Potential Respiration in *Oxyrrhis marina* and *Rhodomonas salina*

Tesis de Master

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ABSTRACT

The Potential respiration in cultures of the heterotrophic flagellate *Oxyrrhis marina* (predator) and on a micro algae *Rhodomonas salina* (prey) was calculated from measurements of the electron transport system (ETS) activity. On *R. salina* these measurements were made during the exponential, stationary and cell-decline phase for a period of 64 days, but in *O. marina* they were only made during the period of starvation (cell-decline phase), for a period of 18 days. Time-courses of Chlorophyll, protein and cell density were compared to the potential respiration (Φ). In *R. salina* the Φ increased during the exponential phase in parallel with the cell- protein, cell number, and chlorophyll *a.* In *O. marina*, separated from its prey, Φ followed a similar pattern with the protein, chlorophyll and cell density. Flow cytometry proved useful not only for cell-enumeration, but also for monitoring population changes in thenon-axenic mixed-population cultures. From the physiological and biochemical measurements it is clear that the predator, *O. marina* was metabolically more active than its prey, *R. salina*. Its Φ was 19 nmO_2 min^{-1} $cell^{-1}$, 20 times higher than the Φ of *R. salina*, with a Φ of only 0,9 nmO_2 min^{-1} $cell^{-1}$. In addition *O. marina* was also 20 richer in protein than *R. salina* (942 versus 47 pg protein/cell). However its protein-specific Φ was effectively the same (12 versus 13 nmO_2 min^{-1} $(protein)^{-1}$, respectively. An interesting finding, that suggests that *O. marina* may be able to function as an autotroph, was the constancy, for 9 days, of the ingested chlorophyll/cell in *O. marina* days after all its chlorophyll-rich prey was consumed.

INTRODUCTION

Life on our planet has been connected to the ocean since the beginning and all the different processes of life can be found in it. In order to understand the ocean as a whole it is important to understand these oceanic processes as much as it is to understand the organisms. Here we focus on one of these processes, respiration, and on two marine organisms, *R. salina*, a photosynthetic flagellate and *O. marina*, a heterotrophic dinoflagellate. *R. Salina* is representative of the phytoplankton which inhabits the oceanic euphotic zone. *O. marina* represents the microzooplankton which can inhabit all oxygenated depths of the world ocean. These plankters not only contribute to the energy transfer of the food web, but also contribute to the CO_2 balance in the ecosystem via their respiration. Enormous numbers of plankton are currently respiring all around the world, consuming great quantities of O_2 and producing equally great quantities of CO_2.

These chemical transformations are important factors in the global cycles of carbon and oxygen and must be accounted for in models of these elements. Accordingly, we need to develop ways to measure and to predict these transformation rates from easily measureable variables. For this reason biological oceanographers study the physiology and biochemistry of oxygen consumption. In these studies they have demonstrated the relationship between oxygen

consumption and the enzymatic respiratory electron transport system (ETS), and learned that the respiratory ETS can be controlled by the availability of substrates (reactants) and changes in temperature (Packard et al. 1996). This control follows the rules of Michaelis-menten kinetics (Cornish-Bowden, 2004) and Arrhenius temperature regulation theory (Arrhenius, 1915 and Martin et al, 2009).

The assay of the ETS is a biochemical technique that measures potential rates of respiration. In addition, it reflects the integrated metabolism of the entire organism (Pomeroy 1974). Ninety % of an organism's oxygen consumption is accomplished by the electron-transport system. It and oxidative phosphorylation take place on the inner mitochondrial membrane of the eukaryotic cells and on the plasma membrane of the prokaryotic cell (Hinckle and McCarthy 1978). The ETS consists of electron carriers that are arranged in four complexes. These are complex I, where nicotinamide adenine dinucleotide (NADH) passes electrons to ubiquinone; Complex II which receives electrons from flavinadeninedinucleotide ($FADH_2$): complex III which receives electrons from ubiquinone (UQ); and complex IV which transfers electrons to O_2 (Lehninger et al. ,2005). The enzymes involved in this pathway are NADH (EC1.6.99.3), NADPH (EC 1.6.99.6), and succinate (EC1.3.99.1) dehydrogenase. (Hernández-León citated in Harris, 2000). Because the ETS assay was designed for a mixed seawater community of eukaryotes and prokaryotes as well as for mitochondrial and microsomal ETS, NADPH is added to satisfy the demands of those prokaryotes that use NADPH in place of NADH.

Since it is an enzyme assay, the method for detecting ETS activity is based on saturating the electron transport systems with NADH, succinate, and NADPH respectively (Rosario-Pinilla, 2002) so that the maximum reaction rate (V_{max}) of the ETS is measured. This is the only way that the measurement of the ETS activity can be reliably reproduced. The assay uses an artificial electron acceptor, a tetrazolium salt called INT (2-(p-iodophenyl)-3-(p-nitrophenyl)- 5 – phenyl tetrazolium chloride), to collect the electrons from NADH, NADPH, and succinate between complex II and complex III in the ETS. This electron acceptor effectively records, stoichiometrally, the transferred reducing equivalents produced in catabolic metabolism. In other words, it is reduced by much the NADH, $FADH_2$, succinate, etc. that is produced in glycolysis, the Krebs Cycle, beta-oxidation, and other metabolic cycles in the cell. Upon reduction, INT changes color from a light yellow to a dark red. This red formazan compound is detected in a spectrophotometer at 490nm (Hernández-León citated in Harris, 2000). Here we use this type of analysis to study the respiratory biochemistry measuring the Φ in *O. marina* and *R. salina*.

Heterotrophic flagellates are an important link in the oceanic food web as they are involved in energy transfer between trophic levels and modulating plankton blooms, Jeong (1999). One of these, *O. marina* (Fig. 1), has been used in diverse studies not only because it is easy to culture, but also because it plays a role in the oceanic ecosystems. Since the 1950s there have been numerous studies using cultures of *O.marina*. Its nutritional requirements and the method for culturing it were worked out by Droop (1959). Clarke and Pennick (1976) studied its morphology and ultrastruture with an electron microscope. Recently Jeong et al (2003) studied its role in controlling red tides. But until now no one has tried to use ETS activity as a proxy for oxygen consumption in cultures of *O. marina.* This was one of the objectives of this study.

Another objective was to investigate the characteristics of the ETS activity in the autotrophic flagellate, *R. salina* (Fig. 2) a microalgae commonly used as a food source in aquaculture (Camino et al.2005). This unicellular flagellate which has a diameter range from 9.2 to 9.9 µm (Valenzuela 2005) is used as prey in many studies because it is easy to grow and to maintain in culture, but few use it as the principal subject of the study. In one of these few studies (Valenzuela, 2005), its growth was characterized in different culture medium. In another study, it was exposed to different light and temperature conditions to check its ability to acclimatize to these conditions (Hammer et al. 2002).

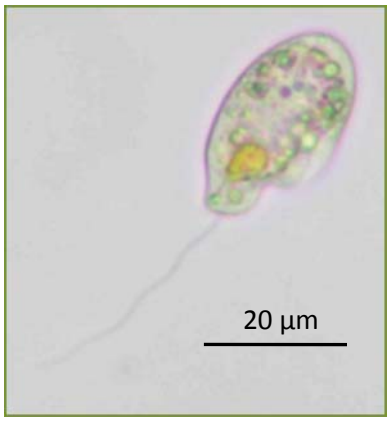

Fig.1 *O. marina.* Photo M. Aristizabal

Fig.2 *R. salina.* Photo M. Aristizabal

MATERIALS AND METHODS

One objective of this study was to measure the Φ in different physiological states of *R. salina* and *O. marina.* To achieve this objective, experiments were conducted on batch cultures of these organisms as they grew through their lag, exponential, steady-state and scenesent growth phases. Triplicate cultures, of *R. salina* and of *O. marina* (at the same temperature condition, but different light conditions) were grown non-axenically in 1.5 L pyrex-glass erlenmyer flasks. From each one of the six cultures flasks cell samples were obtained by filtration for: Chlorophyll, Protein, and ETS activity. , The samples were taken every 2 days for a period of 21 days and every 5 to 7 days for a period of 43 days on the *R. salina* cultures. For *O. marina* they were taken every 2 days for 12 days and then every 3 days for a period of 6 days.

Algal cultures. The microalga *R. salina* and heterophic dinoflagellate, *O. marina* was obtained from the microalgal culture collection of the Instituto de Ciencias del Mar de Barcelona (C.S.I.C., Barcelona, Spain.) Cultures used as inocula were maintained in 2-L glass bottles with filtered and UV irradiated sea water enriched with f/2 media (Guillard, 1975 cited in Andersen, 2005). All bottles and experimental flasks were capped with cotton plugs in order to facilitate gas exchange, to prevent contamination and to limit evaporation. Daily (morning and at night), the flasks were swirled, in order to maintain the cells in suspension. The mother cultures and experimental flasks of *R. salina* were placed at a distance of 15cm from the light. Flasks of *O. marina* were places at a distance of 30 cm from the light. Irradiance was measured by a Radiometer (Data Logger LI-1400).

While the mother culture of *R. salina* was in mid-exponential phase a 40ml inocula was transferred to each of the three (1.5-L) experimental erlenmeyer flasks, containing 700ml (filtered and UV irradiated) sea water enriched with f/2 media (Guillard,1975). These flasks would be used for the *R. salina experiment.* The three flasks were then irradiated continuously at a distance of 15 cm for 24h with two 40W Cool-White light bulbs. The average temperature, salinity and pH were 22.5± 0.5ºC, 38‰ (PSU) and 8, respectively.

A well fed mother culture of *O. marina* was maintained and used to supply 15ml innocula that were transferred to three (1.5-L) experimental Erlenmeyer flasks, containing each flask a population level of *R. salina* of 835,000 cells ml^{-1}. The three flasks were exposed to same temperature conditions as the *R. salina* cultures, but at lower irradiance (0,8 µmol photons m^{-2} s^{-1} (Spherical SPQ). Before starting an experiment with *O. marina,* the heterotroph would eat all the *R. salina* available in its medium. This was verified by microscope in samples taken every two days.

Analytical methods

ETS activity, growth rate, chlorophyll and protein.

ETS activity

The measurement of respiratory ETS activity is an index of the potential respiration in the organisms present in the culture. It can detect the mitochondrial ETS in eukaryots and the plasma ETS in prokaryots, and archaea.

ETS essays on the *R. salina* and *O. marina* cultures were made kinetically using a spectrophotometer (Beckman Du 650), in which the cuvette temperature was controlled by a water bath (22.5ºC). ETS samples (2 ml) from each culture experiment were filtered through 25mm Whatman GF/F filters. Immediately the filter was homogenized for 2 min in a teflon-glass tissue grinder, powered by a drill motor (Einhell SB 701/1) in phosphate buffer (0.05 mol, pH 8.0). Then the homogenates were centrifuge for 2 min at 1000rpm at 2ºC. In 2 cuvettes were added 100µl from the supernatant fluid, 100µl of a 4mM tetrazolium (INT) solution and 300µl ETS reaction mixture containing NADH (1mM) and NADPH (0.343 mM)[*]. In the other two cuvettes were added 100µl from the supernatant, 100µl of INT solution and 300µl of Blank substrate instead of the ETS reaction mixture. Immediately the four cuvettes were placed in the spectrophotometer and monitored at 490nm. Readings were taken every 33 sec for a total of 8 min at 22.5ºC. The slope of this time-series was the INT-formazan production rate per minute which is stoichiometrically related by a factor 2 to ETS activity and by a factor of ¼ to Φ. The details of these calculations were described in Packard and Christnsen (2004). At the same time that the ETS samples were taken, samples were fixed for chlorophyll *a* and protein. In addition, 1ml from each experiment was taken for cell count, using a flow cytometer (Beckman Coulter).

Chlorophyll *a* (chl*a*) Two ml of *R. salina* and *O. marina* were filtered on a Whatman GF/F filter and immediately frozen in liquid nitrogen for 30 min. After about ½ h the sample was transferred to freezer at -80ºC and then stored in darkness. Two months later the filters were analyzed for chlorophyll a, fluorometrically in a Turner-Design fluorometer (10AU- CE) according to the method of Holm-Hansen et al. (1965).

Protein Protein was analyzed by the Lowry method (Lowry et al. 1951) as modified by Rutter (1967). Two ml, from each of the six experimental Erlenmeyer flasks, were taken and filtered on 25mm GF/F filters, then the filters were quick-frozen in liquid nitrogen for 20min, and finally stored at -80ºC.

Cell density Concurrent with the analysis of ETS activity, a 2 ml sample was taken from each flask divided into triplicate subsamples and run through a flow cytometer, (Cell lab Quantas (Beckman-Coulter) equipped with a 488nM laser. *R. salina* was identified from chlorophyll and phycoerythrin fluorescence. Not only cell density, but also the culture evolution was monitored with the flow cytometer. All measurements were corroborated by inverted microscope observations.

Growth rate The average specific growth rate was calculated by linear regression of the natural logarithm of the cell density against time. The exponential growth phase was determined graphically using a GraphPad Prism.

RESULTS

The ETS activity on *R. salina* and *O. marina* is presented as potential respiration (Φ) in units of nanomoles O_2 min^{-1} (ml of culture)$^{-1}$.ETS activity is negligible if substrates (NADPH and NADH) are not added as is shown by SB line with negligible slope Fig. 3 and 4.

[*] The NADH and NADPH concentrations in the stock solutions of the ETS reaction mix were within the range of 0.63-0.78mg/ml and 0.21-0.31 mg/ml, respectively.

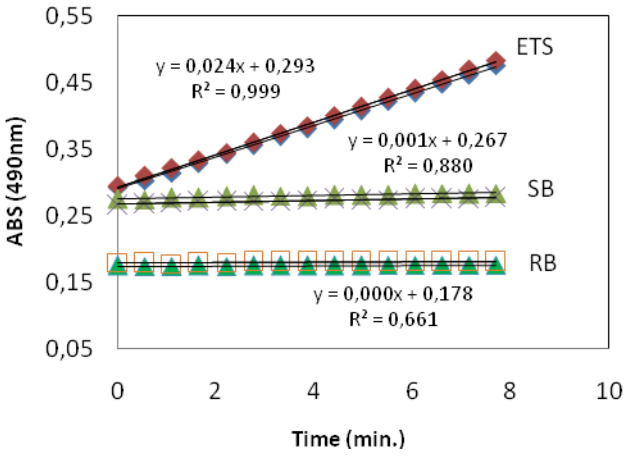

Fig 3. An ETS assay with a substrate blank, and a reagent blank on one of the experiments with *R. salina.* The substrate blank does not contains NADH, NADPH, or succinate , i.e., it is a "zero-substrate" blank. It is equivalent to the ETS assay described by Balmstedt (2000).

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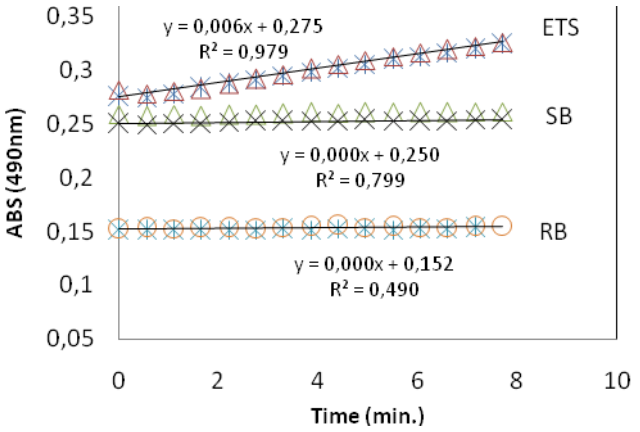

Fig 4. An ETS assay with a substrate blank, and a reagent blank on one of the experiments with *O. marina.* The substrate blank does not contains NADH, NADPH, or succinate , i.e., it is a "zero-substrate" blank.It is equivalent to the ETS assay described by Balmstedt (2000).

Growth in *R. salina* cultures

Nutrients are one of the environmental variables that control the growth of the algae cultures (*R. salina*). These cultures follow three defined phases (Fig 5) exponential phase, stationary phase and cell-decline phase (senescence). As the inoculum was large and the parent culture had been growing under the same conditions, the lag phase is not well defined and lasted only a day. Following this "phase" the exponential phase, representing the maximum growth rate lasted from 8 to 10 days with a average μ = 0,25 day^{-1} (n =3). Then the growth slowed as the nutrients were depleted and the culture entered into stationary phase (19-21days). At the end of this phase one can observe that the density of culture debris (organic, inorganic, and bacterial) becomes as large as the *R. salina* density (Fig. 7). The evolution of the debris on the *R. salina* cultures can be seen in Fig. 6 and 7.

During the cell-decline phase the *R. salina* cell numbers start to decrease and the debris density gets higher (Fig.7). Also the *R. salina* population becomes dispersed from its original location (Region 1) as shown in Fig. 9, 10 and 11 (Quadrant Q2). Under the microscope, the *R. salina* cells move slowly, the aggregates become visible, and the culture´s color turns pink and yellow.

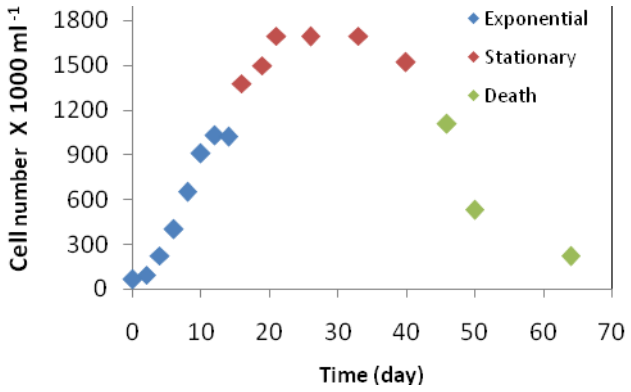

Fig. 5 Growth of cryptophytes *R. salina* in batch culture (Experiment # 2). Different growth phases are show as: ◆ exponential, ◆ stationary, ◆ death.

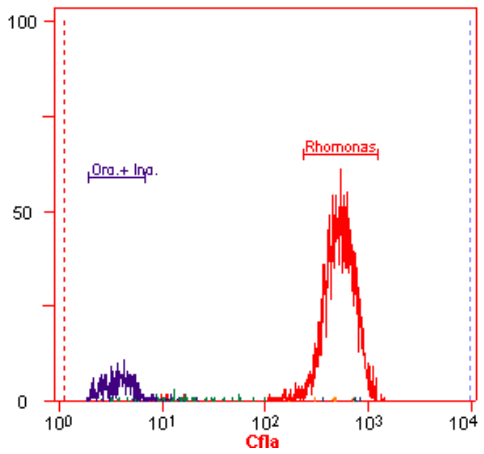

Fig. 6 Single parameter histogram of *R. salina* cell number (136 $X10^3$cell/ml) and debris (16 $X10^3$/ml) at the beginning of the experiment. Cell count on the y-axis and chlorophyll fluorescence (cfla) on the x-axis.

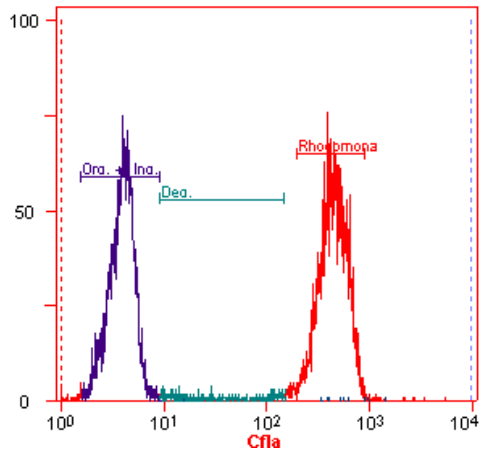

Fig. 7 Histogram on *R. salina* culture from the last days of the stationary phase. Cell count plotted against chlorophyll fluorescence (cfla).

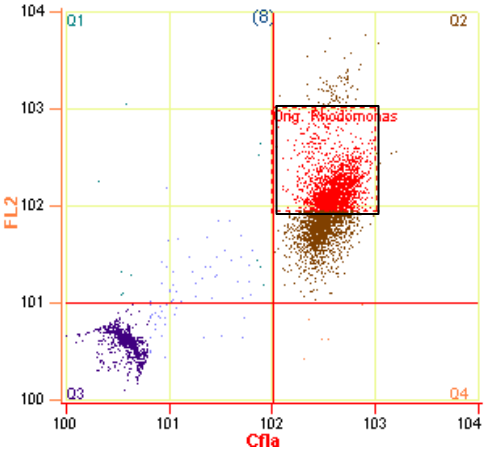

Fig. 10 Histogram of the the first week of the cell-decline phase on the *R. salina* culture. See caption from Fig. 7 for more details on the plot.

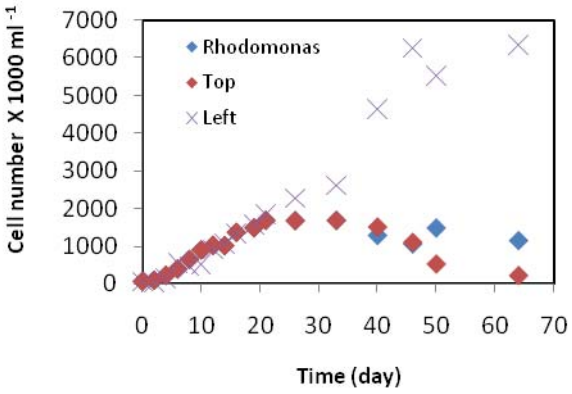

Fig. 8 *R. salina* culture density of the debris and the *R. salina* cells.

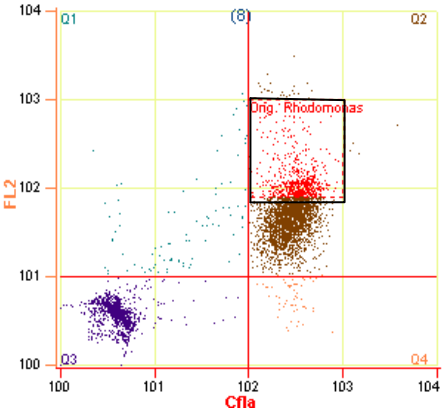

Fig. 11 Histogram of the migration of the *R. salina* culture, during week 2 of the cell-decline phase.The location of the population has now fallen from its original location in Region 1. See caption from Fig. 7 for more details on the plot.

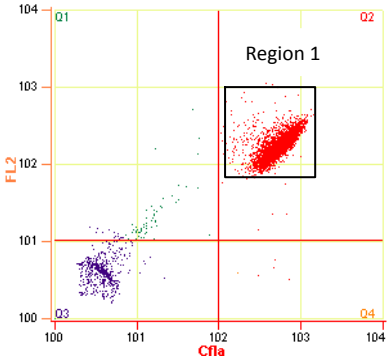

Fig. 9 The plot represents a spatial domain created when the phycoerythrin signal (FL2) is plotted against the Chlorophyll fluorescence (Cfla). The box represents the domain of the original *R. salina* culture

ETS Activity and chlorophyll follow the same pattern as Chl *a* during the exponential phase, and during the following nine days in the stationary phase. While in stationary phase both Chl *a* and ETS activity remain relatively constant. However towards the end of this phase the Chl *a* starts to decrease while the ETS activity, as Φ, increases towards the end of the 12 days in stationary phase (Fig. 12).

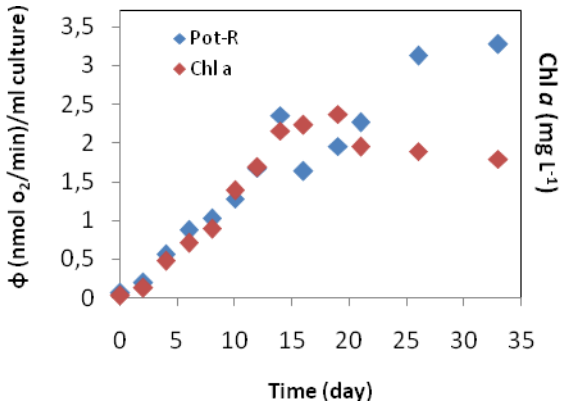

Fig. 12 ETS activity and Chl a in the exponential and stationary growth phasesin *R. salina.*

In all three experiments with *R. salina* the cell specific Φ increased towards the end of the stationary phase and continued to increase all through the cell-decline phases (Fig. 13). This was unexpected because both chlorophyll and ETS activity are good phytoplankton biomass proxies (Packard et al., 1974)

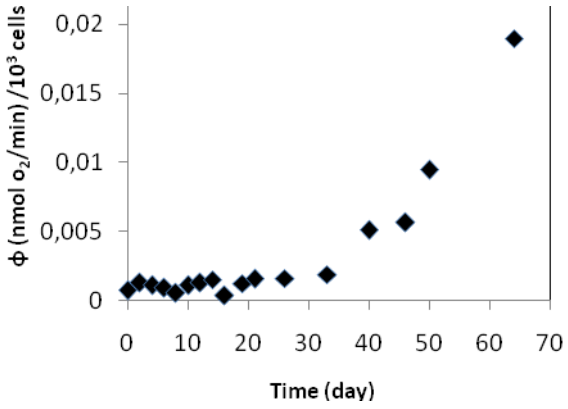

Fig. 13 Cell Φ on a *R. salina* culture.

Cell- protein,in the beginning, started at levels of 0.032, 0.042, 0.05 mg per ml of culture, respectively for the three experiment. Then during the following 12 days the protein increasesd up to an average maximum of 0.13 mg per ml of culture. Afterwards, the protein content decreased during the following three days. There is a slight increasein the next 48 hr, but then the protein was stable for the next 43 days (Fig. 14).

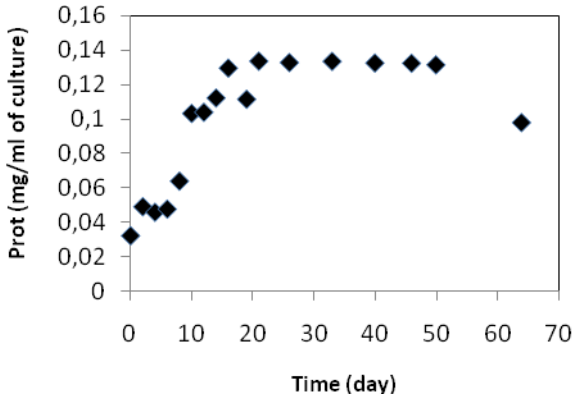

Fig. 14 Protein content during the growth of *R. salina.*

When the Φ is normalized by the protein it increases during the first 6 to 8 days of the cultures. Afterwards, in the following 11 days, the protein-specific Φ plateaus, and then during the last 43 days of the culture it steadily increases (Fig. 15).

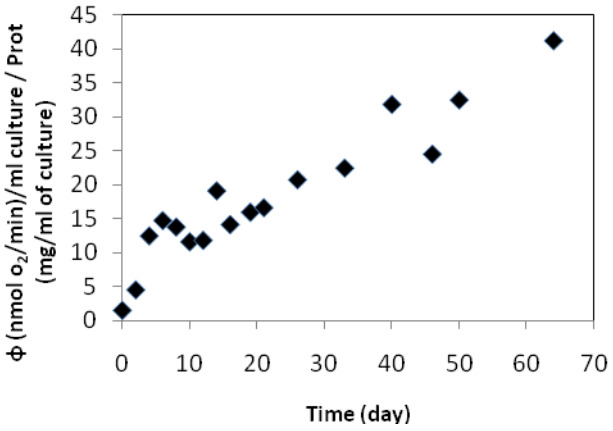

Fig. 15 Protein-specific Φ on *R. salina.*

On the contrary, the Chlorophyll-specific Φ decreases on the first six days and remains constant during the following 11 to 13 days and then increases during the last 12 to 14 days (last days in the stationary phase).

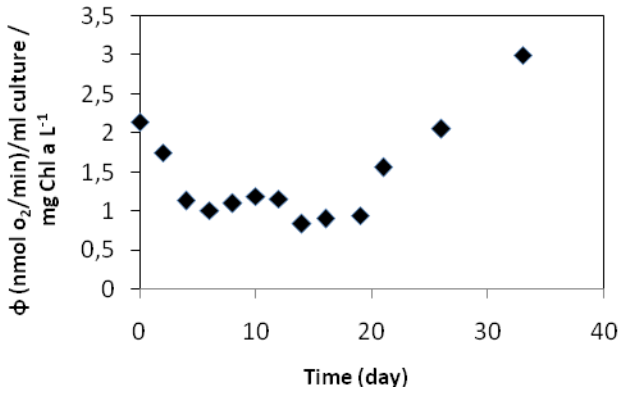

Fig. 16 Chlorophyll-specifc Φ on *R. salina.*

Protein-specific chlorophyll for the three experiments. During the first 6 to 8 days there is a strong increase in the chlorophyll-protein ratio. Then it undulates between 10 and 20 with a single peak around days 19-20. The strong increase in the first 5 days of the cultures may explain the drop in the chlorophyll-specific Φ during the same peeriod.

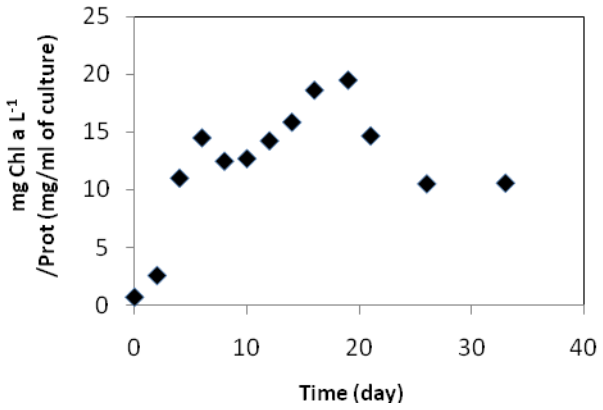

Fig. 17 Protein- specific chlorophyll on *R. salina* during the exponential and stationary phase.

O. marina cultures

The experiment on the *O. marina* culture started as soon as the *R. salina* were absent from the medium. The disappearance was confirmed by observations using an inverted microscope. The cell density of *O. marina* decreased during the first six days. In the following four days, the cell numbers remained stable and continued in this way for another eight days (Fig. 24). At the beginning of the experiment there were three different groups of *O. marina* in the culture as it can be seen on Fig 18 and 19. The region 1 represented the large *O. marina*, region 2 represented *O. marina* smaller than in region 1, but bigger that the ones in region 3.This size difference was also checked using the inverted microscope. Not only were there three different groups of *O. marina,* but also the density of each group was different throughout the experiment. At the beginning of the experiment the distribution between the three groups was 90.99%, 4.53% and 4.32% as shown in regions 1, 2 and 3 (samples from experiment # 1). As the experiment proceeded, the group from the *O. marina* region 1 attained a level of 97,5% while region 2 and 3 decreased lower and lower to 0,25 and 0,37, respectively. The first group to disappear from the histogram was the region 3 group, The region 2 group falls to only 0,27% and group from region 1 continues with 97%, as before.

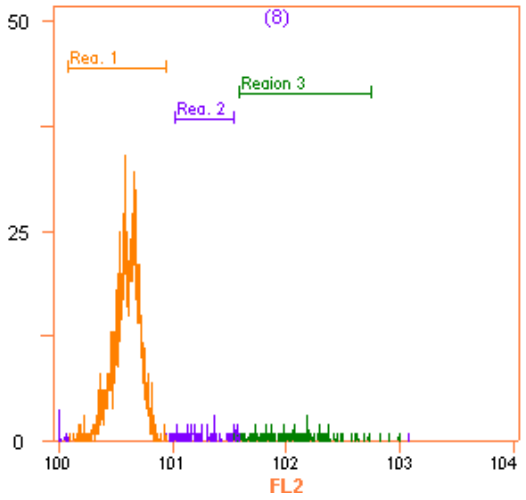

Fig. 18 Distribution of the *O. marina* culture at the beginning of the experiment. Region 1, 2 and 3 representing the three sizes on the *O. marina* cultures, large, middle and small, respectively. Phycoerythrina signal (FL2) plotted.

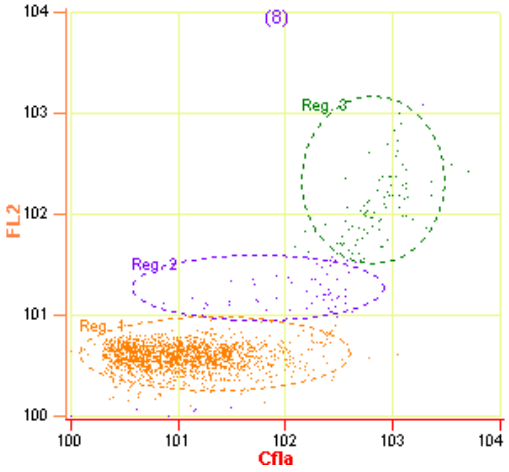

Fig.19 *O. marina* culture at the beginning of the experiment. Three size groups. Phycoerythrina signal (FL2) plotted agains the Chlorophyll fluorescence (Cfla).

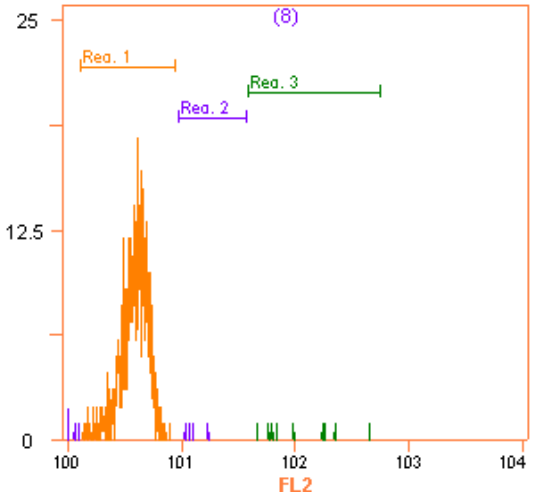

Fig. 20 *O. marina* culture four days from the beginning of the experiment.

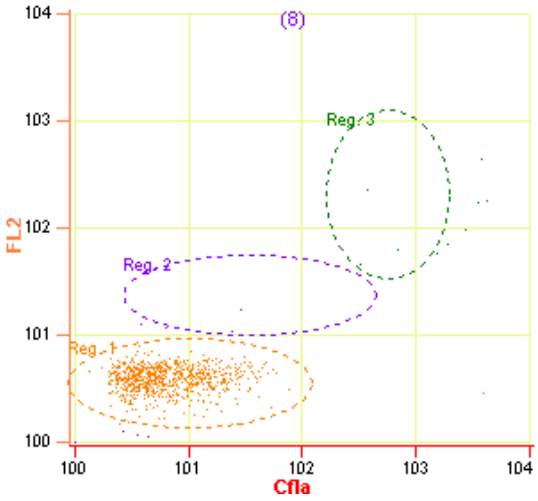

Fig. 21 *O. marina* culture four days from the beginning of the experiment.

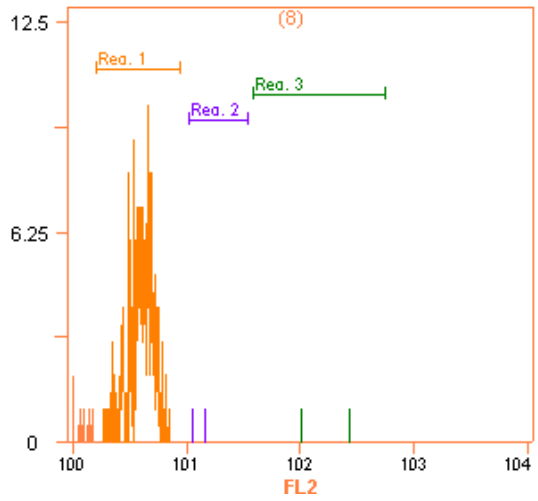

Fig. 22 *O. marina* culture eight days from the beginning of the experiment.

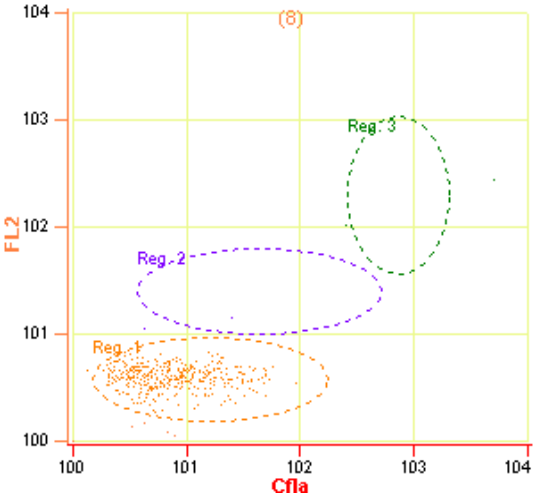

Fig. 23 *O. marina* culture eight days from the beginning of the experiment.

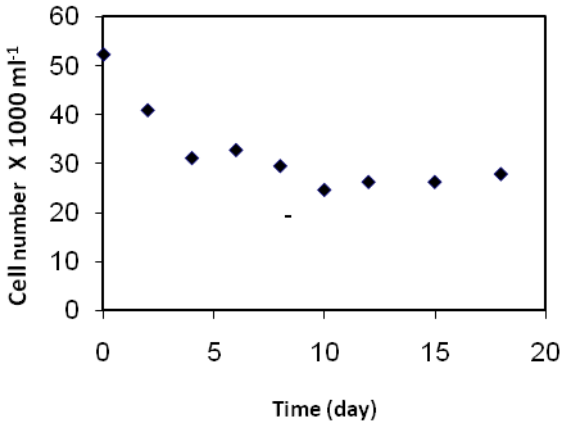

Fig. 24 The cell-decline growth phase of *O. marina* in batch cultures (Experiment # 3).

Φ decreased in the first four days of the experiment, followed by six stable days. The Φ decreased in the following five days, then culture continued for three stables days.

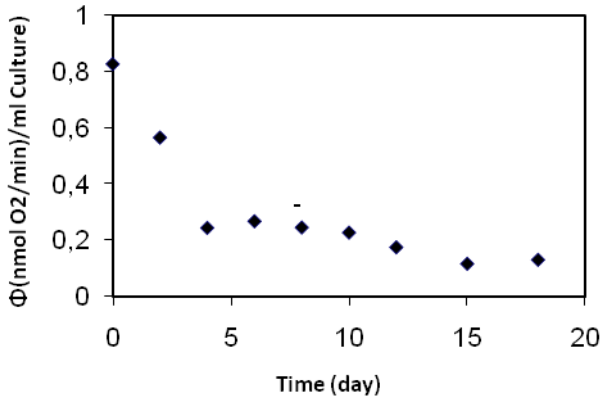

Fig. 25 Growth of *O. marina* in batch cultures (Experiment # 3). The growth phase corresponds to the cell-decline phase.

In the three experimental flasks, the concentration of chlorophyll *a* decreased rapidly during the first four days, following a stable eleven days of chlorophyll *a* concentrations. The chlorophyll *a* follows similar time-course pattern as does the Φ.

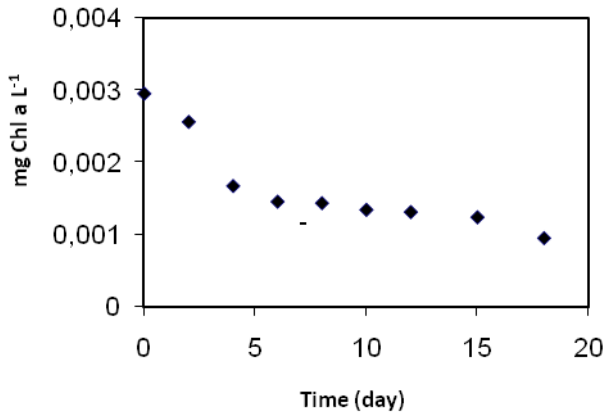

Fig. 26 Chl *a* at the cell-decline phase on *O. marina.*

Protein decrease during the first 4 days, following a stable 5 days of proteins content. Afterwards, the protein content decreased during the following 5 days. There is a slight increasein the next 3 days (Fig. 27).

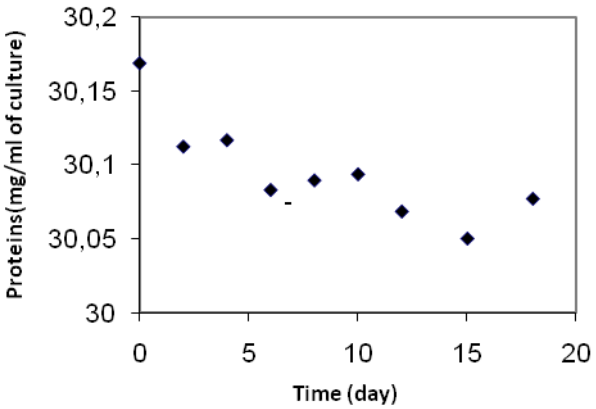

Fig. 27 Protein contents during the cell-decline phase on *O. marina.*

Physiological relationships

R. salina: Chlorophyll-Φ relationship

In plankton ecology studies, the chlorophyll-ETS or chlorophyll-Φ ratio is useful in correcting microzooplankton Φ measurements for the phytoplankton component. From measurements of the chlorophyll contaminated the zooplankton sample and measurements of, the ETS-chlorophyll ratio in phytoplankton, one can calculate and subtract the phytoplankton contribution from the total ETS. Here, in Fig. 28, one can see the relationship between Φ and chlorophyll in *R. salina* experiment #1.The ratio (Φ/Chl) is1.0 nmol O_2 min^{-1}(μg Chl)$^{-1}$, equal to the mean Φ/Chl from the three experiments (*Table I*).

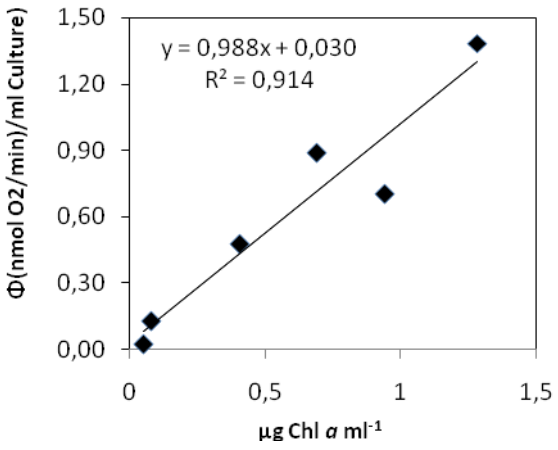

Fig.28 Φ vs chl a inthe exponential phase of the *R. salina* experiments. Here Φ /Chl is 1 nmolO_2/min/µg.

Table I: Chlorophyll-Φ relationship during the exponential phase in the three experiments from the *R. salina* cultures. Note that Φ /Chl is in units of nmolO_2/min/µg.

Exp	Φ/chl	r^2	n
1	0,988	0,914	6
2	0,900	0,984	6
3	1,105	0,988	6

R. salina: Protein-Φ relationship

In biochemical studies of enzyme kinetics, the protein-specific enzyme activity is useful in determining the level of purity of the enzyme, the turnover rate, and the true V_{max} of the enzyme. In addition, this relationship should vary as the *R. salina* cells shift from nutrient–rich to nutrient-starved conditions. Because the ETS complexes on the inner membrane of the mitochondria are constitutive. That is, they should maintain their density (i.e., concentration) regardless of the nutritional state of the cell. If this is the case for *R. salina*, and nutrient-limitation leads to protein loss, then one would expect the Φ-protein ratio to rise during nutrient-limitation. Such studies of protein-specific enzyme activity are rare in research reports on plankton cultures. Here we define the ratio during the nutrient-sufficient phase (exponential) of the *R. salina* cultures, but not for the other phases. The bacterial contamination in these other phases precludes using the data. However, from the exponential phase where bacterial contamination was minimal (Fig.6) the Φ-protein relationship can reliably be attributed to *R. salina*.

The results of the Φ-protein analysis yielded a Φ-protein ratio (example: slope of Fig. 29) for *R. salina* Exp #2 of 10 nmol O_2 min^{-1} (mg protein)$^{-1}$, From *Table II* this represents the low end of the range of Φ-protein in this alga. In Exp-3, at the high end, the ratio was 17.21 nmol O_2 min^{-1} (mg protein)$^{-1}$.

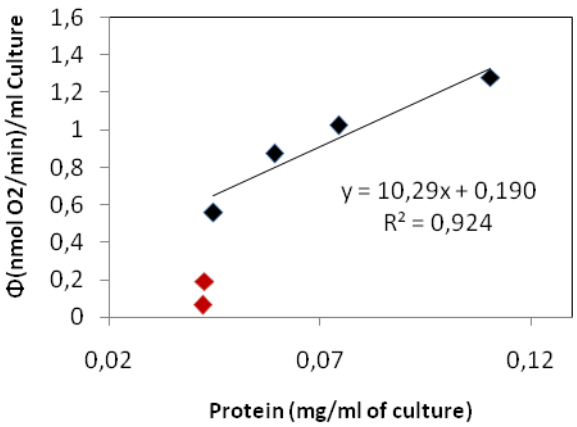

Fig. 29 Φ vs Protein on the exponential phase of the *R. salina* experiments.Here the Φ /protein is 10.3 nmol $O_2$$min^{-1}$ (mg protein)$^{-1}$.

Table II: Protein-Φ relationship during the exponential phase of the *R. salina* cultures. The number of points used in the regression analyses for the determination of the Φ/protein ratio is given by Φ /protein in units of nmol $O_2$$min^{-1}$ (mg protein)$^{-1}$.

Exp	Φ/Protein	r^2	n
1	11,11	0,630	4
2	10,29	0,924	4
3	17,21	0,638	4

R. salina: Cell number-Φ relationship

As above for the protein-Φ relationship, the cell number-Φ relationship is helpful in understanding the nutritionally-driven shifts in a cell's physiological state. Here, however the shift should be different. Because the ETS is constituative, nutrient-limitation should not change the Φ per cell. It should remain constant throughout all phases of the cell's life. Unfortunately, because of the bacterial growth in the later phases of the culture only the data from the exponential phase of the *R. salina* cultures are presented, never the less, documenting this ratio is an important autecological result for *R. salina*. Fig. 30 and *Table III* show that the Φ per cell ratio ranges from 1 to 0.7 femtomol O_2 min^{-1} $cell^{-1}$ and here in all three experiments, all the measurements followed the same trend.

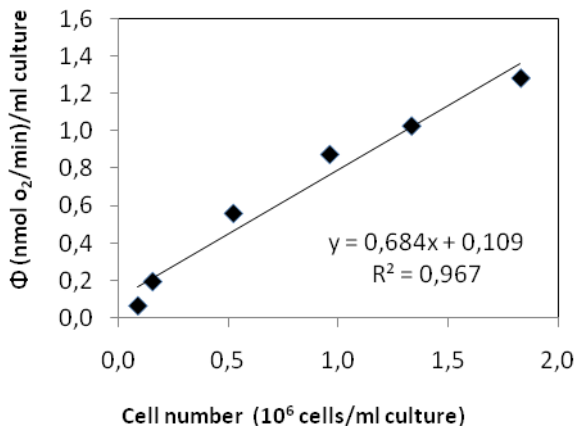

Fig. 30 Φ vs Cell number in the exponential phase of the *R. salina* experiments. The Φ/Cell here is 0,685 fmol O_2min^{-1} cell^{-1}.

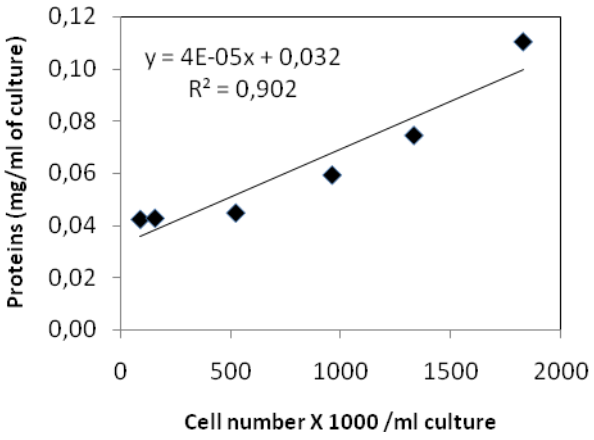

Fig. 31 Protein vs Cell number in the exponential phase of the *R. salina* experiments. The slope here is 40 picog protein per cell.

Table III: Cell number-Φ relationship during exponential phase of the experiment on the R. salina cultures. The original units of the cell number and Φ are the units in Fig.30 so the ratio is in femtomol O_2 min^{-1} cell^{-1}.

Exp	Φ/cells	r²	n
1	0,986	0,707	6
2	0,684	0,987	6
3	1,107	0,904	6

Table IV: Protein per cell during the exponential phase of the R. salina experiments. The units are pg protein/cell.

Exp	protein /cell	r²	n
1	70	0,839	6
2	40	0,902	6
3	30	0,566	6

R. salina: Protein per cell relationship

One would expect that if nutrients are available to the alga, the cell-protein in the culture would also increase with growth (Fig. 31). Accordingly, in the exponential phase, while the culture is nutrient sufficient, the protein should increase. Upon nutrient exhaustion, the protein in the culture should decrease. However, on the basis of the flow cytometry data which indicates increasing bacterial contamination after the exponential phase, the data from these latter phases were excluded. Only the data from the exponential phase are presented here.

The mean protein/cell ratio for the exponential phase in the three experiments is 47 pg/cell (*Table IV*).

R. salina: Chlorophyll- cell number relationship

If there is a direct relationship between the chlorophyll a and the nutrient concentration, one could expect that high nutrient levels available to the cells in exponential phase would translate to high chlorophyll concentrations. The relation between nutrients and chlorophyll can be seen in Fig. 12 In the exponential phase when the nutrients are available, the chl increases. Later, when the nutrients are limiting (12-35 days) the chlorophyll plateaus and then decreases.

In the three experiments done on the *R. salina* cultures, chlorophyll a cell^{-1} increases during the exponential phase. This increase parallels the cell density increase as shown in Fig. 32. The average ratio (Chl/cell #) is 0,969 pg/cell (*Table V*).

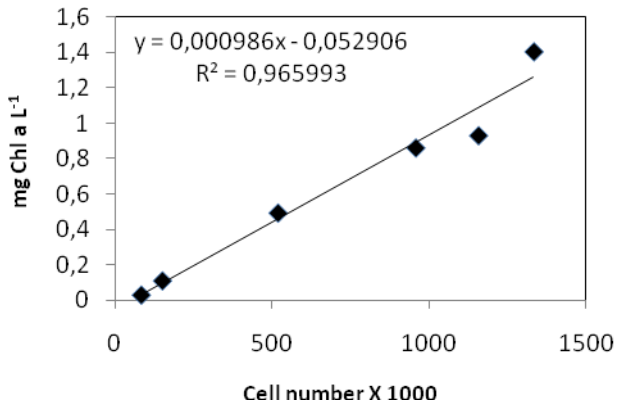

Fig. 32 Chlorophyll *a* vs Cell number in the exponential phase of the *R. salina* experiments. Here the chlorophyll per cell was 0.986pg Chl/ cell.

Table V: Cell number-chlorophyll relationship during exponential phase of the experiment on the R. salina cultures. The units for chl/cells are pgchl/cell

Exp	chl /cell	r²	n
1	0,963	0,810	6
2	0,986	0,965	6
3	0,958	0,955	6

O. marina: Protein-Φ relationship

As mentioned above, the protein-specific enzyme activity is useful in determining enzyme kinetic parameters. In *O. marina* the Φ-protein ratio should tend to rise when all the *R. salina* have been eaten and the *O. marina* begins to lose protein during starvation (Fig. 27). Unfortunately, cannibalism precluded the possibility to observe this shift. Here we documented the Φ-protein ratio in the *O. marina* population after they had consumed the *R. salina* and while it was entering a cannibalistic phase. We called it a cell-decline phase rather than a death phase because the surviving cannibals were very much alive. Even though the protein declined in the culture (Fig. 27) the cells were hardly dying. In any case, the protein-specific Φ ranged from 12,35 to 12,34nmol O_2 min^{-1}(mg protein)$^{-1}$as shown in Fig. 33 and *Table VI*.

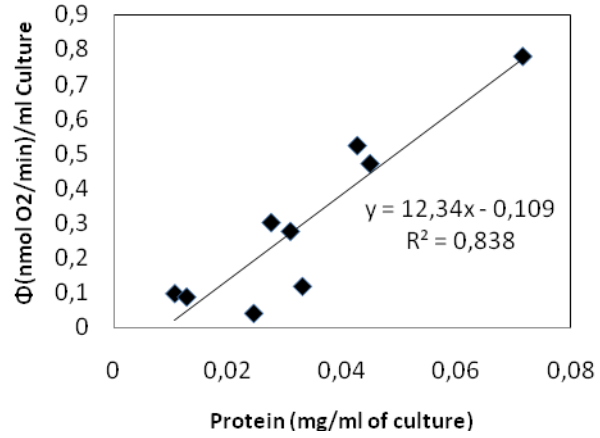

Fig. 33 Φ vs Protein on the cell-decline phase of the *O. marina* experiments. Here the Φ/protein is 12,34nmolO_2/min)/(mg protein).

Table VI: Protein-Φ relationship on *O. marina* during the cell-decline phase of the experiments. The units for protein are mg/ml of culture and Φ units are (nmolO_2/min)/(mg protein). *The protein data for this experiment were erroneous and not included.

Exp	Φ/Protein	r^2	n
1	12,35	0,662	9
2	12,34	0,838	9
3	*	*	*

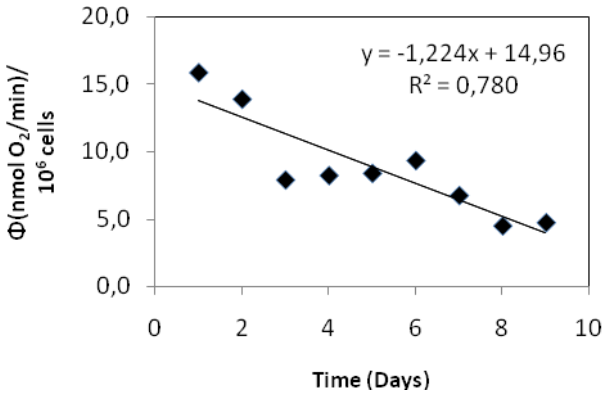

Fig.34 Cell specific Potential respiration on a *O. marina* culture.

O. marina: Cell number-Φ relationship

In O. marina, after consuming all the *R. salina*, the cell number-Φ relationship should remain constant, but did not, it increased (Fig. 35).

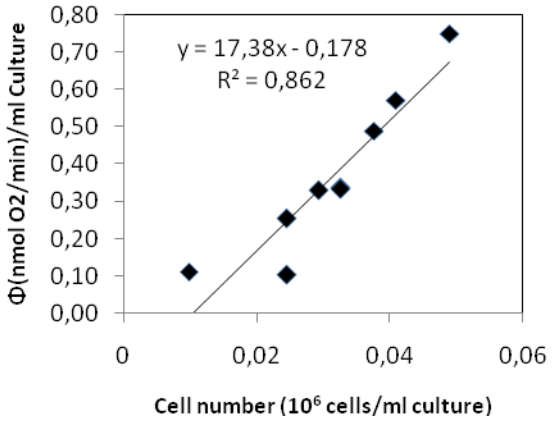

Fig. 35 Φ vs cell number in the cell-decline phase of the *O. marina* experiments. The /cell is 17.4 fmol O_2 min^{-1} $cell^{-1}$.

Table VII: Φ/cell in *O. marina* cultures. The units of the cell number are (10^6 cells/ml culture); Φ units are ($nmolO_2$/min)/(mlculture).Φ/Cell is thus given in units of fmol O_2 min^{-1} $cell^{-1}$.

Exp	Φ/Cell	r²	n
1	17,38	0,862	9
2	15,55	0,829	9
3	25,34	0,943	9

O. marina: Cell number - protein relationship

Here the protein per cell in the recently well-fed *O. marina* population ranges from 1060 to 826pg protein/cell (*Table VIII and* Fig 36) an order of magnitude higher than in their algal prey (*Table IV*).

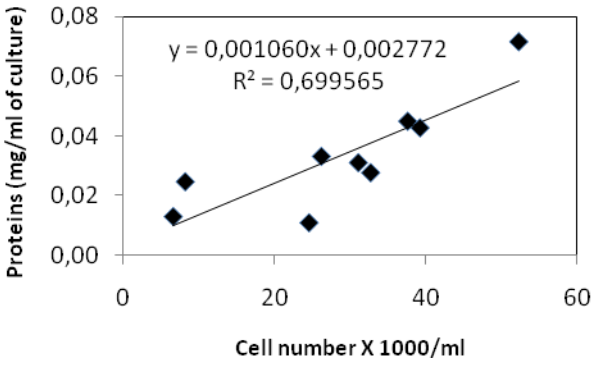

Fig. 36 protein vs cell number on the cell-decline phase of the *O. marina* experiments. From the regression here the protein/ cell for this experiment is 1060 pg/cell (Table VIII).

Table VIII: Cell number-protein relationship on *O. marina* during the cell-decline phase experiments. The units for protein/cell are pg/cell. *The protein data for experiment #3 were erroneous and not included.

Exp	Protein /Cell	r²	n
1	824	0,4	9
2	1060	0,69	9
3	*	*	*

O. marina: Cell number - chl relationship

O. marina is heterotrophic and does not contain chloroplasts. Its pigmentation is caused by the pigments in its algal prey. Never the less, after feeding on *R. salina* it contains measureable chlorophyll (Fig. 37 and *Table IX*). If the chloroplasts of the *R. salina* were digested then the chlorophyll/cell in *O. marina* should decrease. It does not (Fig. 38). This suggests that the dinoflagellate maintains the *R. salina* chloroplasts to harvest the photosynthate the chloroplasts may produce upon illumination. In this case *O. marina* would be acting like the ciliate *Mesodinium rubrum*. In any case the ratio chl/cell number in *O. marina* range from 38 – 63pg/cell (*Table IX*) in the three experiments.

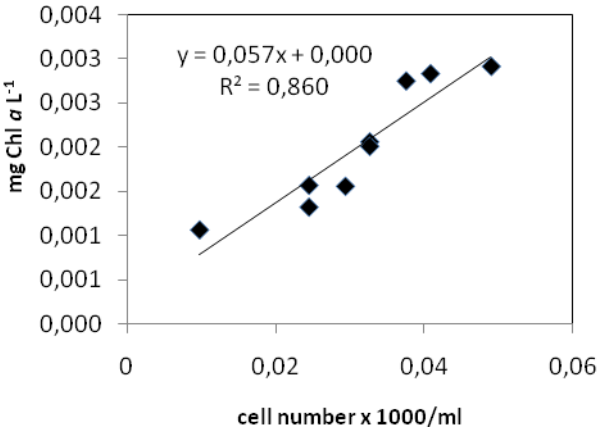

Fig. 37 Chlorophyll vs cell numberin the cell-decline phase of *O. marina*. Here the regression yields a chlorophyll per cell value of 57.2 pg/cell.

Table IX: Cell number-chlorophyll a relationship in *O. marina* during its cell-decline phase. The units for chl/cell are pg/cell.

Exp	chl /Cell	r²	n
1	57	0,86	9
2	38	0,72	9
3	63	0,55	9

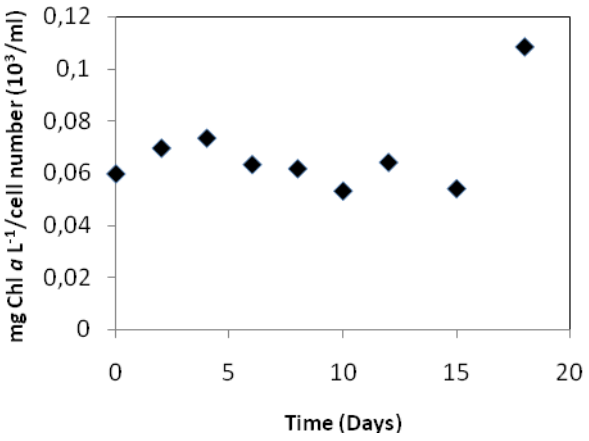

Fig. 38 Cell specific chlorophyll on a *O. marina* culture.

Discussion

ETS activity has never been measured in *R. salina* and *O. marina* before. However, chlorophyll *a* and protein have been measured frequently in both organisms (Hammer et al., 2002); Lafearga-De la Cruz (2005); Seixas (2009) and so we are able to compare our measurements with others in the literature (Table X). They compare favorably and lend confidence to the results. Seixas et al (2009) found that the protein/cell in *R. salina lens* was 65.0 ± 2.5 picog protein/cell (calculated from their phycoerythrin/cell data.). This is within the range in Table IV of 30-70 pg protein/cell for our *R. salina.* The chlorophyll per cell for all three experiments (*Table V*) was about 1 pg/cell comparing favorably with the value of 1.35 pg/cell obtained by LaFarga et al. (2005).

To evaluate the Φ measurements made here one must turn to the research of Hammer et al.(2002). They found that the respiration per chl *a*, at an irradiance of 30 µmol photons m^{-2} s^{-1} and 20° C was 0,62 $nmolO_2(\mu gchla)^{-1}min^{-1}$ and here Φ at an irradiance of 31.5 µmol photons m^{-2} s^{-1} and 22.5°C was 1,0$nmolO_2(\mu gchla)^{-1}min^{-1}$. If our value of Φ was corrected to 20° C it will be about 0,89 $nmolO_2(\mu gchla)^{-1}min^{-1}$.

Table X:

Species	Medium	Chl/cell (pg/cell)	Pro/cell (pg/cell)	References
R. salina	f/2	0,969	46,6	This paper
R. salina	f/2	1,1 – 1,7		Lafarga et al, 2005
R. salina lens	*		65,0±2,5	Seixas et al, 2009
R. salina sp	f/2		7,6-19,1	Valenzuela et al, 2005

* Nutrient saturated conditions.

Here it is appropriate to point out that these types of basic physiological measurements made on oceanographically significant organisms are important to anchor, in biological reality, measurements that are made at sea. This research is an early step in defining some basic biochemical and physiological parameters associated with respiration that will facilitate the interpretation of future open ocean field measurements.

This research project fortuitously, was able to employ flow cytometry in characterizing the microbial populations inhabiting the *R. salina* and the *O. marina* cultures. Flow cytometry analysis permits the verification of biological conditions in the culture during an experiment. This is particularly important when working with mixed populations in non-axenic cultures. The flow cytometer views of the changing cell-distribution patterns can help in the interpretation of physiological and biochemical measurements made on the mixed population (Figs. 6-7 and 9-11). In the batch culture experiments done here, nutrients were added only in the beginning. This

procedure guaranteed that, after the initial nutrient uptake, the changes in both the dissolved and particulate organic and inorganic components in the flasks were caused by internal predation, growth, and death of the different populations of bacteria, *R. salina*, and *O. marina*. Flow cytometry measurements made during these experiments provided data on the changing cell-density of *R. salina.* This in turn, made it possible to view the different growth phases of *R. salina* (Fig. 5). Furthermore, it made it possible to track bacterial cells mixed with organic and inorganic debris present in the experimental flask and compare this progression with *R. salinas* and *O. marina* cell-densities though out the experiment (Fig. 6-11).

The histograms in Fig. 6 show the low levels of bacteria and culture debris (organic and inorganic particles) in the beginning of the experiment. In Fig. 7 one can see that by the end of the stationary phase the bacteria and debris density is as high as the cell-density of the *R. salina* population. Under these conditions any measurement made on the culture would reflect the biochemical and physiological properties of both the bacterial and either the *O. marina* or the *R. Salinas* populations. Since the bacteria population is uncharacterized, such measurements are meaningless and cannot be reported. For this reason the work reported here is based only on the exponential growth phase when the biological contamination of the cultures was minimal.

After stationary phase, when the culture enters the cell-decline phase, the cell density of the *R. salina* decreases. In the histogram in Fig 9 .the region 1 represents the location of the *R. salina* at the beginning of the experiment. This location in the FL2-Cfla domain is the reproducible “home” of the R. salina population in the early days of the experiment. As the culture ages the *R. salina* population-location migrates. The other histograms (Fig. 10 and 11) show this dispersion of the *R. salina* cells. Using the inverted microscope one can see the cultures deteriorating. The cells move slower, they form aggregates, and many *R. salina* become immobile.

From the flow cytometer results, the *R. salina* cell-density decreases after day 35 while at the same time the debris density (including bacteria) increases. As a result, the ETS activity, protein and chlorophyll *a* during the following days cease to track one another.

Analysis of ETS activity, protein and chlorophyll a during the exponential, stationary and cell-decline phase reveals that during the 64 days of the experiment the ETS activity tracks protein (Fig. 14). This is expected because ETS activity is a ubiquitous property of living cells regardless of whether they are bacteria, algae, or protozoans. During the same 64 days (Fig. 12) the ETS and Chlorophyll *a* track each other only during the exponential phase. In the stationary phase when the ETS activity is significantly influenced by the growing bacterial (without chlorophyll) the ETS activity continues to rise while the chlorophyll falls. If the ETS activity were only associated with the *R. salina* cells it would have tracked the chlorophyll *a* as well as the *R. salina* cell-density. But, as one can see in Fig 12, this was not the case because the bacteria captured on the filters along with the *R. salina* cells inflates the ETS signal, but not the chlorophyll one. At the beginning of the experiment when the bacterial population is low, the filters capture only *R. salina* cells and very few bacteria. However, as time passes and the culture reaches the stationary phase the bacterial population rivals that of the *R. salina.* As a result the ETS activity and the chlorophyll are determined principally by the *R. salina* cells only in the beginning. Afterwards the *R. salina* cells continue to determine the chlorophyll signal, but the ETS signal is determined by both the *R. salina* and the bacteria. This explains the poor relationship between the ETS activity and the chlorophyll *a* during the end of the stationary phase and during the cell-decline phase.

In the *O. marina* cultures was not possible to measure the ETS activity, chlorophyll *a*, and protein concentrations on a complete growth curve because of the presence of *R. salina*, (*O. marina´* food), in the experimental flasks. The *O. marina* culture was started by a small inoculum in order to have a similar *O. marina´* population size. The experiment was started two days after *R.*

salina disappeared from the medium. This period in the *O. salinas* culture after the *R. marina* cells disappear is consider a cell-decline phase (no food available)and the Φ decreased as the chlorophyll *a*, protein and cell density decreased on the first four days, After these four days, the density, chlorophyll a, and protein stabilized (Fig. 24, 26 and 27).

CONCLUSION

1. The ETS analyses on the *R. salina* and *O. marina* cultures, demonstrate that without substrate addition (NADPH and NADH), the ETS activity is negligible (Fig. 3 and 4). This result argues that the zero-substrate tetrazolium reduction assay of Balmstedt (2000) can not accurately detect respiratory ETS activity.

2. Flow cytometry analysis of the size spectrum the particles and cells in a culture (Figs. 6-7; 9-11) provides valuable information on the characteristics of population growth. This information is important for interpreting the results and also for planning future experiments.

3. *O. marina* was metabolically more active than *R. salina*. Its Φ was 19 nmO_2 min^{-1} $cell^{-1}$in contrast to 0,9 nmO_2 min^{-1} $cell^{-1}$It was also richer in protein than *R. salina*: 942 versus 47 pg protein/cell.

4. O. marina maintains the level of ingested chlorophyll for days suggesting a type of symbiosis with the *R. salina* chloroplasts.

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