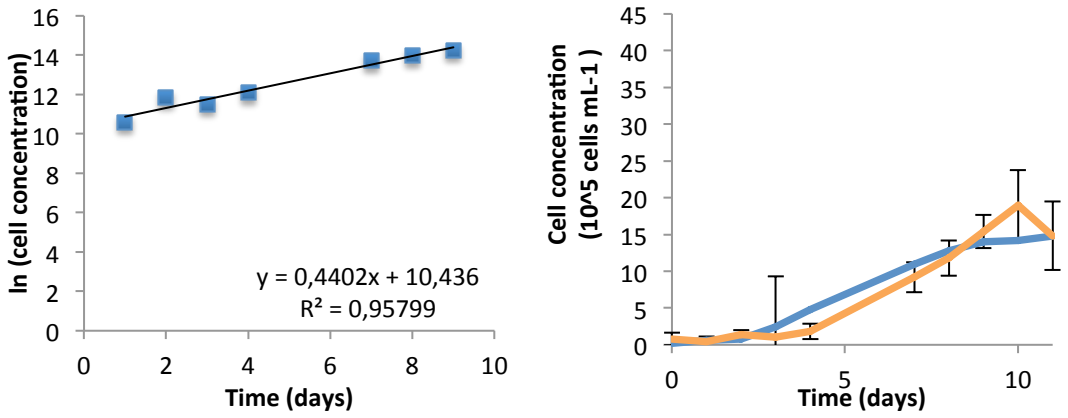


***Figure 17.*** *Flask with 6 mL of the clean nutrient solution _ non moving cells with lugol, _ moving cells without lugol.*

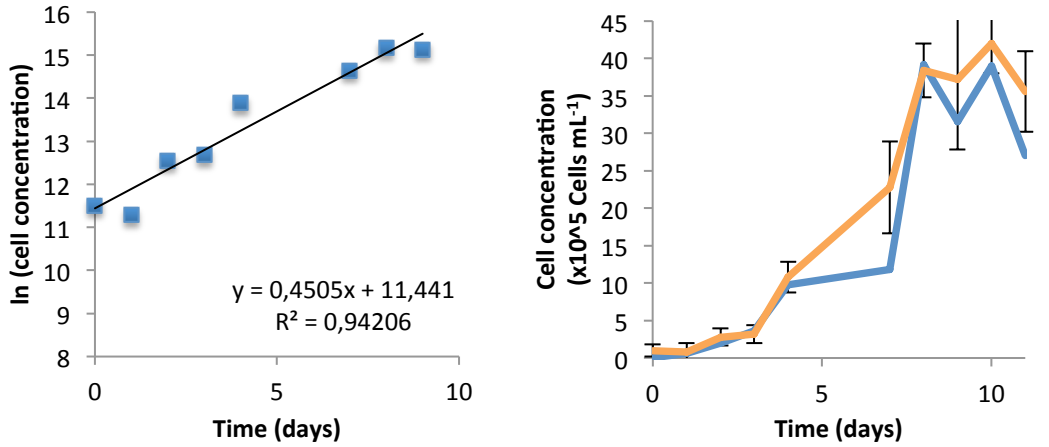


***Figure 18.*** *Flask with 2 mL of the clean nutrient solution _ non moving cells with lugol, _ moving cells without lugol.*

***Table 3.*** *Growth rate (μ), doubling time ($t_d$) , and maximal biomass concentration ($X_{max}$) of Rhodomonas salina grown in flask cultures at Marine Station.*

| | 6mL precipitated solution | 2mL precipitated solution | 2mL clean solution | 6 mL clean solution |
|---|---|---|---|---|
| **μ (day-1)** | 0.57 | 0.62 | 0.45 | 0.44 |
| **$t_d$ (day)** | 1.2 | 1.1 | 1.5 | 1.6 |
| **Xmax (cell mL$^{-1}$)** | 3080000 | 3680000 | 4200000 | 1900000 |

Relative to the flask culture grown at Fjord Lab higher cell concentration and growth rates were obtained in flask cultures grown at Marine Station. Best growth was obtained in flask cultures supplied with 2mL of nutrient solution. The clean one gave a higher maximal cell concentration and the precipitated one gave better doubling time and growth rate. To stress the cells sufficiently to see if and

how the second unknown species grow up, a flask with a low growth rate was used for the next experiment.

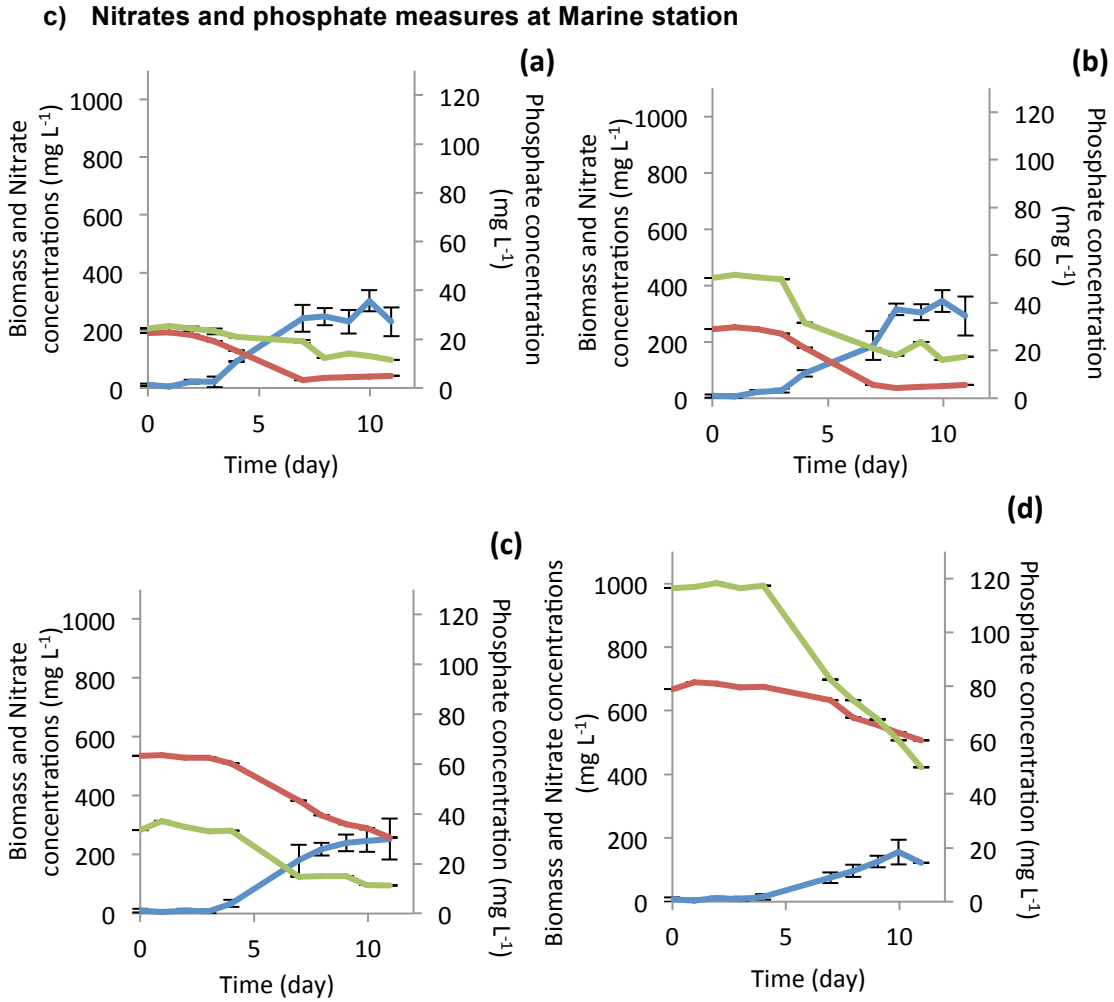


***Figure 19.*** ***Study of growth of Rhodomonas salina in flask cultures at Marine Station. (a) 2 mL of precipitated nutrient solution, (b) 2 mL of clean nutrient solution, (c) 6 mL of the precipitated nutrient solution, (d) 6 mL of clean nutrient solution.*** **_ nitrate curve, _ phosphate curve, _ biomass curve**

The phosphate and nitrate concentrations were equal during the lag phase in every flask culture. During exponential growth phase nutrients were taken up needed to enable the cells to divide. When the stationary phase was reached the substrates uptake stopped. Considering an interaction between the seawater and the analytical method for nitrate, we may assume that the flask cultures containing 2 mL of nutrient solution were nitrogen depleted. We can also see that the initial phosphate concentrations from the precipitated nutrient solutions are lower than the concentrations in the flask culture supplied with the clean solution. We can therefore suppose that the precipitation is made of phosphate.

***Table 4.*** ***Growth rate, doubling time, biomass yields of nitrate and phosphate, and uptake rates of the flask cultures grown at the Marine Station.***

| | 2mL of the precipitated solution | 2mL of the clean solution | 6 mL of the precipitated solution | 6 mL of the clean solution | Theory (Lafarga De-la-cruz, 2006) |
|---|---|---|---|---|---|
| **$\mu$ ($d^{-1}$)** | 0.62 | 0.45 | 0.57 | 0.44 | 0.68 |
| **$t_d$ (d)** | 1.12 | 1.54 | 1.21 | 1.58 | 1.01 |
| **$Y_{x/n}$ ($g\ g^{-1}$)** | 1.51 | 1.46 | 0.88 | 1.09 | 1.70 |
| **$Y_{x/n}$ ($g\ mol^{-1}$)** | 0.05 | 0.04 | 0.03 | 0.03 | 0.03 |
| **$q_n$ ($g\ g^{-1}\ d^{-1}$)** | 0.41 | 0.31 | 0.65 | 0.40 | 0.37 |
| **$Y_{x/p}$ ($g\ g^{-1}$)** | 19.50 | 9.48 | 10.97 | 2.64 | 26.00 |
| **$Y_{x/p}$ ($g\ mol^{-1}$)** | 0.21 | 0.10 | 0.12 | 0.03 | 0.27 |
| **$q_p$ ($g\ g^{-1}\ d^{-1}$ )** | 0.08 | 0.15 | 0.08 | 0.41 | 0.025 |

The flask culture supplied with 2 mL of the precipitated solution grew at almost the same rate as found by Lafarga De-la cruz. Biomass yields of nitrate and phosphate are also close to her values. However, the nitrate and phosphate specific uptake rates are better than Lafarga De-la-cruz values. The efficiency of the *Rhodomonas salina* to uptake nitrate and phosphate is better than what we expected for the 2 mL of precipitated nutrient solution. The yields stay the same as expected because even if the cell can uptake efficiently nutrients, they can't use more than a certain amount of it. We can suppose so that the cells used the maximum nutrient amount that they can with the nutrient solutions of 2mL, and it used less nutrients with the nutrient solutions with 6 mL because there is too much of it.

### d) Nitrate and phosphate measures at Fjord lab

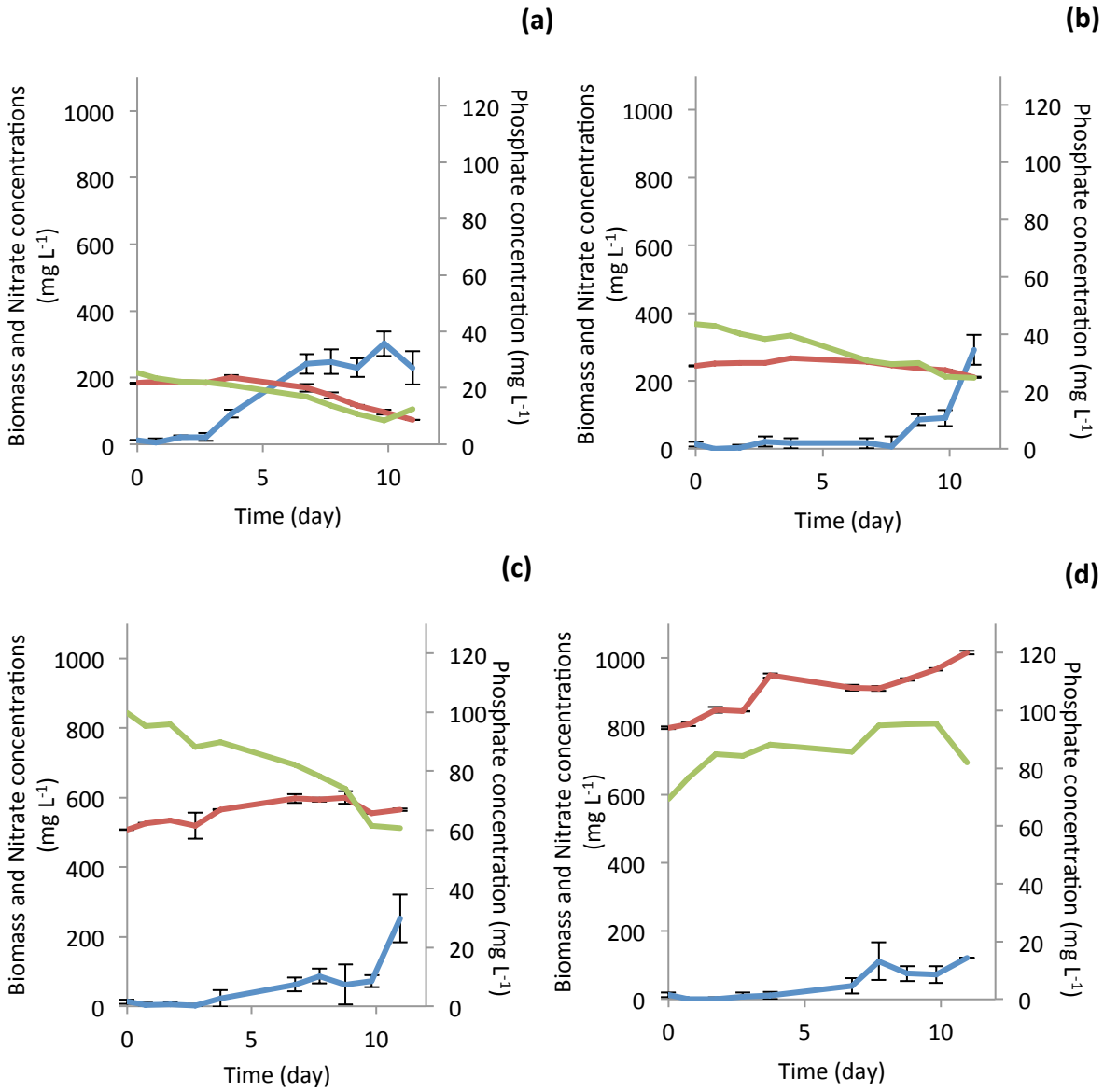


***Figure 20.*** ***Study of growth of Rhodomonas salina in flask cultures at Fjord Lab. (a) 2 mL of precipitated nutrient solution, (b) 2 mL of clean nutrient solution, (c) 6 mL of the precipitated nutrient solution, (d) 6 mL of clean nutrient solution.***

**_ nitrate curve, _ phosphate curve, _ biomass curve**

At the Fjord Lab the light limitation didn't enable the flasks to grow fast enough to see an uptake of nutrients. Indeed, we can see that the initial nutrient concentrations are constants. We can see in the flask cultures with 6mL of nutrient solution and the flask cultures with 2 mL of the clean nutrient solution that the exponential phase is just beginning at the end of cultivation. The only relevant results and calculations of specific uptake rates, yields and growth rates are about the flask with 2 mL of precipitated nutrient solution.

***Table 5.*** ***Yields of nutrients and kinetic parameters of the flasks from the Fjord lab.***

| | 2mL of the precipitated solution | 2mL of the clean solution | 6 mL of the precipitated solution | 6 mL of the clean solution | Theory (Lafarga De-la-cruz, 2006) |
|---|---|---|---|---|---|
| $\mu$ ($d^{-1}$) | 0.38 | 0.38 | 0.34 | 0.33 | 0.68 |
| $t_d$ (d) | 1.82 | 1.83 | 2.07 | 2.08 | 1.01 |
| $Y_{x/n}$ ($g\ g^{-1}$) | 2.08 | 8.16 | -4.21 | -0.49 | 1.70 |
| $Y_{x/n}$ ($g\ mol^{-1}$) | 0.06 | 0.25 | -0.13 | -0.01 | 0.03 |
| $q_n$ ($g\ g^{-1}\ d^{-1}$) | 0.18 | 0.05 | -0.08 | -0.68 | 0.37 |
| $Y_{x/p}$ ($g\ g^{-1}$) | 17.86 | 14.91 | 6.12 | -8.55 | 26.00 |
| $Y_{x/p}$ ($g\ mol^{-1}$) | 0.19 | 0.16 | 0.06 | -0.09 | 0.27 |
| $q_p$ ($g\ g^{-1}\ d^{-1}$) | 0.12 | 0.55 | -0.69 | 0.06 | 0.025 |

In Table 5 it is visible that the values obtained are lower than the values found by Lafarga De-la-cruz, the light condition in the Fjord Lab strongly affects phosphate and nitrate uptake and yields which are lower than in the other lab.

### e) Investigation of the effect of the pH on growth

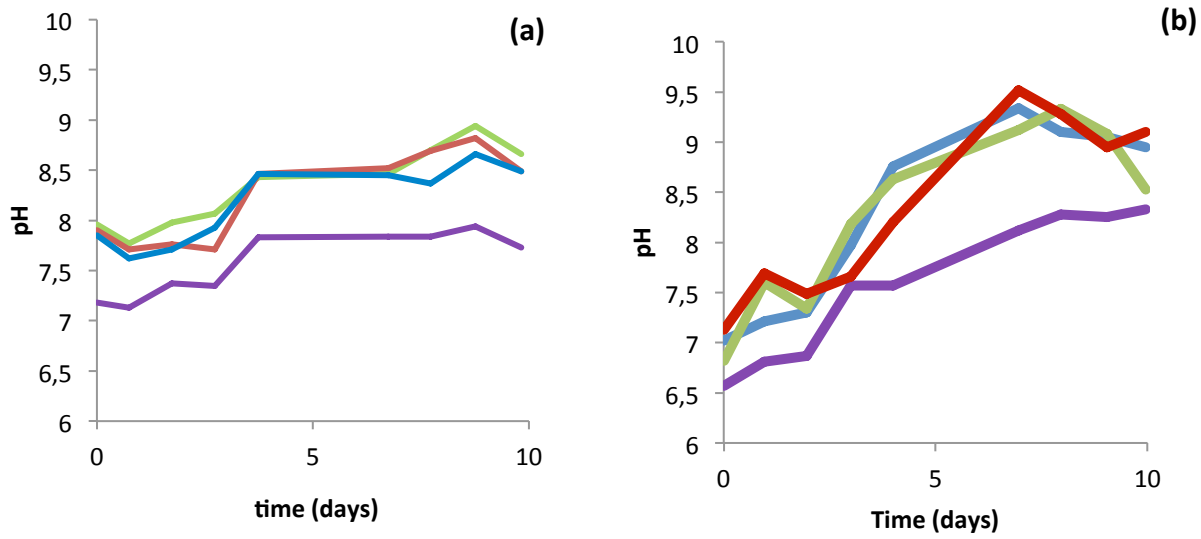


***Figure 21.*** ***PH variations, (a) Fjord Lab flask cultures. (b) Marine Station flask cultures. _ flask culture with 2 mL of precipitated nutrient solution, _ flask culture with 2 mL of clean nutrient solution, _ flask culture with 6 mL of precipitated nutrient solution, _ flask culture with 6 mL of clean nutrient solution.***

The pH increases in almost every flask culture. The only different is the flask with 6mL of the clean main solution what has a lower pH than the others.

When cells are consuming nitrate and phosphate, they catch a proton and the medium becomes more alkaline. That's why the pH increases in correlation to the growth phases of the algae. It may also mean that *Rhodomonas salina* in the flask culture with 6 mL of the precipitated nutrient solution is not as able as the other cultures to take up nutrients, probably due to the high concentration of nutrients, however, the flask culture with 6 mL of the clean nutrient solution seems not to be influenced by high

concentration of nutrients. It is also obvious that the 2 mL flask cultures are not influenced by concentrations of nutrients at all.

**f) Study of the cells mobility**

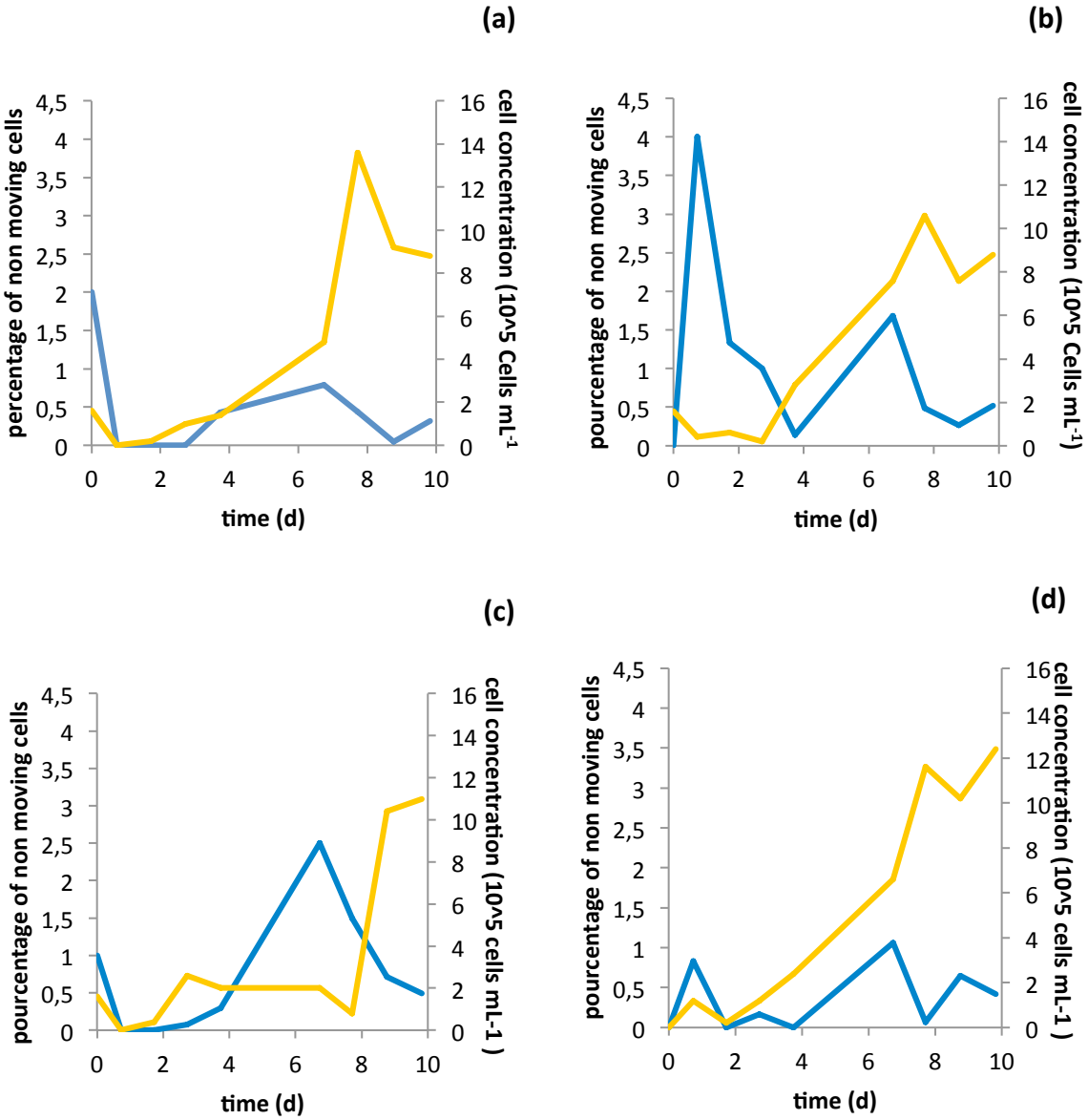


***Figure 22.*** ***Cells mobility of the flask cultures. (a) flask with 6 mL of the clean nutrient solution. (b) flask with 6mL of the precipitated nutrient solution (c) flask with 2 mL of the clean nutrient solution.(d) flask with 2 mL of the precipitated nutrient solution. _ growth curve, _ mobility curve***

The reason why we obtain percentages higher than 100% is that the cell numbers are approximated. Too low quantities of cells were counted to get precise results. Despite of that we can get the variation in mobility. Mobility of the cells is correlated to the different reproduction stages of the algae. First, algae stopped moving to synthesize enzymes responsible for the substrate uptake (lag phase), then move again, that's why we can see a peak of non moving cells around the first day of growth. At day four every cell has synthesized the compounds they need to grow, they are completely adapted to the medium. Since that day, cells started to stop moving because they are about to divide their selves and lose their flagella (encystment). At day seven almost every cells has stopped moving. Then they divided resulting in more and more mobile daughter cells. At the end of the exponential phase, almost

every cell was moving. Nitrate became limiting so they cannot divide anymore around the ninth day. It was noticed that the flask cultures with the precipitated nutrient solution needed more time to adapt itself to the medium than the flask cultures with the clean nutrient solution. Indeed, more cells stopped moving when 6 mL were used rather than 2 mL of nutrient solution.

## 2. Variation of the growth depending on the pH

### a) Growth study

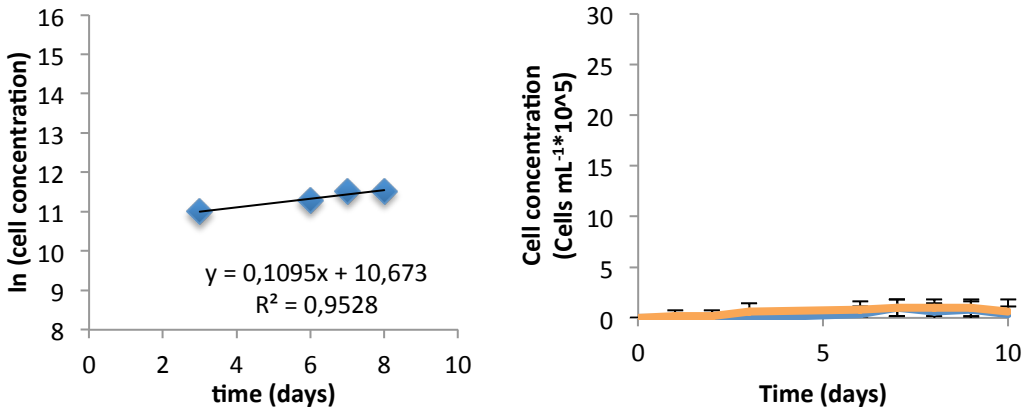


***Figure 23. Growth of the flask culture at pH 5. _ non moving cells with lugol, _ moving cells without lugol***

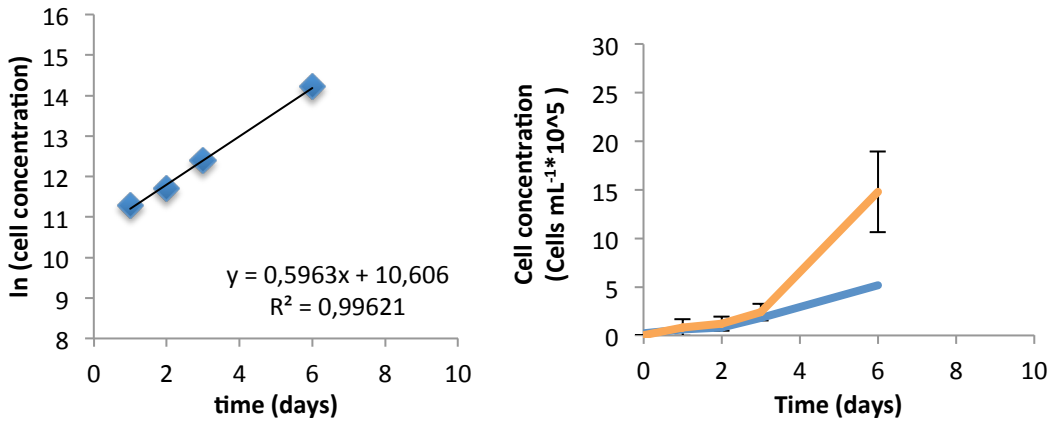


***Figure 24. Growth of the flask culture at pH 6. _ non moving cells with lugol, _ moving cells without lugol***

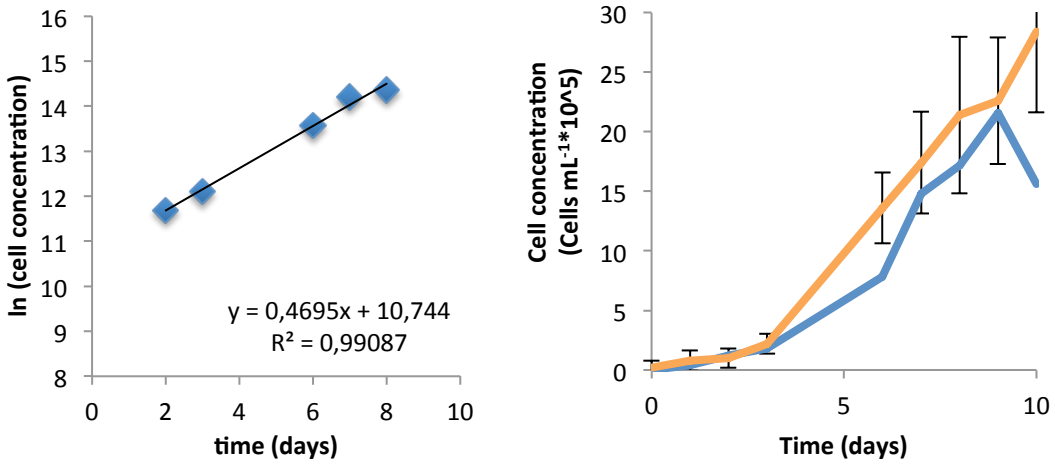


*Figure 25. Growth of the flask culture at pH 7. _ non moving cells with lugol, _ moving cells without lugol*

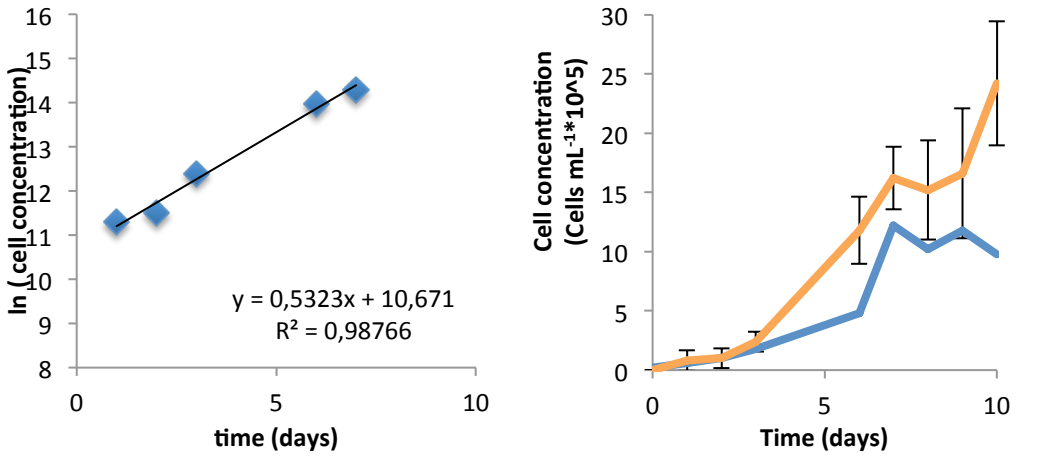


*Figure 26. Growth of the flask culture at pH 8. _ non moving cells with lugol, _ moving cells without lugol*

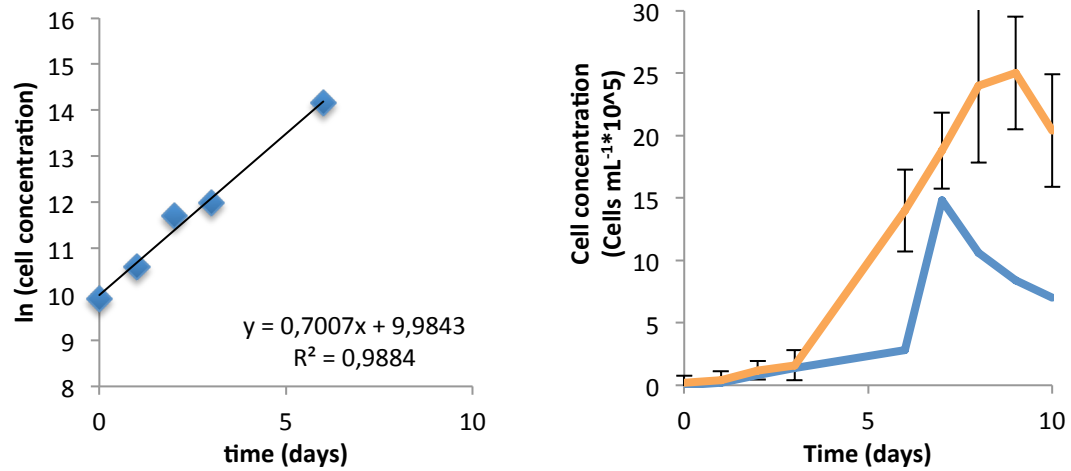


*Figure 27. Growth of the flask culture at pH 9. _ non moving cells with lugol, _ moving cells without lugol*

***Table 6.*** ***Kinetic parameters of the flask with different pH***

| initial pH of the flask cultures | 5 | 6 | 7 | 8 | 9 |
|---|---|---|---|---|---|
| μ (day-1) | 0.11 | 0.60 | 0.47 | 0.53 | 0.70 |
| $t_d$ (day) | 6.4 | 1.2 | 1.5 | 1.3 | 1 |
| Xmax (cell $mL^{-1}$) | 100000 | 1480000 | 2840000 | 2420000 | 2500000 |

The best growth rate has been obtained with an initial pH of 9 which actually grow at a pH between 7.5 and 9, while the others grow at a lower pH at the beginning and a higher pH at the end. Even if the light might be the limiting factor, the flask cultures started at a pH around 9 with 2 mL of the clean nutrient solution is the one which overtake the growth rate found by Lafarga De-la-cruz, and reached a maximal biomass concentration of 2500000 cells mL-1 .

There is no growth in the flask culture started at pH 5, the medium is too acid. The pH is still acid in the flask culture with pH 6 but *Rhodomonas salina* apparently grew well. The culture has been stopped before the end of the exponential phase because it was needed to inoculate the photo-bioreactor.

**b) Investigation of the effect of pH on growth**

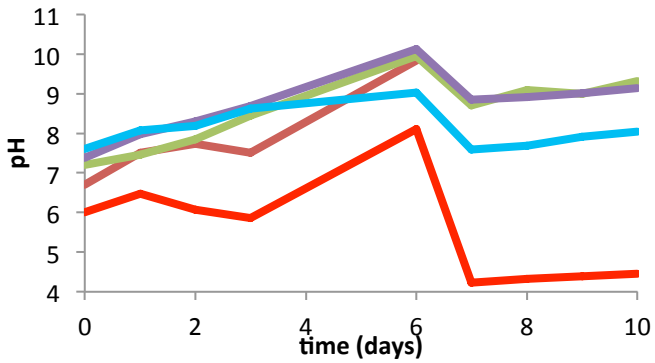


***Figure 28.*** ***PH variation of the flask cultures. _ flask at pH 5, _ flask at pH 6, _ flask at pH 7, _ flask at pH 8, _ flask at pH 9.***

The pH has been adjusted below the pH expected to prevent an increasing during the autoclavation of the medium. During autoclavation $CO_2$ was released from the medium and the pH subsequently increased. Even when the pH did not match the expected the difference between the flask cultures was still significant to see how *Rhodomonas salina* behaves at different pH conditions.

At day six, nutrients were added visible in Fig. 19 by an decrease in pH. The pH further increased the same way for each culture except for the flask started at pH 5. The flask started at pH 9 grew at best at the pH expected: around 8 – 8.2 as also found by Lafarga De-la-cruz.

**c) Study of the nutrient consumption**

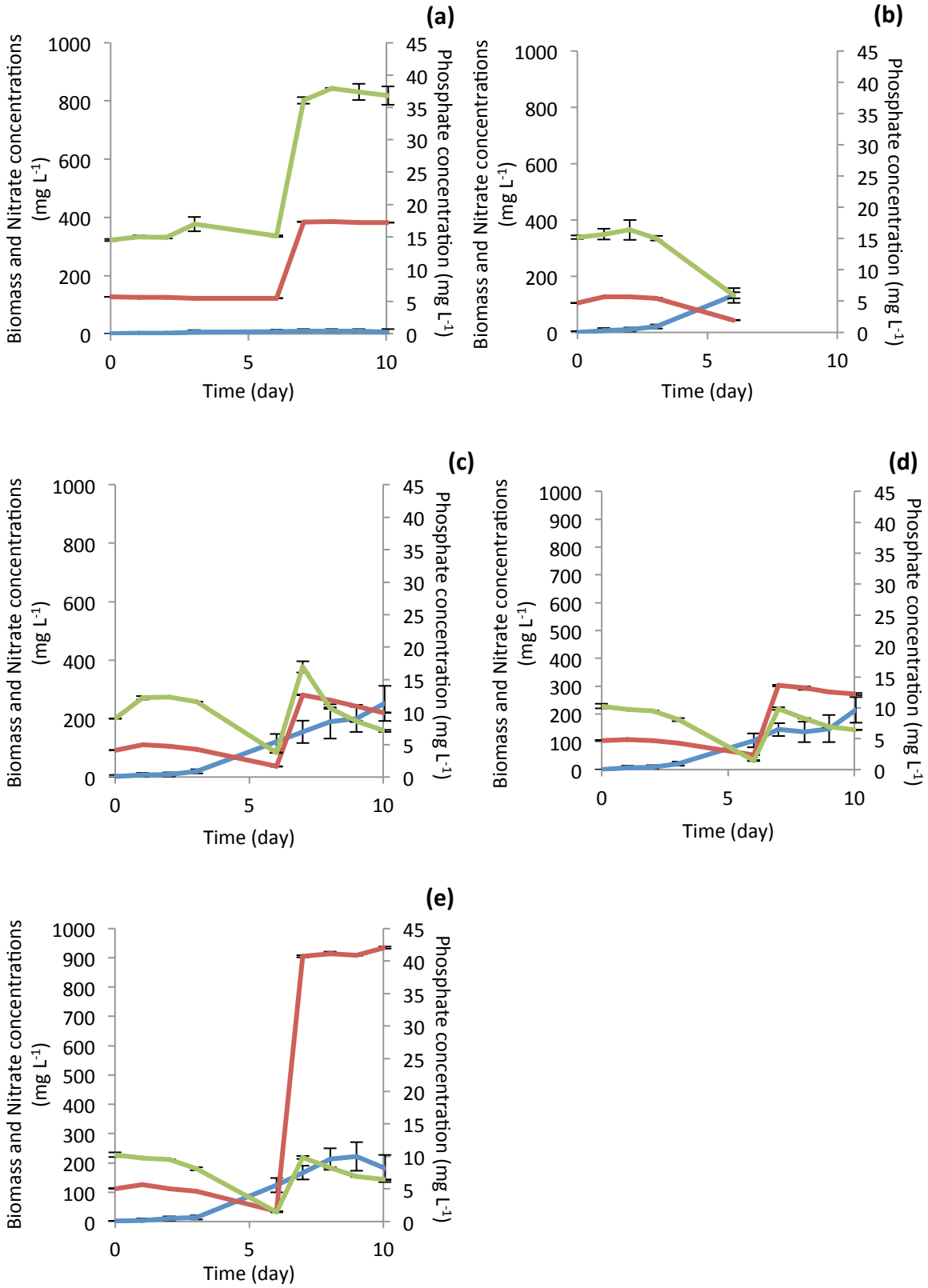


***Figure 29.*** ***Investigation of growth and nutrient uptake of Rhodomonas salina grown in flask cultures at different pH. (a) pH 5 flask. (b) pH 6 flask. (c) pH 7 flask. (d) pH 8 flask (e) pH 9 flask*** **. nitrate curve, _ phosphate curve, _ biomass curve.**

***Table 7.*** ***Kinetic parameters of the flasks cultures with different pH.***

| | pH5 | pH6 | pH7 | pH8 | pH9 | Theory (Lafarga De-la-cruz, 2006) |
|---|---|---|---|---|---|---|
| **$\mu$ ($d^{-1}$)** | 0.11 | 0.60 | 0.47 | 0.53 | 0.70 | 0.68 |
| **$t_d$ (d)** | 6.33 | 1.16 | 1.48 | 1.30 | 0.99 | 1.01 |
| **$Y_{x/n}$ ($g\ g^{-1}$)** | 1.56 | 2.12 | 2.14 | 2.00 | 1.58 | 1.70 |
| **$Y_{x/n}$ ($g\ mol^{-1}$)** | 0.05 | 0.03 | 0.03 | 0.03 | 0.025 | 0.03 |
| **$q_n$ ($g\ g^{-1}\ d^{-1}$)** | 0.07 | 0.28 | 0.22 | 0.27 | 0.44 | 0.37 |
| **$Y_{x/p}$ ($g\ g^{-1}$)** | -11.33 | 14.13 | 22.66 | 11.88 | 14.89 | 26.00 |
| **$Y_{x/p}$ ($g\ mol^{-1}$)** | -0.12 | 0.15 | 0.239 | 0.13 | 0.16 | 0.27 |
| **$q_p$ ($g\ g^{-1}\ d^{-1}$)** | -0.01 | 0.04 | 0.02 | 0.04 | 0.05 | 0.025 |

With no biomass of the flask 5, we can see the nutrient rates without the influence of *Rhodomonas salina*'s growth. The flask 9, has been overflowing with the flask, that's why when we added nutrients the concentration of nitrates is so high. The best results of nitrates yields have been obtained with the flasks of initial pH 6, 7 and 8.

We cannot use the results for the flask with the initial pH 5 because there is no biomass. In all the other flasks, we can see that the biomass nitrate yields are very high comparing to the theory. The nitrate give a good productivity of biomass. The phosphate doesn't, because the yields are low. The specific uptake rates show good capacities of uptake for the nitrates and very good ones for the phosphates.

We can also see on Fig 19 that when the pH exceed 9, or when it is below 8, the results of uptake rates and growth are lower

We can conclude that the *Rhodomonas salina*, in light limiting conditions and at a pH between 8 and 9, uptake easier phosphates than nitrates. We know that the phosphate is stored and used less that nitrate for the growth. Our results confirm thanks to the yields of biomass that the nitrates are responsible for the good productivity.

d) **Study of the cells mobility**

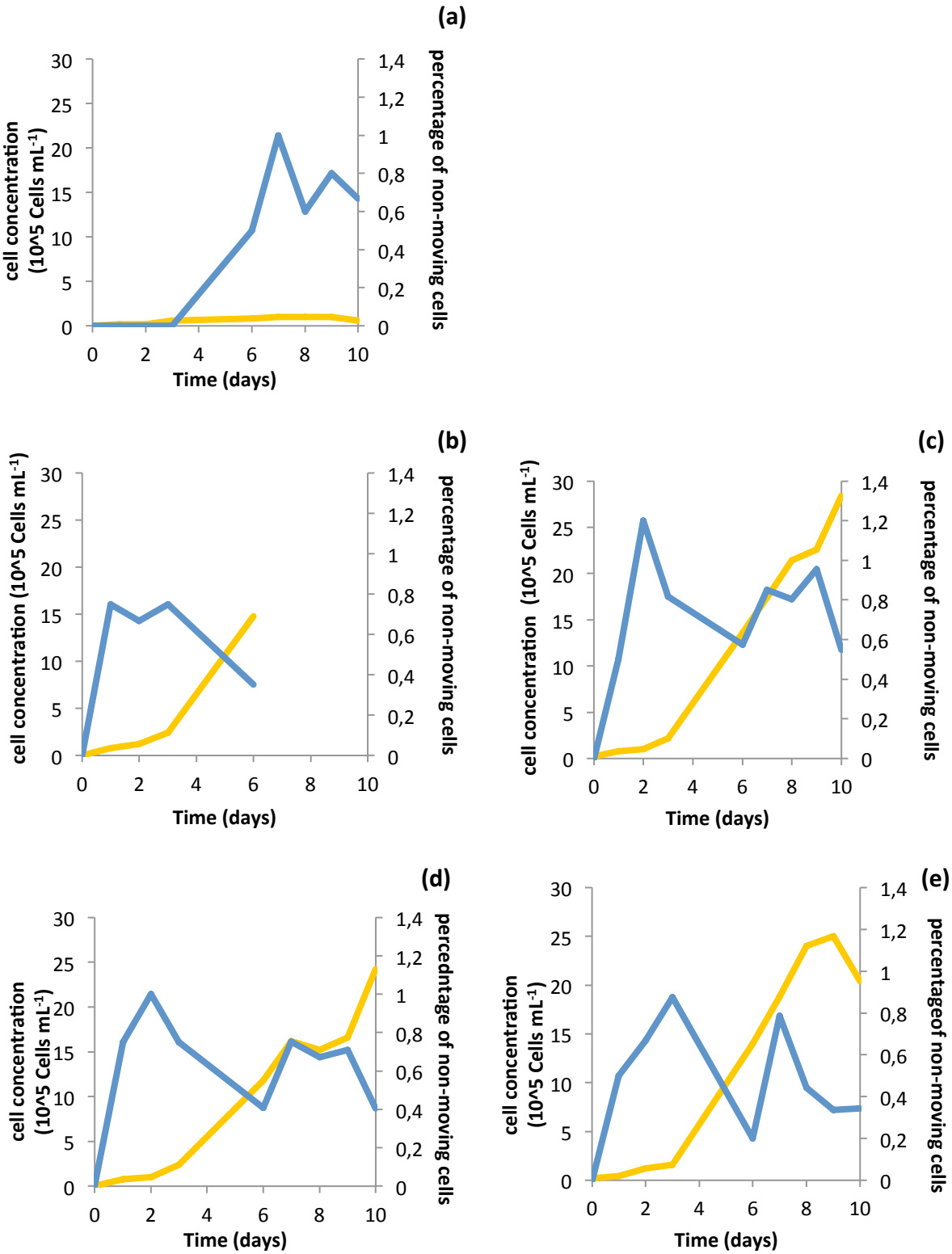


***Figure 30. Investigation of mobility of Rhodomonas salina in flask cultures. (a) pH 5 flask. (b) pH 6 flask. (c) pH 7 flask. (d) pH 8 flask (e) pH 9 flask. _ growth curve, _ mobility curve***

At first, some algae stop moving to adapt its metabolism to the medium, and then move again, that's why we can see a peak of non moving cells around the first days of growth. Around the second or third

day, cells finish to synthesize enzymes they need to uptake the substrates, etc… Then they start moving again. The sixth day nutrient has been added, and then it stops again moving to adapt itself to the new medium. Around the eighth day, cells stop moving progressively to divide; it is the beginning of the exponential phase. Afterwards, the daughter cells rate increasing make the percentage of non moving cells becoming lower.

The biomass of the flask at pH 5 is too low to have relevant data of mobility.

## II) Batch cultures

In order to see the uptake of nitrate and phosphate, and increase in algal cell concentration, *Rhodomonas salina* was grown in batch cultures carried out in a photo-bioreactor described in Material and Methods.

### 1. First experiment

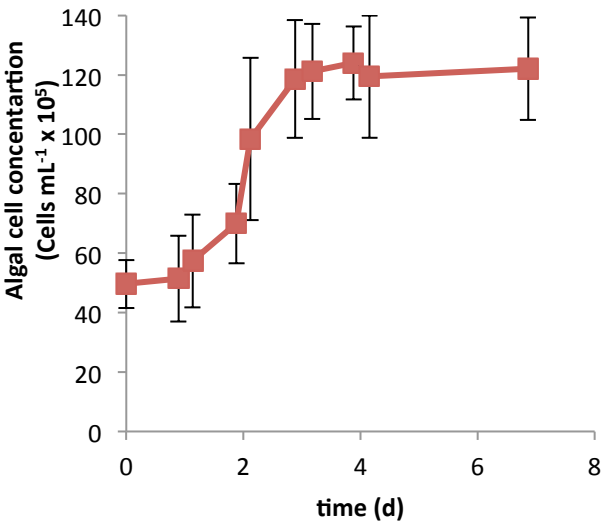


***Figure 31.** Increase in algal cell concentration of R. salina grown in a batch culture.*

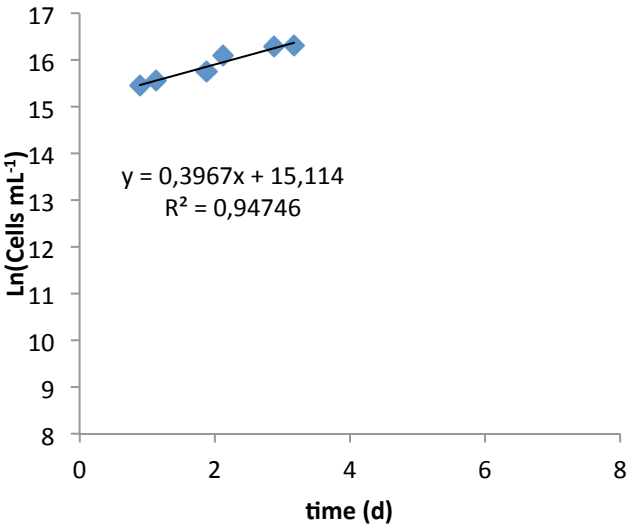


***Figure 32.** Natural logarithm of algal cell concentration between Days 1 and 3.*

Increase in algal cell concentration clearly showed the four growth phases (lag-, exponential growth-, stagnation-, and stationary phase) expected (Fig. 31). The exponential phase starts after Day 1 until Day 3. Natural logarithm of algal cell concentration is illustrated in Fig. 32. The growth rate determined as the slope of the regression line was 0.39 $day^{-1}$ corresponding to a doubling time of 43 h. The maximum biomass concentration reached was around $12*10^6$ $cells.mL^{-1}$ after 4 days of cultivation. The obtained growth rate is slower compared to 0.65 $day^{-1}$ found by Lafarga-De la Cruz et al. (2006) under best light conditions. The maximum concentration of $12*10^6$ $cells.mL^{-1}$ resulted in insufficient light supply what may be the reason for the stagnation of growth after day 4 (Hammer et al., 2002) when nitrate and phosphate were still present.

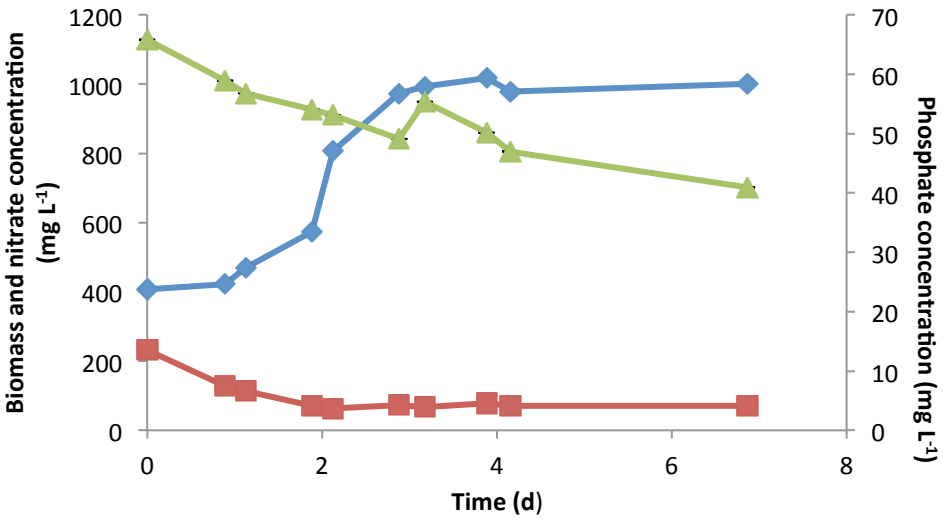


***Figure 33.** Increase in biomass concentration (calculated from the algal cell concentration) (◆), and decreases in nitrate (■), and phosphate (▲).*

Nitrate and phosphate concentrations decreased during the first 3 days from 230 to 70 $mg.mL^{-1}$ and from 70 to 50 $mg.mL^{-1}$, respectively. Lafarga-De la Cruz et al. (2006) found similar results and explained them by a direct correlation of the growth rate to irradiance level and nutrient concentration. Nitrate concentration was never depleted, but stationary phase is normally explained by lack of nutrients (Fogg and Thake, 1987). A high consumption of nutrients may be related, according to Mc Carthy (1981), to an increase in the activity of the enzymes responsible for uptake and assimilation.

Yields of biomass of nitrate and phosphate are respectively 3.72 and 23.89 $g_{biomass}.g_{nutrient}^{-1}$, what is in agreement to the results of Lafarga-De la Cruz et al. (2006) (1.7 and 25 $g_{biomass}.g_{nutrient}^{-1}$). Specific uptake rates of nitrate and phosphate are 0.09 and 0.16 $g_{nutrient}.g_{biomass}^{-1}.d^{-1}$, respectively. The difference between the results found by Lafarga-De la cruz et al. (2006)(0.4 and 0.025 $g_{nutrient}.g_{biomass}^{-1}.d^{-1}$) can be explained by the differences in growth conditions of the stock culture and different light conditons.

During the first week, almost no appearance of a second strain was noted and it was concluded to be a sign of good culture conditions for *R. salina*.

A second batch culture was performed at a lower initial cell concentration to obtain a faster growth rate without light limitation and realized by dilution of a previous batch culture.

## 2. Second experiment

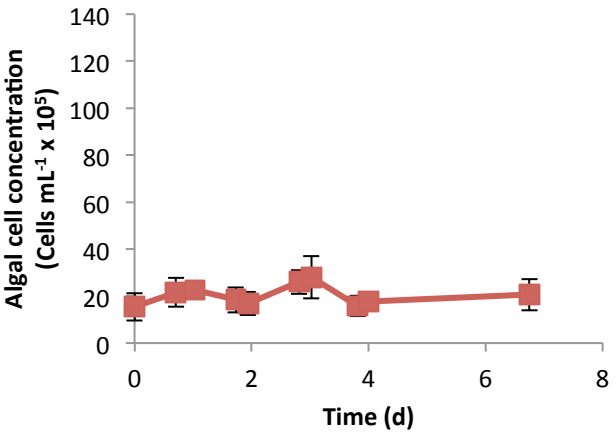


***Figure 34.*** *Increase in algal cell concentration of R. salina grown in a batch culture.*

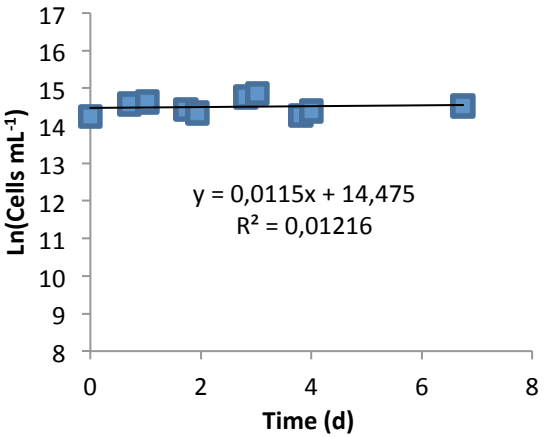


***Figure 35.*** *Natural logarithm of algal cell concentration.*

No growth of R. salina was observed during one week of cultivation (Figs. 34 and 35). The lack of growth may be a consequence of the 3 days of nutrient depletion the cells experienced until the new batch culture was started. When the new batch culture was started a second algal species likely to be present from the beginning in the stock culture of *R. salina* grew up shortly exponentially, visible as green cell with a different morphology than *R. salina* (Fig. 36).

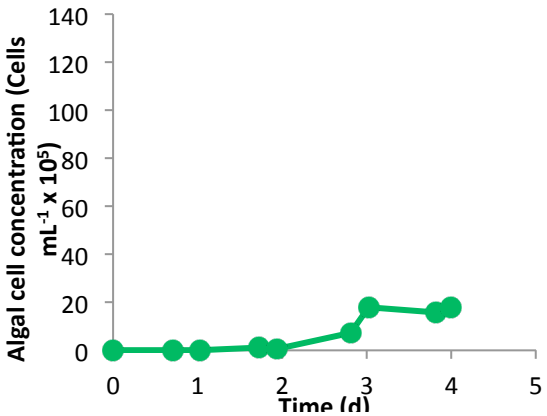


***Figure 36.*** *Increase in algal cell concentration of a second algal species.*

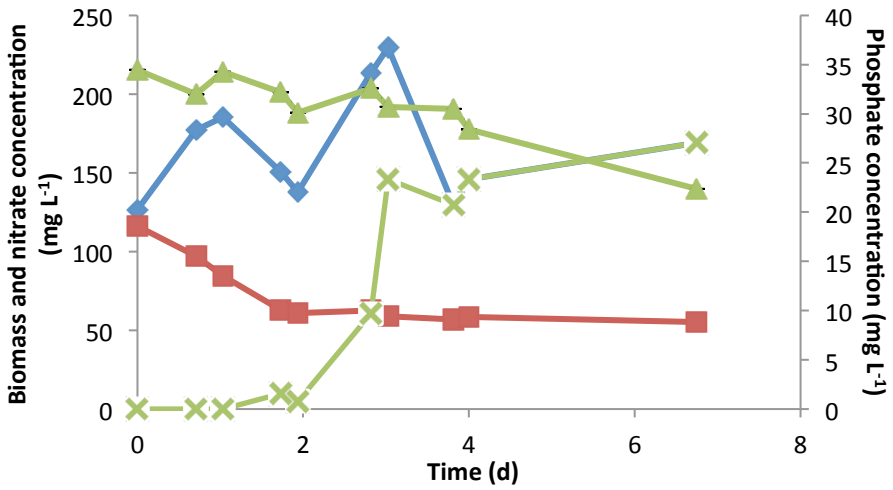


***Figure 37.*** ***Second batch culture of R. salina. Increase in biomass concentration (calculated from the algal cell concentration) (◆), green cells concentration (×), and decrease in nitrate (■), and phosphate (▲).***

Uptake of nutrients differs from the first batch culture. Phosphate concentration was very variable, likely due to the two different species growing. The phosphate uptake may therefore be different for the two species. Nitrate decreased in two different linear phases as for the first experiment but it was not related to the growth of the unknown species (Fig. 37).

Biomass obtained at the end of second week was not used to start a continuous culture, and a third batch culture was performed using a new stock culture of *R. salina*.

### 3. Third experiment

A third batch culture was started with 0.5L of a *R. salina* stock culture grown as flask culture. In order to avoid again a nutrient limitation the culture was regularly fed with additional nutrients.

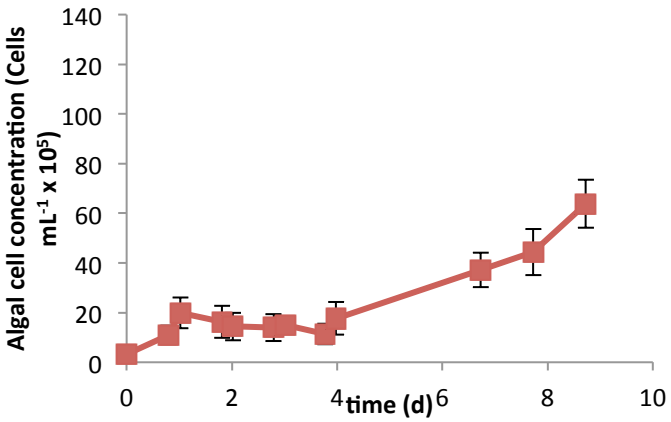


***Figure 38.*** ***Increase in algal cell concentration of R. salina grown in a batch culture.***

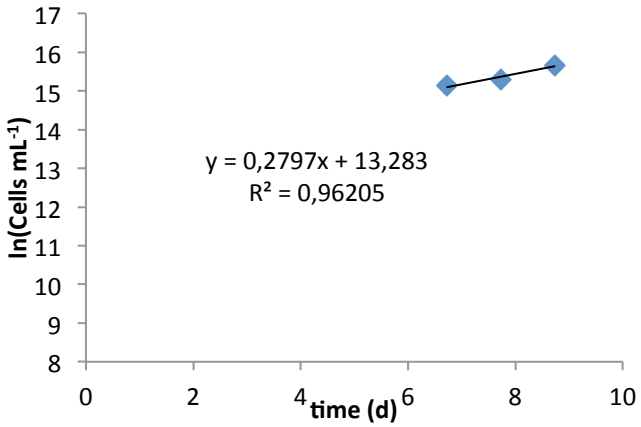


***Figure 39.*** ***Natural logarithm of algal cell concentration between Day 6 and 9.***

The third batch culture was performed to get sufficient biomass to start a continuous culture and the mussel experiment. The batch was started with an initial algal cell concentration of 320000 cells $mL^{-1}$, and nutrients have been added to the culture at Days 2, 4, and 8. Two exponential growth phases were observed. The first between days 0 and 1 to an algal cell concentration of 2 million cells.$mL^{-1}$ and a second between days 7 and 9 from day 7 to day 9 resulting in 6.5 million cells $mL^{-1}$ (Fig. 38).

After Day 9 the culture was changed to a continuous and the dilution rate was adjusted according to the growth rate (0.28 $day^{-1}$) found between Days 7 and 9 (Fig. 39) (Fogg and Thake, 1987).

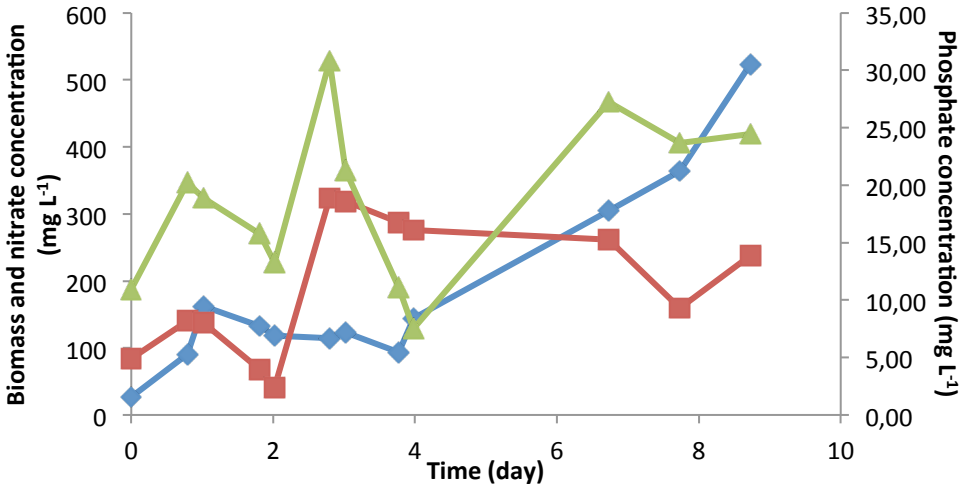


***Figure 40.*** ***Third batch culture of R. salina. Increase in biomass concentration (calculated from the algal cell concentration) (◆), and decrease in nitrate (■), and phosphate (▲).***

Three peaks of phosphate concentration were observed at Days 3, 7 and 9 corresponding to the additions of nutrients. There is no peak of nitrate concentration at Day 7 probably because it was already decreased when the sample was taken. At the beginning, nutrients were used for the first growth. The first addition at Day 2 was used for lag phase of the second growth to synthetize new enzymes. The second addition was used by the cells to start the new growth. The last addition at Day 8 allowed the cells to keep growing exponentially.

Because of these additions of nutrients it was not possible to calculate yields of biomass.

### III) Continuous flow culture

After the cell concentration reached approximately 6.5x$10^6$ cells $mL^{-1}$ the batch culture was changed to a continuous flow culture. The continuous culture provides the possibility to maintain a stable biochemical composition over time in order to feed the mussels.

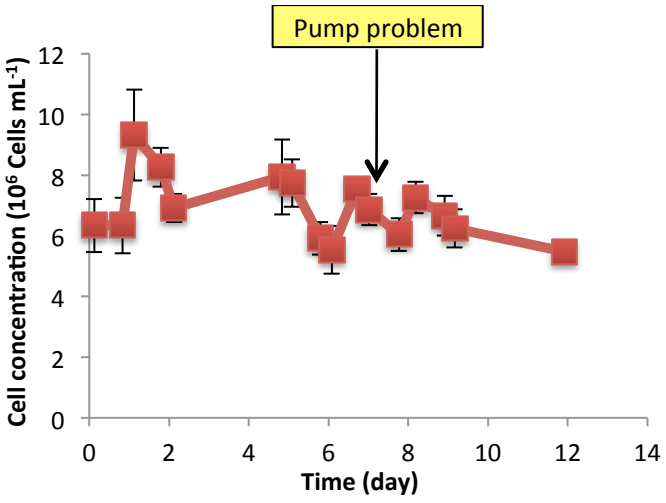


***Figure 41.*** ***Continuous flow culture. Cell concentration of R. salina.***

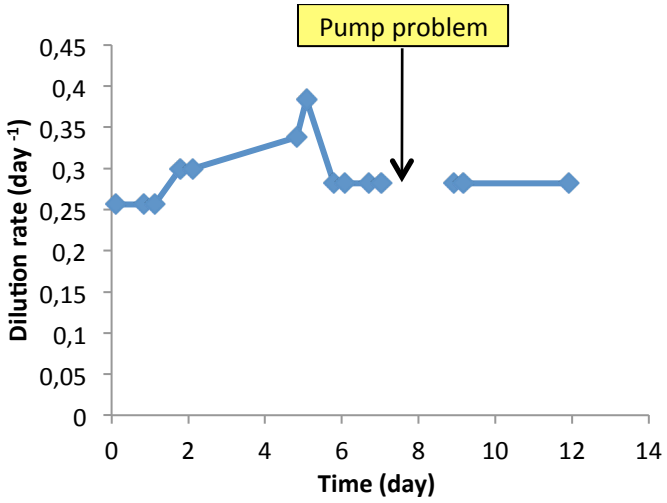


***Figure 42.*** ***Dilution rate used for the continuous flow culture.***

Fig.41 shows the cell concentration of Rhodomonas salina grown in a continuous culture cultivated at a certain dilution rate (Fig. 42). The average cell concentration was about $7x10^6$ cells $mL^{-1}$ as also obtained in batch cultures. Eriksen et al.(1998) obtained using the same reactor a higher cell concentration of about $10x10^6$ cells $mL^{-1}$. Ups and down of the cell concentration during continuous production are explainable by the changes in dilution rates (Fig. 42).

Between Days 6.7 and 9 the pump did not pump fresh medium to the reactor and the dilution rate consequently 0 (Fig. 42). The cell concentration however, was unaffected (Fig. 41).

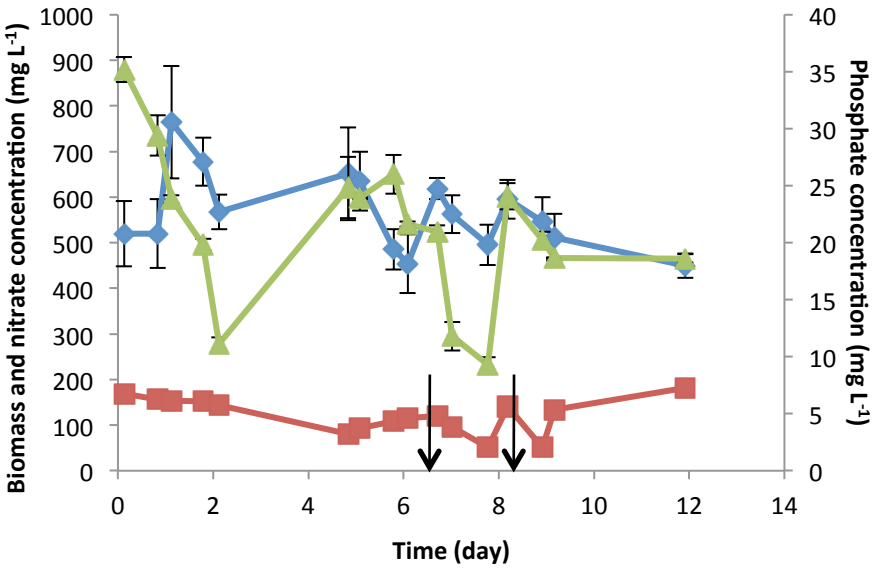


***Figure 43.*** ***Kinetic study of the continuous culture of R. salina. Concentration of biomass (◆), nitrate (■) and phosphate (▲).***
***Each point is the average of two measurements. Arrows indicate periods where the dilution rate was zero.***

Nitrate and phosphate uptake is correlated to the growth of *R. salina*. In fact, when the concentration of biomass increase, the nitrate and phosphate concentration decrease as found in batch cultures performed. The concentrations of nitrate and phosphate (Fig.43) are correlated to the biomass concentration except during the periods where the dilution rate was stopped. That means that the cells took up the nitrate and phosphate present in the culture and the concentrations subsequently decreased.

***Table 8.*** ***Growth and uptake rates obtained in the continuous flow culture of R. salina and compared to the values obtained from the batch culture.***

| D=0.28 $day^{-1}$ | **Continuous flow culture** | **Batch culture** |
|---|---|---|
| | From 5.8 to 9.2 (day) | |
| $Y_{X/NO3}$ (g $g^{-1}$) | 6.9 ± 3.2 | 3.7 |
| $q_{NO3}$ (g $g^{-1}$ $d^{-1}$) | 0.05 ± 0.02 | 0.09 |
| $Y_{X/PO4}$ (g $g^{-1}$) | 10.1 ± 7.4 | 23.9 |
| $q_{PO4}$ (g $g^{-1}$ $d^{-1}$) | 0.04 ± 0.03 | 0.16 |

In Table 8, the growth rates and uptakes rates obtained in the continuous flow culture and batch culture are listed. Even when the pump did not supply the reactor with fresh medium it was assumed for the calculations that there is, except for phosphate, no dramatic influence on concentrations and an average was formed before and after the pump problem between days 5.8 and 9.2. The dilution rate was assumed to be constant at 0.28 $day^{-1}$.

The yield of biomass of nitrate ($Y_{X/NO3}$) for the continuous flow culture is higher than the yield obtained from the batch culture. Conversely, the specific uptake rate of nitrate is lower for the continuous flow culture than for the batch culture.

The yield of biomass of phosphate ($Y_{X/PO4}$) for the continuous flow culture is lower than the yield obtained from the batch culture. Conversely, the specific uptake rate of phosphate is higher for the continuous flow culture than for the batch culture.

To conclude, we can say that the *R. salina* needs more nitrate and less phosphate in the continuous culture compared to the batch culture. But a lot of parameters can affect these results: the dilution rate for example but also the light limitation may strongly influence the uptake of nitrates, while pH, temperature, and $CO_2$ were controlled.

## IV) Bioenergetic studies on Mytilus edulis

***Table 9.*** ***Comparison of growth (G), increase in length (L) and dry weight (W), filtration rate (F), and weight specific growth rate (μ) of mussels fed in the laboratory and mussels grown in Kerteminde Bay. The growth experiment run for 21 days in the Kerteminde Bay (estimated cell concentration: 2500 cells $mL^{-1}$) and 9 days at a cell concentration of 8119 ± 3328 cells $mL^{-1}$ at the beginning and around 5000 cells $mL^{-1}$ at the end for the laboratory experiment.***

| | $L_0$ mm | $L_1$ mm | $W_0$ mg | $W_1$ mg | $F_{est}$ mL $h^{-1}$ | $F_{act}$ mL $h^{-1}$ | $G_{est}$ mg $day^{-1}$ | $G_{act}$ mg $day^{-1}$ | $\mu_{est}$ % $day^{-1}$ | $\mu_{act}$ % $day^{-1}$ |
|---|---|---|---|---|---|---|---|---|---|---|
| Kerteminde Bay | | | | | | | | | | |
| 20 mm | 22.69 ± 2.3 | 26.58 ± 1.9 | 14.1 ± 6.4 | 134.6 ± 43.1 | 447 | 956 | 1.74 | 5.74 | 2.34 | 10.74 |
| 30 mm | 28.62 ± 1.2 | 32.97 ± 1.4 | 55.1 ± 20.1 | 273.5 ± 92.0 | 1100 | 1572 | 2.77 | 10.40 | 1.69 | 7.63 |
| Laboratory | | | | | | | | | | |
| | 29.99 ± 1.3 | 30.41 ± 1.0 | 61.9 ± 11.1 | 67.7 ± 18.2 | 1188 | 1737 | 18.72 | 0.65 | 28.89 | 0.998 |

After being fed for 3 weeks in Kerteminde Bay, the mussels kept have grown significantly (Table 9) from 22.69 ± 2.3 mm to 26.58 ± 1.9 mm (gain of approximatly 3.89 mm) for the 20 mm mussels and from 28.62 ± 1.2 mm to 32.97 ± 1.4 mm (gain of approximatly 4.35 mm) for the 30 mm mussels.

Dry weight of soft parts increased from 14.1 ± 6.4 mg to 134.6 ± 43.1 mg (gain of approximatly 120.5 mg) for the 20 mm mussels and from 55.1 ± 20.1 mg to 273.5 ± 92.0 mg (gain of approximatly 218.4 mg) for the 30 mm mussels.

As expected by the size, a difference in filtration rate was observed: the filtration capacity increased with shell length and weight and accordingly the estimated growth rate also increases since the mussels ingest more food.

Although the 20 mm mussels were apparently growing slower than the 30 mm, their weight specific growth rate was higher.

A significant change in dry weight of soft parts was not observed for the laboratory experiment due to the short time period and to the limitation in food supply. The cell concentration obtained from the bioreactor wasn't high enough to feed 35 mussels. The actual growth rate and the weight specific growth are very low compared to the mussels grown in Kerteminde Bay.

Theorically, the estimated growth rate and the weight specific growth rate are much higher because the *Rhodomonas salina* concentration used is higher, the mussels have therefore more available energy for growth.

For the field study, the estimated filtration rate and growth rate were widely underestimated whereas these values are usually overestimated. These approximations are more likely the consequences of underestimations of the cell concentration in seawater. The concentration was not measured directly at the mussel farm but at the entrance of the Kerteminde harbor located 1-2 km away.

***Table 10. Condition index of the mussels grown in Kerteminde Bay (20 mm and 30 mm) and in laboratory at different time ($t_0$ and $t_1$).***

| Condition index (mg $cm^{-3}$) | | |
|---|---|---|
| | $t_0$ | $t_1$ |
| Kerteminde Bay | | |
| 20 mm | 1.21 | 7.17 |
| 30 mm | 2.35 | 7.63 |
| Laboratory | | |
| Laboratory | 2.30 | 2.41 |

As shown in Table 10, the condition index (CI), which gives an indication of the mussel density, increases with the growth for each group of mussels. It means that the growth is not exclusively dedicated to shell growth but also to the increase of the dry weight.

The smaller mussels CI seem to increase more than the bigger mussels (30mm).

We can notice here that although the 30 mm mussels from the field are close to the ones from the laboratory regarding the filtration rate and size, their condition index is much higher.

# CONCLUSION

In order to study the bioenergetics and growth behaviour of *Mytilus edulis*, it is necessary to study growth characteristics of *Rhodomonas salina*, the algae used to feed the mussel. In this goal, four different experiments have been realised.

*R.salina* growth experiments were performed in flasks, simulating batch cultures, in different conditions of lightning, nutrient, and pH. It resulted that the algae can't have an optimal growth with too much nutrient in the medium, it grows better with a low phosphate rate. Good kinetics parameters can be reached with 40 µmol $s^{-1}$ $m^{-2}$, even if it is supposed to be a light limited condition according to several publications. The best growth rate of the experiment (0.70 $day^{-1}$) is higher than the values found by Lafarga De-la-cruz in 2006. Moreover, the algae managed to grow at a pH until 9 with good kinetics parameters.

In parallel, a batch culture has been performed in a photo-bioreactor with optimal growth conditions according to literature in order to obtain a biomass concentration of about 10 million cells $mL^{-1}$ to start the continuous culture. It resulted that *R.salina* is very sensitive to the variations of conditions especially when the biomass concentration is very high and that the inoculum was probably containing another species what was able to disturb the algae growth. In the end, a biomass concentration of 6.5 million cells.$mL^{-1}$ was obtained to start the continuous culture.

A continuous culture is started in the photo-bioreactor from the previous batch culture in exponential growth phase with a dilution rate of 0.28 $day^{-1}$. The experiment is performed for a week during what the harvest biomass is used to feed blue mussels. The dilution rate had to be changed during the experiment in order to keep a constant growth.

An aquarium of clear seawater is filled with 35 *M.edulis*. The growth and bioenergetics behaviour of the mussel are studied by putting in the aquarium a constant amount of *R.salina* produced in the continuous culture. It's been seen an increase of weight and length with the growth.

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# Appendix 1 – Medium recipe for algae production

The medium used for the algae production is composed of a main solution, a trace metal solution and a vitamin solution.

**Main solution** (for 1000 mL)

$NaNO_3$: 100g
$Na_2EDTA$: 45g
$H_3BO_3$: 33.5g
$NaH_2PO_4$, H2O: 20g
$FeCl_3$, 6 $H_2O$: 1.3g
$MnCl_2$, 4 $H_2O$: 0.36g
Trace metal solution: 1 mL
*Filled up with distilled water.*

**Trace metal solution** (for 100 mL)

$ZnCl_2$: 2.1g
$CoCl_2$, 6 $H_2O$: 2.0g
$CuSO_4$, 5 $H_2O$: 2.0g
$(NH_4)6Mo_7O_{24}$, 4 $H_2O$: 0.9g
*Filled up with distilled water and 0.1 N HCl.*

**Vitamin solution** (for 100 mL)

Thiamin dichloride $B_1$: 200 mg
Biotin: 50 mg
$B_{12}$: 10 mg
*Filled up with distilled water.*

## Appendix 2 – Calibration curve for nitrate and phosphate

**Figure 1:** Calibration curve for nitrate obtained at 220 nm with $NaNO_3$.

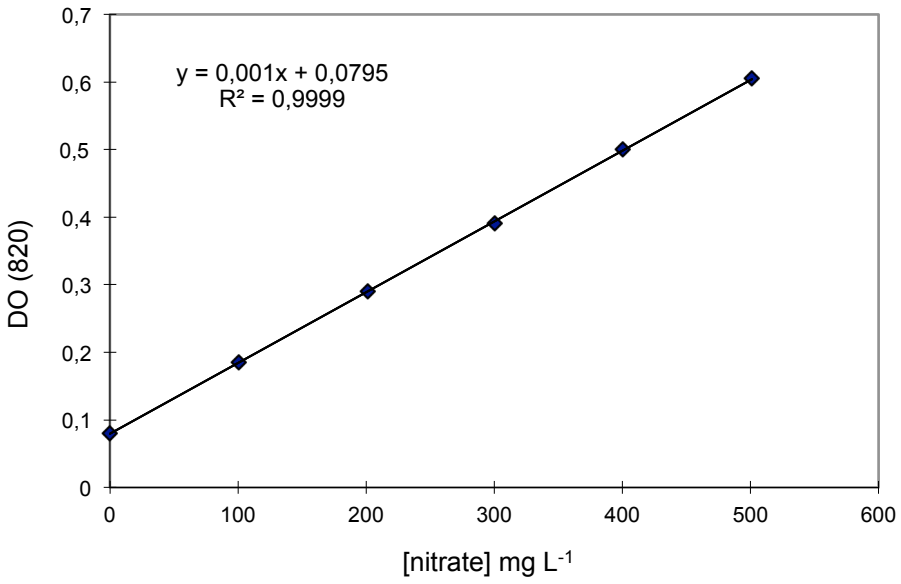
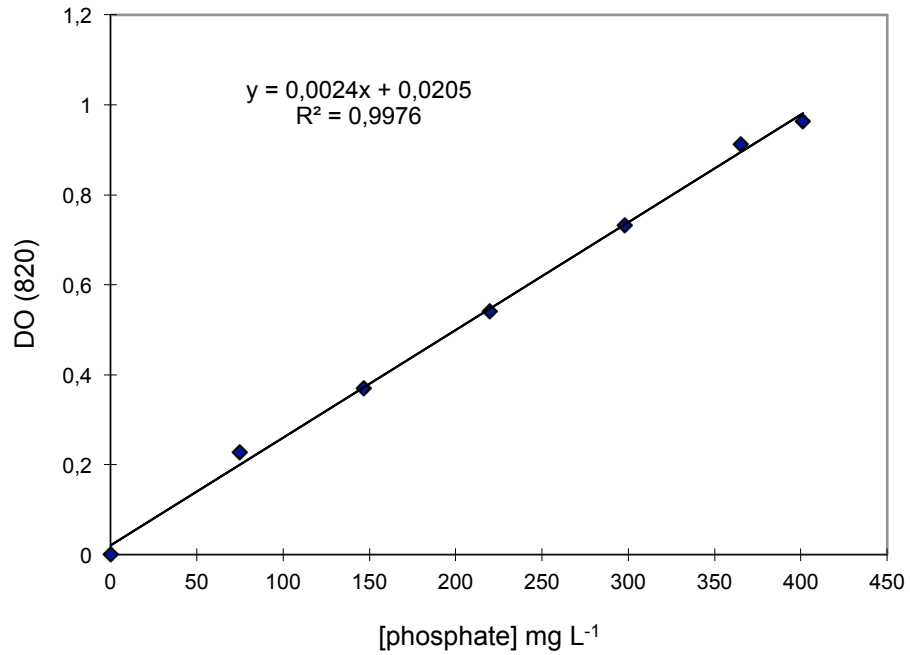
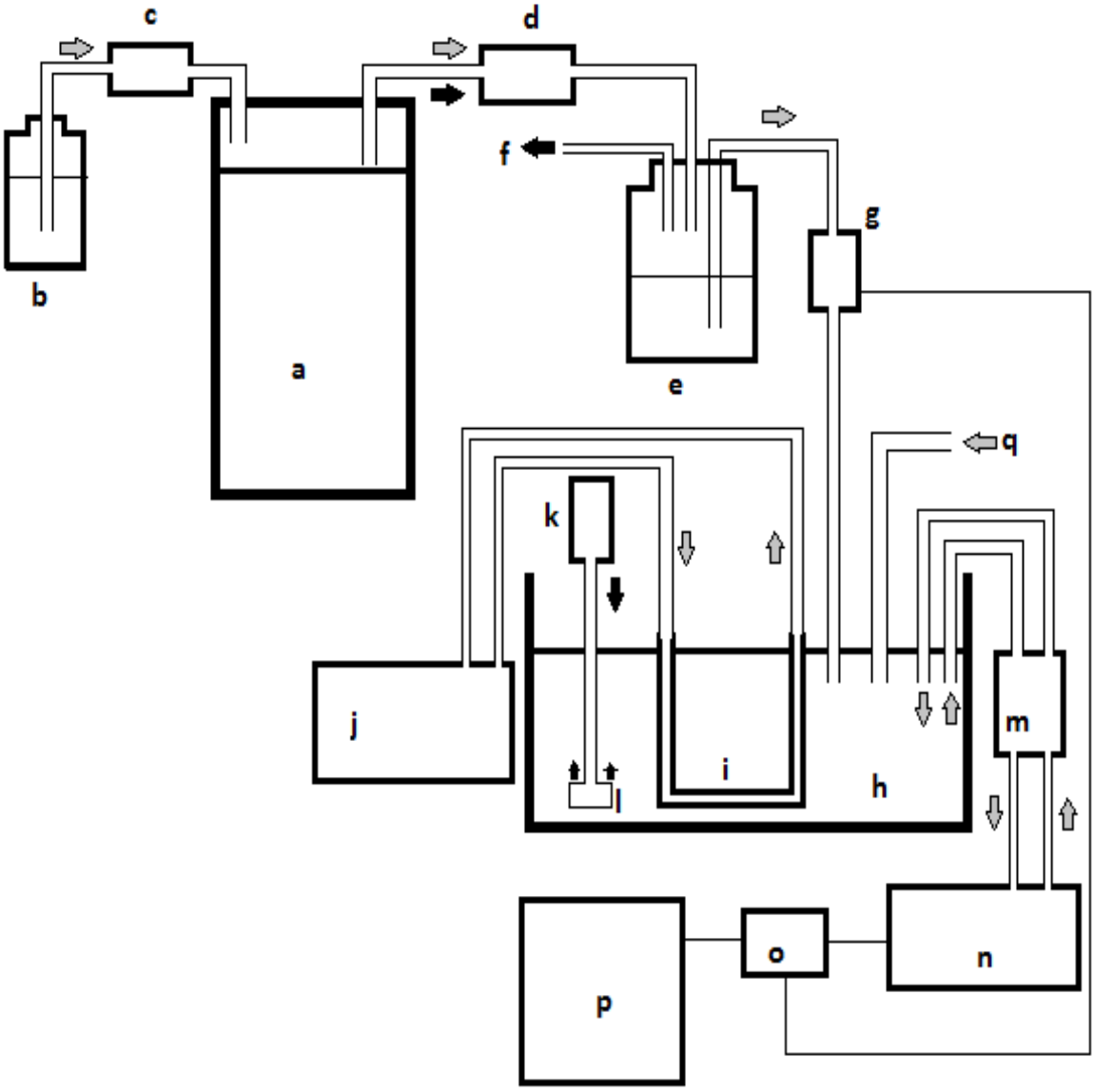



**Figure 2:** Calibration curve for phosphate obtained at 820 nm with $NaH_2PO_4$.



## Appendix 3 – Scheme of the mussel experiment




***Scheme of the mussel experiment supplied with algal biomass produced in a continuous flow culture***

a. bioreactor
b. nutrient solution
c. liquid pump
d. air and liquid pump
e. harvested biomass
f. recycled air
g. liquid pump
h. mussel aquarium
i.cooling tube
j. water bath
k. air pump
l.air diffuser
m. liquid pump
n. fluorometer
o. data acquisition system
p. computer
q. biofiltrated sea water

**gas flow**

**liquid flow**