Behavior of respiratory metabolism and pyridine nucleotide levels in *Oxyrrhis marina* during starvation.

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Running head: Respiratory metabolism and PNs in *O. marina*.

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Abstract

Respiration in eukaryotes is a physiological process that occurs wherever the oxygen and organic matter are present. Respiration is oxygen consumption (R_{O2}) catalyzed by the respiratory electron transport system (ETS) enzymes. The physiological R_{O2} rate depends directly on the enzymatic control of the respiratory ETS activity. According to Packard et al. (1996), we argue that substrate availability is the most probable regulatory mechanism controlling this activity. The major ETS substrates are the pyridine nucleotides (PNs) and they occur as nicotine adenine dinucleotide (NAD) and as nicotine adenine dinucleotide phosphate (NADP). Up to now, no studies have quantified their intracellular concentrations in a marine dinoflagellate. *Oxyrrhis marina* is a microheterotrophic marine dinoflagellate which has been widely investigated. Nevertheless, no study has characterized its respiratory metabolism from measurements of oxygen consumption (R_{O2}) and ETS activity. For this reason, our main objectives were 1) to measure R_{O2} and ETS activity in *O. marina* and to determine their behavior during starvation, and 2) to quantify the PN levels during the same period. To achieve these objectives, we fed *O. marina ad libitum* and then we monitored the ETS, R_{O2}, protein and PN levels for 17 days.

Our results showed a divergence of R_{O2} and ETS activity during the experimental time, as the R_{O2} decreased and the ETS activity remained nearly constant. The intracellular PN concentrations decreased in a manner parallel to the R_{O2}. In fact, we observed that both parameters were well-correlated. Finally, we concluded that the difference in PN concentration between well-fed and starved organism supports the use of this methodology in measuring changes in PN levels in *O. marina*.

Keywords: Respiration, ETS activity, pyridine nucleotides, NADt and NADPt, *Oxyrrhis marina*.

INTRODUCTION

Respiration is a fundamental measurement of the physiological activity of organisms. It is a ubiquitous process that occurs at all depths and in all regions of the oceans where oxygen and organisms are present (Packard and Williams, 1981). Despite the importance of respiration rate as a key component in the estimation of the carbon flux in the biosphere, its magnitude remains uncertain (del Giorgio and Duarte, 2002). This lack of information is grounded in the difficulty in quantifying the *in situ* respiration rates in different areas and depths with currently available techniques. Moreover, direct measurements of respiration are accomplished by quantifying the oxygen consumption (R_{O2}) rate in incubated organisms. This is tedious and time consuming work that diminishes the raw data acquisition rate and in oceanography the data acquisition rate is a major consideration in planning research.

For this reason, Packard et al (1971) devised a biochemical assay to estimate the R_{O2} rates by measuring the activity of the key enzymes involved in the process, in this case, the respiratory electron transfer system (ETS) enzymes. The ETS is composed of pyridine nucleotide dehydrogenases, flavin nucleotide dehydrogenases and cytochrome oxidoreductases arranged in four complexes (Lehninger et al., 2005), which together account for more than 95 % of all aerobic and anaerobic respiration. This enzymatic system is embedded in the inner mitochondrial membrane in eukaryotic cells and in the plasma membrane in prokaryotic cells. The ETS assay of Packard et al (1971) was designed to measure, under substrate saturation, the maximum activity of these enzymes, i.e., the potential respiration (ϕ). Additionally, it was designed to detect the ETS activity in all organisms from bacteria to zooplankton so as to serve as a measure of ϕ in a parcel of seawater as well as to reflect the integrated metabolism of the entire organism when measured on an individual (Pomeroy, 1974).

Due to the strong causal biochemical relationship between the physiological R_{O2} and the ETS activity, the control in the former should be determined by the control in the latter. Thus, Packard et al. (1996) argued that the most quantitative regulatory mechanism

controlling the respiratory ETS activity is the substrate availability. The ETS substrates are the pyridine nucleotides (hereinafter, PNs) and the succinate. However, the PNs play the dominant role. They occur in oxidized and reduced form as nicotine adenine dinucleotide (NAD^+, NADH) and as nicotine adenine dinucleotide phosphate ($NADP^+$, NADPH). Both PNs are ubiquitous in living cells (Penfound and Foster, 1996; Kurnasov et al., 2003) and they play a key role in providing reducing equivalents for hundreds of cellular reactions. NAD(H) is mainly used catabolically by the enzymes that catalyze substrate oxidation, while NADP(H) is mainly used anabolically by enzymes involve in substrate reduction (Pollack et al., 2007). Their major biological functions are modulating energy metabolism, reductive biosynthesis and antioxidation processes (Ying, 2008). Furthermore, recent studies show that NAD(H) and NADP(H) play important roles in cell signaling in animals, plants and fungi (Hunt et al, 2004; Berger et al., 2004; Ziegler, 2005).

As a consequence of the impact of PNs on almost every metabolic pathway, they have been widely investigated in biomedicine and in plant and animal physiology. Thus, in the 50's and 60's, Chance et al. (1955; 1962) and Lowry et al. (1961) developed different techniques to measure the amount of PNs in cells, fluorometrically and spectrophotometrically, respectively. After that, a large number of relevant publications appeared applying these techniques to study the behavior of animal physiology under different physiological conditions (Mayevsky and Chance, 2007 and reference therein; Zerez et al, 1984, among many others). The knowledge of PN levels in plants is more recent and is increasing steadily with studies on the role of PNs in mitochondria (Moller and Rasmusson, 1994) and their levels and redox state in cells (Agius et al., 2001; Hagerdon et al., 2004) under different metabolic situations (Kasimova et al., 2006; Noctor et al., 2006). More recently, Wos and Pollard (2009) demonstrated the usefulness of measuring NADH for estimating the bacterial metabolic activity in heterogeneous bacterial ecosystems.

Despite the well-described importance of PNs in other fields, few attempts have been made to characterize their role in marine organisms. Roy and Packard (1998) measured the amount of $NADP^+$ in the marine bacterium *Pseudomonas nautica* and compared it with

isocitrate dehydrogenase activity. On the other hand, Steigenberger et al. (2004) attempted to estimate marine primary production with the NADPH fluorescence. However, to our knowledge, no work has described the intracellular PN levels in marine organisms or has compared them with respiration rates.

In the present laboratory study we have focused on the respiratory metabolism of the marine dinoflagellate O*xyrrhis marina*. This microzooplankter was chosen, rather than a phytoplankter or metazoan, to avoid the problems that the photosynthetic ETS or a longer life cycle might have added. Additionally, increasing evidence demonstrates the importance of microzooplankton in the marine foodwebs as they are involved in energy transfer between trophic levels (Calbet and Landry, 2004). The ubiquitously distributed *O. marina* has extensively served as a model organism to examine the response of the heterotrophic dinoflagellates to a variety of factors, such as salinity (Droop, 1959), temperature (Kimmance, 2001), or food concentration (Flynn and Davidson, 1993), or an interactions of the last two (Kimmance et al., 2006). Likewise, Jeong et al. (2003) have described *O. marina's* role in controlling red tides. Nevertheless, no attempt has been made to determine the respiratory metabolism in *O. marina* using the oxygen consumption and the ETS activity.

For this reason the main objectives of this work are: 1) to characterize the respiratory metabolism in *O. marina* with measurements of oxygen consumption and ETS activity during a food deprivation period, and 2) to explore the role of PNs levels in controlling the cellular respiration rates during the same starvation period.

METHODS

Culture conditions

The microalga *Rhodomonas salina* and the heterotrophic dinoflagellate *O. marina* were obtained from the microalgal culture collection of the Institute of Marine Sciences of Barcelona (ICM-CSIC, Spain). Batch cultures of both organisms were grown non-axenically in 1L Erlenmeyer flasks with filtered and UVA irradiated seawater enriched with f/2 medium

(Guillard, 1975; Andersen, 2005). The temperature, pH and salinity conditions were the same for both cultures (22.5ºC ± 0.5 ºC; 8; 38 psu, respectively). Light for culture maintenance, provided by fluorescent cool white light bulbs, was continuous. However, the light intensity for the different species was different: the Rhodomonas cultures were exposed to high light conditions (31.5 µmol photons $m^{-2}s^{-1}$) while the dinoflagellates were cultured at dim light (0.8 µmol photons $m^{-2}s^{-1}$). Irradiance was measured using a radiometer (Data Logger LI-1400). All experimental flasks were capped with cotton plugs in order to allow gas exchange and to avoid contamination and evaporation. Twice a day, the flasks were gently swirled to maintain the cells in suspension. A well fed mother culture of *O. marina* was used to supply inoculums for the experimental cultures. The culturing protocol is described more fully in Aristizabal (2009).

Experimental design

Triplicate cultures of *O. marina* were maintained in three 1 L pyrex glass Erlenmeyer flasks maintained under the experimental culture conditions described above. Each flask, containing approximately the same population level of *R. salinas*, was inoculated with the same volume of the well-mixed mother culture of *O. marina*. During the 21 days of the experiment, no more food was added.

The sampling began when the amount of the *R. marina* in the culture was negligible. This was verified by daily microscope examination of the samples. Hereafter, cell samples were taken every two days for measurements of chlorophyll, protein, ETS activity, PN levels and respiration. At the same time, a 1 ml aliquot from every experimental flask was taken, fixed with lugol (4%), and counted using a double counting chamber (Neubauer) to estimate the cell number in the cultures.

Respiration measurements

In the present work, the experimentally determined R_{O2} refers to the O_2 consumption by the *O. marina* culture. The R_{O2} units are nmol O_2 min^{-1} l^{-1}. A 6-channel oxygen sensor (mod 928, Strathkelvin Instruments) was used to record changes in the dissolved O_2 concentration

during incubation times ranging from 1 to 2 hours. Three of the 6 channels measured samples (2ml) from each culture experiment while the other 3 channels measured filtered seawater as a control. All the respiration measurements were realized in darkness and under temperature controlled conditions (22.5ºC), using a waterbath (J.P. Selecta).

Biochemical Sampling

Samples for the biochemical parameters were filtered through 25 mm Whatman GF/F filters. For the chlorophyll, protein and ETS activity measurements filtered volumes were 2 ml, while for PN they were 20 ml. Experimental volumes for the PN samples were verified by previous experimentation (data not shown). Filters were folded to an eighth, blotted thrice to remove excess seawater, placed in a capped cryovial and immediately frozen in liquid nitrogen for 30 minutes. Afterwards, they were transferred to the freezer at - 80ºC and stored in the darkness until measurement. Seawater for both the ETS and PN filter blanks was drawn from the sample filtrates.

Analytical methods

ETS activity measurements. ETS assays were determined following the methodology described by Packard et al. (1996). The frozen filters were thawed for a few seconds at room temperature before the analysis. They were homogenized in 1 ml of phosphate buffer (0,05 M; pH 8) for 2 min in a Teflon- glass tissue grinder, powered by a drill motor (Einhell SB 701/1) at 1500 rpm. Then, the homogenates were centrifuged for 2 min at 2000 rpm at 2ºC. The supernatant fluids were kept on ice until analyzed and the pellets were discarded. The measurements were made kinetically using a spectrophotometer (Beckman Du 650), in which the temperature was controlled by refrigerated water bath held at 22.5ºC. In 2 plastic cuvettes of 1-cm light path, 100 µl of the supernatant, 100 µl of 4mM p-iodonitrotetrazolium violet (INT) solution and 300 µl of the ETS reaction mixture were added. This reaction buffer contained NADH and NADPH within the range of 0.63-0.78 mg/ml and 0.21-0.31 mg/ml, respectively. Additionally, duplicate blanks were performed containing the same volumes of supernatant and INT solution, but with 300 µl of substrate blank (buffer without ETS substrates) instead of

the ETS reaction mixture. Immediately, the 4 cuvettes were placed in the spectrophotometer and the formation of formazan was monitored at 490 nm for 8 minutes. The formazan production rate is stoichiometrically related by a factor of 2 to ETS activity and by a factor of 0.5 to ϕ. The details of these calculations were described in Packard and Christensen (2004). Results are reported as ϕ in nmol O_2 min^{-1} l^{-1}.

Pyridine nucleotides measurements. The pyridine nucleotide assay that we used was developed by Wagner & Scott (1994) and largely used in clinical analysis (Umemura and Kimura, 2004; Rogers et al., 2009). We adapted it to fit our needs.

I) Extraction. Filters were thawed at room temperature and immediately homogenized for 2 minutes in a Teflon- glass tissue grinder at 1500 rpm. The homogenization process was accomplished with the samples kept in an ice bath. Filters were ground up in 2 ml of an alkaline extraction solution containing 20 mM nicotinamide, 20 mM sodium bicarbonate ($NaHCO_3$) and 100 mM sodium carbonate (Na_2CO_3). Then, the homogenates were centrifuged for 2 min at 3000 rpm and 2ºC. The supernatants were immediately placed on ice and protected from light. The same extraction lysates were analyzed for NADt, NADPt, NADH, NADPH. Measurements of the total pyridine nucleotides pool (NADt, NADPt) were performed by measuring the spectrophotometric changes in absorbance (570 nm) during a cycling reaction with the unheated extracts. To quantify the concentration of the reduced forms (NADH, NADPH), 1 ml aliquots of the extracts were incubated at 60ºC during 30 min and then were assayed using the same cycling reaction. Thereby, the heat destroys the oxidized forms while has no effect on the reduced forms (Wagner & Scott, 1994).

II) Cycling reaction for determination of NADt and NADH. The reactions in the NAD cycling buffer were as follows:

(1) Ethanol + NAD^+ → Acetaldehyde + NADH + H^+ (catalyzed by ADH)

(2) NADH + PES_{ox} → NAD^+ + PES_{red} (non enzymatic)

(3) PES_{red} + MTT → MTT- formazan + PES_{ox} (non enzymatic)

One hundred microliters of the heated/unheated extracts were added to 800 µl of ice-cold, freshly prepared NAD cycling buffer and incubated in the dark at 37ºC for 5 min. The NAD cycling buffer contained 100 mM Tris-HCl (pH 8.0), 0,5 mM thiazolyl blue (MTT), 1 mM phenazine ethosulfate (PES), 0,2 mg/ml alcohol dehydrogenase (ADH) and 1% bovine serum albumin (BSA). Following temperature equilibration, 100 µl of ethanol was added to the sample. The sample was mixed and then centrifuged (16000g, 30 s) to remove any insoluble materials. The change in absorbance at 570 nm (corrected for turbidity) was monitored over 3 min at 37ºC in a thermoregulated cuvette holder in a spectrophotometer. This absorbance data were corrected for turbidity by subtracting the absorbance at 750 nm.

III) Cycling reaction for determination of NADPt / NADPH. The reactions in the NADP cycling buffer were as follows:

(4) Glucose-6-P + $NADP^+$ → 6-phosphogluconate + $NADPH+H^+$ (catalyzed by G6P DH)

(5) NADPH + PES_{ox} → $NADP^+$ + PES_{red} (non enzymatic)

(6) PES_{red} + MTT → MTT- formazan + PES_{ox} (non enzymatic)

One hundred microliters of the heated /unheated extracts were added to 800 µl of freshly prepared NADP cycling buffer (4ºC) and then incubated at 37ºC, in the dark, for 5 min. The NADP cycling buffer contained 100 mM Tris-HCl (pH 8.0), 0,5 mM thiazolyl blue (MTT), 2 mM phenazine ethosulfate (PES), 5 mM Na_4EDTA and 1.3 IU/ml of glucose-6-phosphate dehydrogenase (G6P DH). Following incubation, 100 µl of 10 mM glucose-6-phosphate was added to the sample. The sample was mixed and the change in absorbance at 570 nm (corrected for turbidity) was measured over 3 min at 37ºC.

IV) Standard curves. Each standard curve was constructed from measurements of the velocity of MTT-formazan production by the enzyme cycling system, starting at seven different NAD and NADP concentrations, ranging from 0 to 200 nM. Figure 1 shows the example for the NADPt. Note that the formazan production rate is defined by the slope. A curve of slopes versus NADH and NADPH concentrations (Fig. 2) was then used to quantify the amount of pyridine nucleotides present in the samples. The standards solutions were prepared by the addition of the appropriate quantity of pyridine nucleotides to 1ml of

extraction buffer. One hundred microliters of these solutions were assayed as described above for the NADt and NADPt. The commercially available NADH and NADPH are not entirely pure and, in addition, they oxidize during storage. For this reason, a standard curve for the reduced nucleotides was also done.

Protein measurements. Protein was analyzed by the Lowry method (Lowry et al., 1951) as modified by Rutter (1967). The units are milligrams of protein per liter of culture. Bovine serum albumin (BSA) was used as a standard.

Chlorophyll *a* measurements. Chlorophyll *a* concentration was determined fluorometrically according to the method described by Holm-Hansen et al. (1965) and reported as micrograms of chlorophyll per liter of culture.

RESULTS

Time profiles of protein, cell number, chlorophyll *a*, ETS activity and respiration in batch cultures of *O. marina*

The experimental observations show the time course of the dinoflagellate cultures during the food deprivation period (Fig. 3). The experiment began when the number of *R. salina* cells in the medium was negligible. This was verified by inverted microscope observations and by measurements of the chlorophyll present in the cultures (Fig 3C). The protein (Fig. 3A) and the potential respiration (Fig. 3D) were nearly constant during the experiment. On the contrary, the cell number and R_{O2} (Fig. 3B and 3E, respectively) decreased exponentially. In fact, both cell number and R_{O2} decreased to more than 50 % of their initial values in the first nine days of the experiment. The difference between the behavior of the respiration and the potential respiration was striking.

Cell-specific time courses were calculated for R_{O2}, ϕ and protein measurements (Fig. 4). The R_{O2} per cell decreased from 300 fmol $O_2 h^{-1} cell^{-1}$ under well-fed conditions to 50 fmol $O_2 h^{-1} cell^{-1}$ by the end of the experiment when the cells were starved. By sharp contrast, the

opposite pattern was observed in the specific ϕ and protein content. Thus, ϕ increased from 200 fmol $O_2h^{-1}cell^{-1}$ to 800 fmol O_2h^{-1} $cell^{-1}$ during the starvation period. Similarly, the protein per cell increased from 0.4 ng $cell^{-1}$ to 3.0 ng $cell^{-1}$.

R / ϕ ratios in relation to starvation and cell size.

The ETS activity measurements provide potential respiration rates of ϕ. To calculate the *in vivo* R_{O2} in the ocean, calibration studies such as that of Arístegui and Montero (1996) or del Giorgio (1992) are used. Nevertheless, Hernández- León and Gómez (1996), in equating ETS to ϕ, pointed out that the R_{O2}/ϕ ratios are affected by different biological and physical factors, i. e., temperature, quantity/quality of food and size. As we measured the R_{O2} and ϕ at the same temperature during the entire experiment, the R/ ϕ ratios were not affected by this factor. Our results showed an exponential decline of the R/ ϕ ratio with the time of starvation (Fig. 5A).The same pattern was observed when comparing the R/ ϕ ratio with the cell size (Fig 5B), considering the amount of protein per cell as a proxy for the cell size.

PN levels and R_{O2} during starvation

The PNs are the substrates for the ETS, but they are also involved in many other enzymatic reactions. We expected the intracellular PN concentrations in *O. marina* to be affected by the food availability as in marine bacteria (Packard et al., 1996). As the organic molecules available for obtaining metabolic energy become exhausted, the intracellular PN levels should decrease. Our data support this prediction since the protein-specific PN levels decreased exponentially with the increase in time of starvation (Fig 6). The general features of the NADt (Fig. 6A) and the NADPt (Fig. 6B) time courses were similar, although the concentration of NADt was two-fold higher than that of NADPt. The mean values of intracellular NADt ranged from 500 pmol mg^{-1}prot, at well fed conditions, to 70 pmol mg^{-1} prot, at starvation. The mean values of intracellular NADPt ranged from 220 pmol mg^{-1}prot, at well fed conditions, to 70 pmol mg^{-1} prot, at starvation. The data for the reduced PN are not shown because they were not conclusive.

The R_{O2} time course (Fig. 3D) tracked both NADt and NADPt time courses (Fig 6) throughout the entire experiment. Figure 7 shows the correlation between R_{O2} and the NADt and NADPt, which are described by the following regression equations: R_{O2} = 0,028 (NADt) + 2,80 (n = 23; r^2 = 0,75), and R_{O2} = 0,06 (NADPt) + 3,15 (n = 23; r^2 = 0,83).

DISCUSSION

Behavior of the time profiles during starvation

The features of the respiratory metabolism of *O. marina* throughout the starvation period have been documented in the present work. Our experimentally determined respiration rates showed an exponential decrease with increasing starvation time. Moreover, these R_{O2} rates, as normalized per cell, were almost 6 times higher in well fed organisms than in starved ones. The same pattern with R_{O2} was observed by Kiørboe et al. (1985) in the copepod *Acartia tonsa* and by Lampert (1986) in the cladoceran *Daphnia magna*. Thus, the recently fed copepods respired more than 4 times the rates of the starved individuals (data taken from their figure 6). The difference in the rates between their study and ours might be dues to the different experimental times. They measured respiration for 4 days, while our experiment lasted 17 days. In the same manner, a rapid decrease in respiration was found after food deprivation in *D. magna*. Additionally, Grigoriou and Richardson (2009) observed that the decreasing trend in respiration also holds during starvation for larger animals. They observed that the R_{O2} declined by 35 % from the initial value after 27 days of food deprivation in the cuttlefish *Sepia officinalis*.

On the other hand, the protein content and ϕ remained constant throughout the entire food deprivation period. This result is consistent with that obtained by Packard et al. (1996) in the marine bacteria *Pseudomonas nautica*. The parallel behavior of these two parameters was expected as the ETS activity has a long history of being a good index of the living biomass.

The protein-per-cell time course showed an increase on the intracellular protein content during starvation. Using the protein-per-cell measurement as a proxy of the cell size, we observed that the largest cell sizes are found at the end of the experiment. Previous laboratory work reported by Aristizabal (2009) supported these results. Using flow-cytometry, she distinguished three different sizes in the culture of *O. marina* during the experiment. As the food deprivation time increased, the contribution of the larger size fraction to total dinoflagellate population increased. Moreover, this increase in size was verified using the inverted microscope.

Variability of R / ϕ during starvation and with cell size.

The ratio of respiration to potential respiration (R/ ϕ) is an index of the physiological state (Christensen et al., 1980). Historically, because ETS activity was often reported in the literature in oxygen units rather than electron units (μmoles ē), R_{O2}/ ϕ appears as R_{O2}/ ETS. The variability in this ratio has been widely addressed in cultures of bacteria, phytoplankton and zooplankton under laboratory controlled conditions (Packard 1985; del Giorgio, 1992), and in natural marine samples taken in different regions of the oceans (Arístegui & Montero, 1995). Hernández-León and Gómez (1996) reported that this variability was impacted by temperature, food quantity/quality and size. In their investigation, they used primary production as a reference for the food quality and/or quantity. In this manner, they obtained higher values of the ratio in samples with higher primary productions rates, while the ratio decreased when the primary production was lower. The observations of these authors were confirmed by this study, where the R/ϕ ratio decreased more than 75 % from well-fed to starve organisms. In addition, they obtained, as we do, high values of the R/ϕ ratio in the smallest size fractions. Eventually, the decreases in the R/ϕ ratio during starvation and with increasing cell size are due to the decline in respiration rates rather than in the ETS activity, as the latter remained almost constant during the entire experiment.

Very often in the literature, there are found R/ϕ ratios higher than 1 (Aristegui and Montero, 1995; Hernández- León and Gómez, 1996). Since the theoretical maximum value of 1 indicates that the organisms or the community fractions are respiring at their maximum

capacity, ratios higher than 1 raise an alarm. During our experiments, we found higher values in well-fed organism and in the smaller cell sizes. This suggests that there are some other oxygen consuming pathways in the cells for which the ETS does not account. Martinez et al. (2010) arrive at a similar conclusion with their respiratory studies of *Artemia*. Needless to say, further investigation is needed to improve the knowledge on this respect.

Relationship of PN levels and respiration rates under starvation

In the present work, we have applied a spectrophotometric method to determine the intracellular total pool (oxidized and reduced) of NAD and NADP (NADt, NADPt) in a marine organism. This methodology is based on the use of coupled enzyme activities (i.e., rates) rather than measuring direct concentrations to determine intracellular substrates. As stated by Lowry et al. (1961), when the intracellular concentration of these substrates is low, amplification by enzymatic cycling increases the detection and gives surprisingly reproducible results.

To our knowledge this is the first determination of intracellular NADt and NADPt concentrations in a marine dinoflagellate. Roy and Packard (1998) determined the $NADP^+$ level in the marine bacterium *Pseudomonas nautica* and they found that, after the C-source (pyruvate) was exhausted, the $NADP^+$ levels remained constant. This fact seems to be in disagreement with our results, but it was not. The enzyme that they studied (isocitrate dehydrogenase, IDH) has two substrates, the isocitrate and $NADP^+$. Their results showed that the isocitrate played the major role in controlling the *in vitro* IDH activity. In our case, the main substrate of the ETS enzymes is the NADH. For this reason, if we compare the results obtained for the isocitrate and for the NADt, they follow similar trends. On the other hand, an attempt to measure the marine primary production using the blue-fluorescence of the NADPH was made by Steigenberger et al. (2004). Nevertheless, they found that the cell densities in the oceans were too small for biomass/photosynthetic measurements to be made by remote sensing using fluorescence lidar.

The concentrations of the PN have been largely investigated in other fields. For instance, Umemura and Kimura (2005) reported a value of 4 nmol mg^{-1} prot for the NADt in

mouse cell monolayers. In isolated mitochondria from tubers of different plant species, the values for NADt ranged from 0,93 to 3,70 nmol mg^{-1} prot (Agius et al., 2001). In our case, the NADt concentration ranged from 0,5 to 0,1 nmol mg^{-1} prot. The difference in the concentrations might be due not only to the difference in type of organisms but also to the physiological state in each experiment.

Our results represent the first demonstration that the total PN levels decrease in a marine dinoflagellate during food starvation. This information is unique in the marine science literature. It supports the idea introduced by Packard et al. (1996) to explain respiratory decreases in marine bacteria and also supports their respiration model based on substrate (i.e., NADH and NADPH) limitation. In this model, they argued that the *in vivo* potential respiration is controlled by the maximum rate capacity of the enzymes involved in the ETS (V_{max}) and by the availability of their substrates.

Finally, we observed a high correlation between the respiration rates measured as oxygen consumption (R_{O2}) and the NADt and NADPt concentration (r^2=0,75 and r^2=0,83, respectively). These results provide more robustness to the respiration model mentioned above.

CONCLUSIONS

(1) The parallelism between the behavior of potential respiration (ETS activity) and biomass (protein) over a long period of starvation argues that the ETS enzyme complexes in O. marina's mitochondria are constitutive.

(2) Divergence of respiration and potential respiration during starvation in the marine dinoflagellate *O. marina* has been demonstrated and documented here.

(3) The fall in the respiration during the onset of starvation suggests that respiration is substrate limited during this period.

(4) Total PNs and respiration are well correlated during starvation in a marine dinoflagellate. This observation supports the use of a respiration model based on substrate limitation.

(5) The fact that six-fold difference in the PN was measured argues that our methodology is sensitive enough to detect real physiological PN changes in dinoflagellates.

Future work:

- Develop the methodology to differentiate the oxidized from the reduced forms of the two PNs.
- Adapt this methodology to the spectrofluorometer so as to gain at least 10-fold sensitivity.
- Learn how to study "well-fed" *O. marina* without the contaminating influence of the food organism.

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Figures legends

FIG. 1. MTT- formazan formation time courses by the enzyme cycling reaction with starting NADP concentrations of 0 (●), 8,75 (o), 17,5 (▼), 35 (Δ), 70 (■), 100 (□), 200 (♦) nM. Each data point represents the mean of triplicate analysis.

FIG. 2. Standard curves were obtained by plotting the slopes of the velocity curves (Fig. 1) against the PN concentration. **(A)** Standard curve for the NADt (●) and NADH (o). The regression equations were slope = 3,291 $x10^{-4}$ NADt + 2,987 $x10^{-3}$ (r^2 = 0,97 ; n = 21) and slope = 2,88 $x10^{-4}$ NADH - 1,788 $x10^{-3}$ (r^2 = 0,99 ; n = 21). **(B)** Standard curve for the NADPt (▲) and NADPH (Δ). The regression equations were slope = 1,04 $x10^{-3}$ NADPt + 6,944 $x10^{-3}$ (r^2 = 0,99 ; n = 21) and slope = 8,45 $x10^{-4}$ NADPH + 8,318 $x10^{-3}$ (r^2 = 0,99; n = 21). Slopes were measured as change in absorbance at 570 nm per minute and the nucleotides were measured in the nanomolar range.

FIG. 3. Time profiles of: (**A**) protein; (**B**) cell number; (**C**) ETS activity; and (**D**) respiration in *O. marina*'s cultures during the experiment. Each data point represents the mean of triplicates analysis (n = 27). Vertical bars are SDs. When no bar is evident, the deviation is smaller than the size of the symbol.

FIG. 4. Cell specific time profiles of: (**A**) respiration; (**B**) ETS activity; and (**C**) protein. The regression equations were R_{O2} = -0,015 (starved time) + 0,30 (r^2 = 0,81); ETS = 0,04 (starved time) + 0,06 (r^2 = 0,93); Protein = 0,16 (starved time) + 0,07 (r^2 = 0,84). Each data point represents the mean of triplicates analysis (n = 27). Vertical bars are SDs.

FIG. 5. Variation of R/ETS ratios with (**A**) time of starvation, (**B**) cell size. The regression equations were R_{O2} = 2,01 - 0,7 ln (starved time) (r^2 = 0,86), and R_{O2} = 1,1 - 0,59 ln (cell size) (r^2 = 0,55). The ratio R/ETS is unitless.

FIG. 6. Experimental observations of the protein specific intracellular levels of (**A**) NADt and (**B**) NADPt. The relationships of NADt and NADPt concentrations with the days of starvation showed a Spearman correlation coefficients of -0,90 (n = 27, p < 0,001) and -0,95 (n = 27, p<0,001), respectively.

Fig. 7. Correlations of R_{O2} time course with (**A**) NADt time course; (**B**) NADPt time course. The Spearman correlation coefficients were 0,87 and 0,91 (n = 23, p < 0,001) for NADt and NADPt, respectively.

Figures

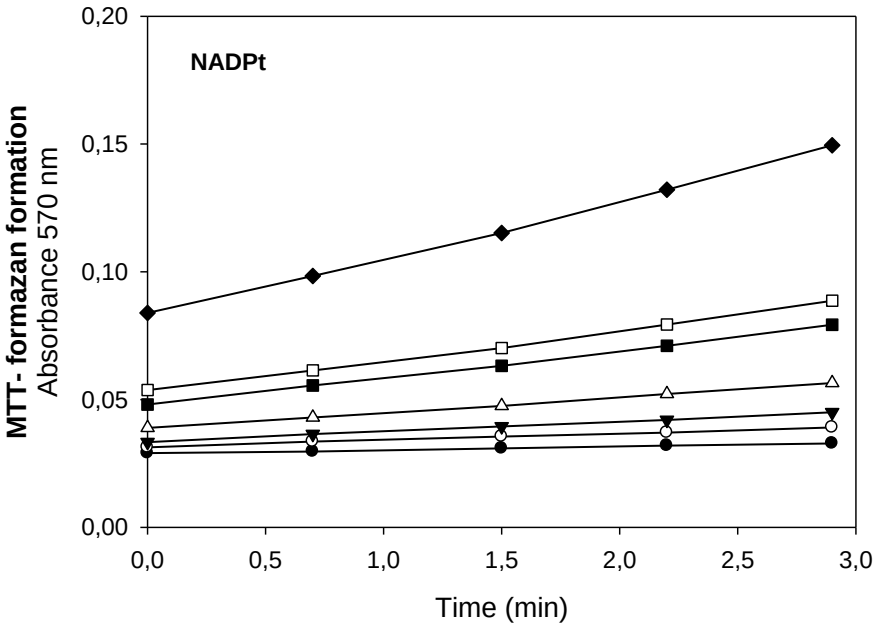


Figure 1.

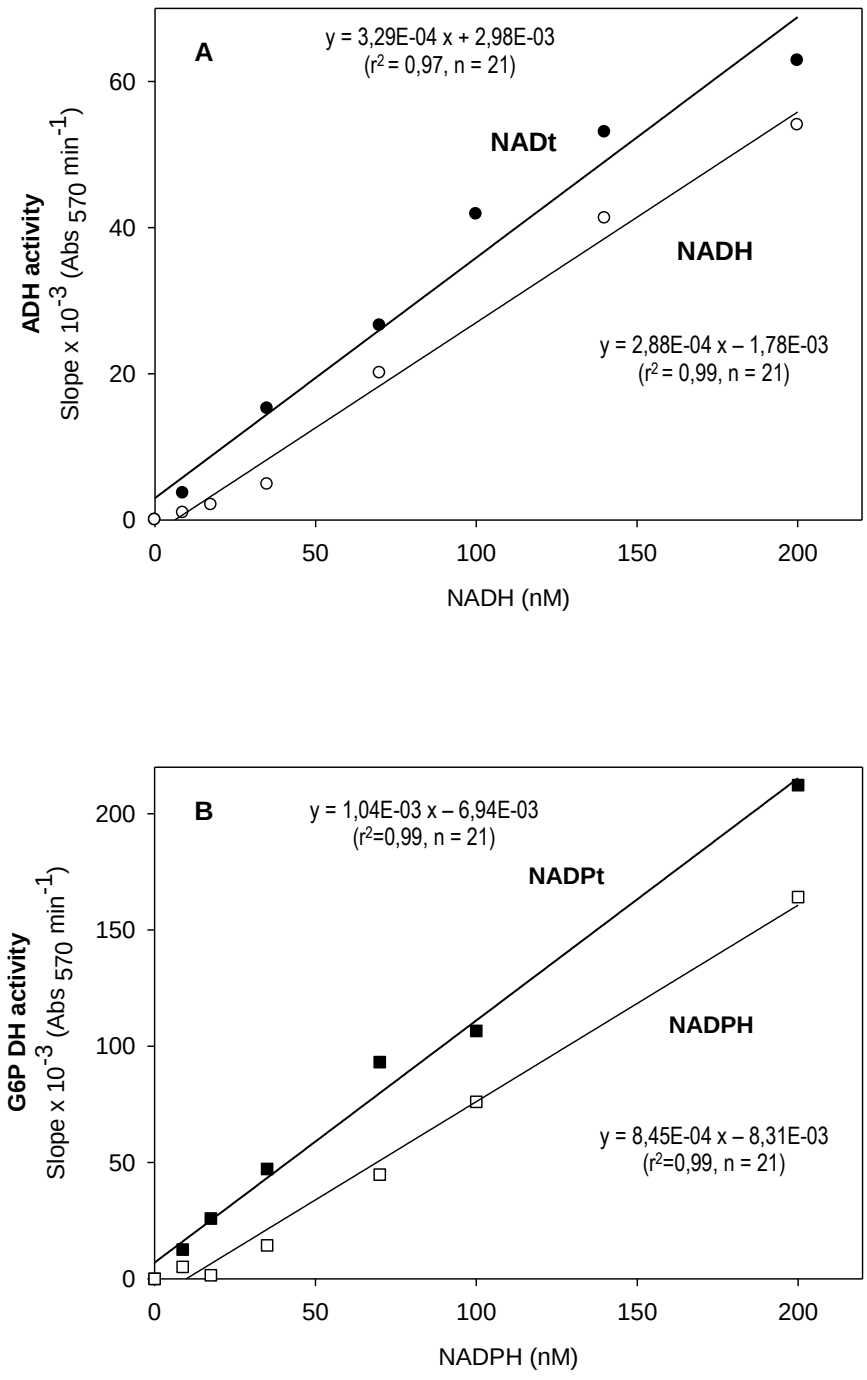

A
y = 3,29E-04 x + 2,98E-03
(r2 = 0,97, n = 21)
NADt
NADH
y = 2,88E-04 x – 1,78E-03
(r2 = 0,99, n = 21)
ADH activity
Slope x 10-3 (Abs 570 min-1)
NADH (nM)
0
20
40
60
0
50
100
150
200
B
y = 1,04E-03 x – 6,94E-03
(r2=0,99, n = 21)
NADPt
NADPH
y = 8,45E-04 x – 8,31E-03
(r2=0,99, n = 21)
G6P DH activity
Slope x 10-3 (Abs 570 min-1)
NADPH (nM)
0
50
100
150
200

Figure 2.

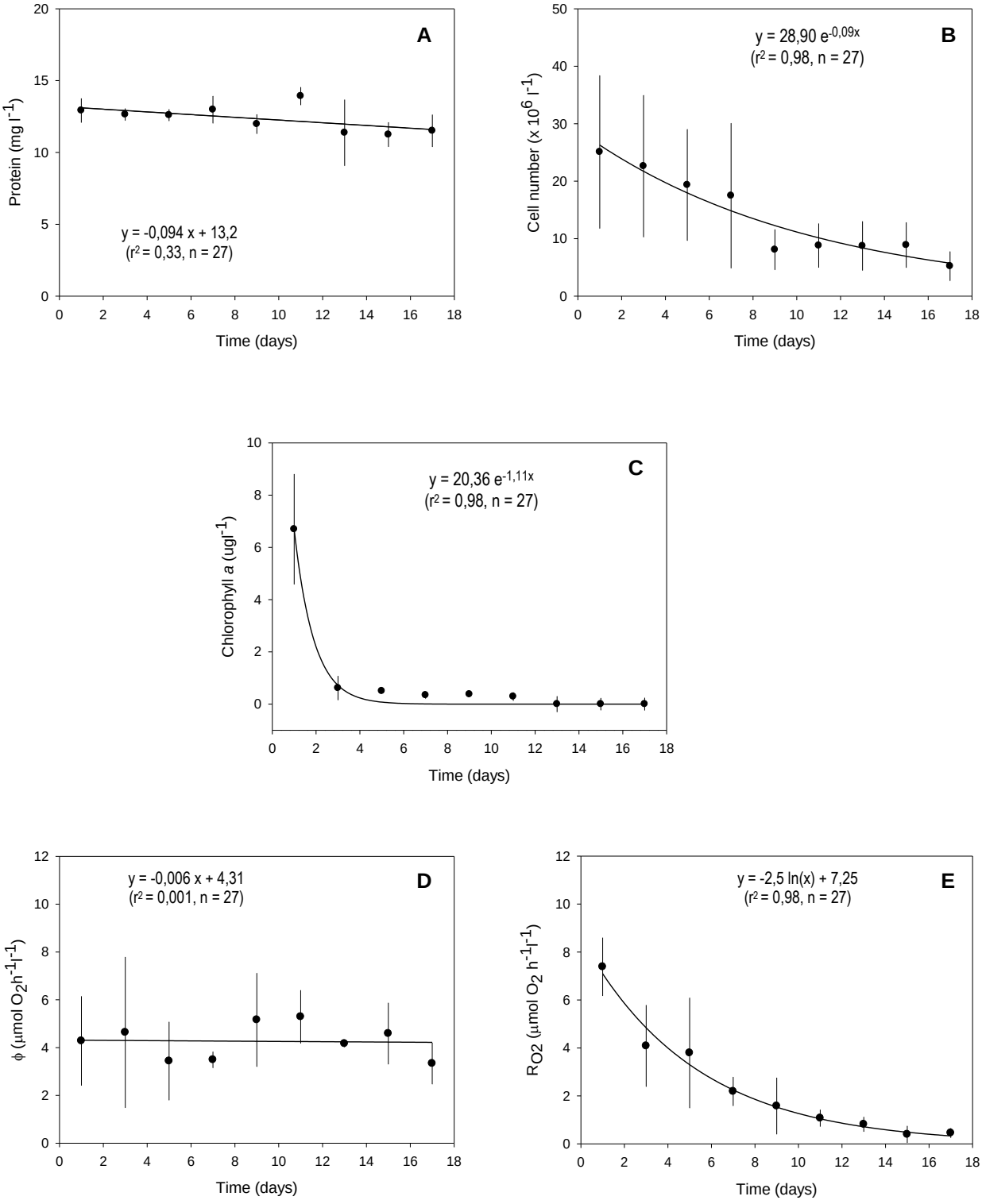

A
Protein (mg l-1)
y = -0,094 x + 13,2
(r2 = 0,33, n = 27)
Time (days)
B
Cell number (x 10^6 l-1)
y = 28,90 e-0,09x
(r2 = 0,98, n = 27)
Time (days)
C
Chlorophyll a (ugl-1)
y = 20,36 e-1,11x
(r2 = 0,98, n = 27)
Time (days)
D
ϕ (µmol O2h-1l-1)
y = -0,006 x + 4,31
(r2 = 0,001, n = 27)
Time (days)
E
RO2 (µmol O2 h-1l-1)
y = -2,5 ln(x) + 7,25
(r2 = 0,98, n = 27)
Time (days)

Figure 3.

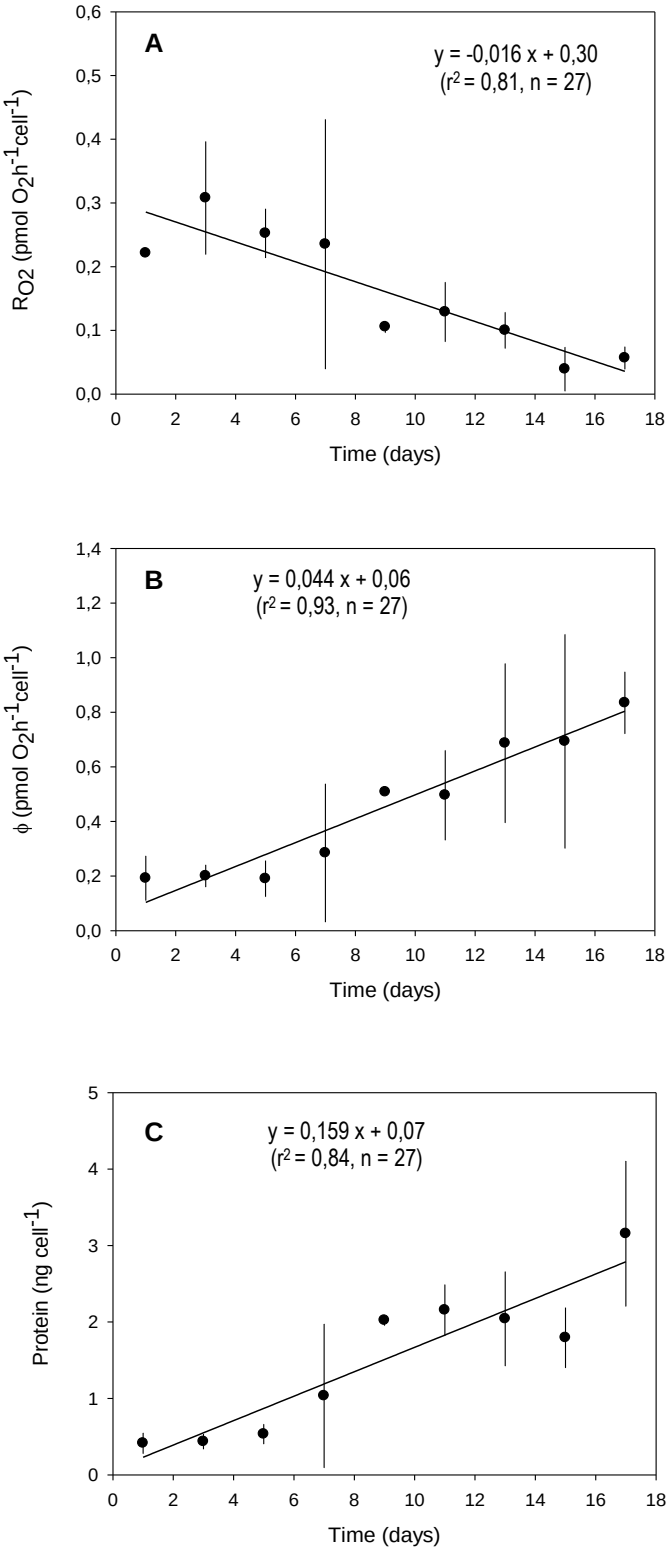
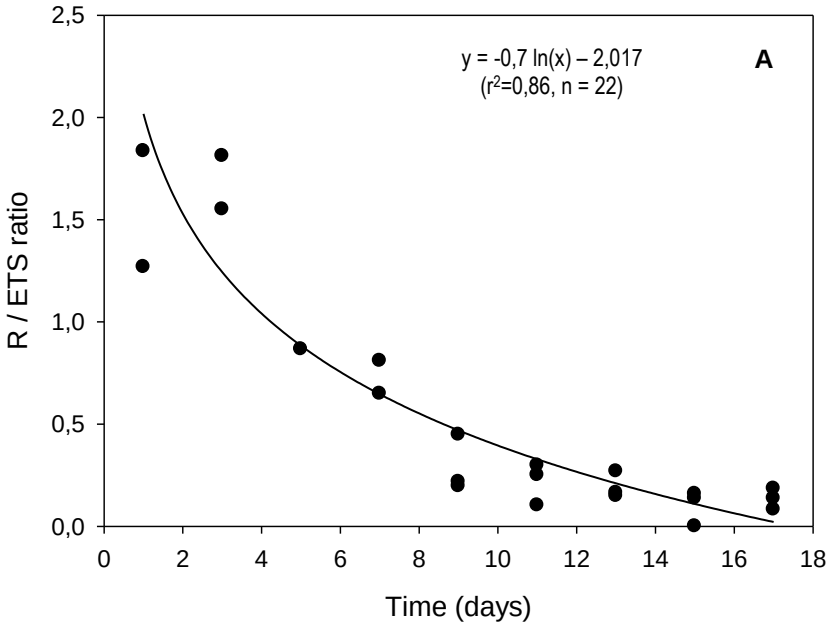

A
y = -0,016 x + 0,30
(r² = 0,81, n = 27)
RO2 (pmol O2h-1cell-1)
Time (days)
B
y = 0,044 x + 0,06
(r² = 0,93, n = 27)
ϕ (pmol O2h-1cell-1)
Time (days)
C
y = 0,159 x + 0,07
(r² = 0,84, n = 27)
Protein (ng cell-1)
Time (days)

Figure 4.


y = -0,7 ln(x) – 2,017
(r²=0,86, n = 22)
A
R / ETS ratio
Time (days)
2,5
2,0
1,5
1,0
0,5
0,0
0
2
4
6
8
10
12
14
16
18

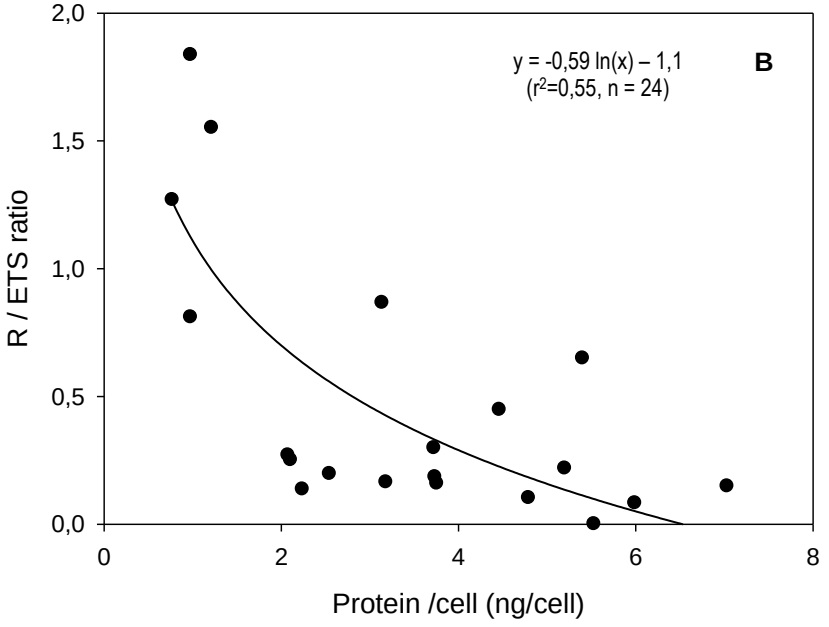
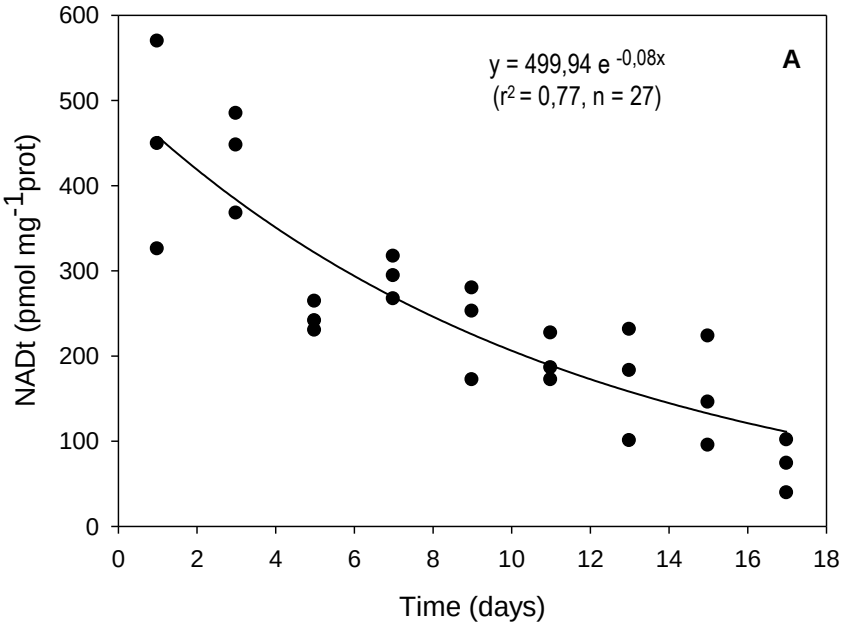

y = -0,59 ln(x) – 1,1
(r²=0,55, n = 24)
B
R / ETS ratio
Protein /cell (ng/cell)
2,0
1,5
1,0
0,5
0,0
0
2
4
6
8

Figure 5.


y = 499,94 e -0,08x
(r2 = 0,77, n = 27)
A
NADt (pmol mg-1prot)
Time (days)
0
100
200
300
400
500
600
0
2
4
6
8
10
12
14
16
18

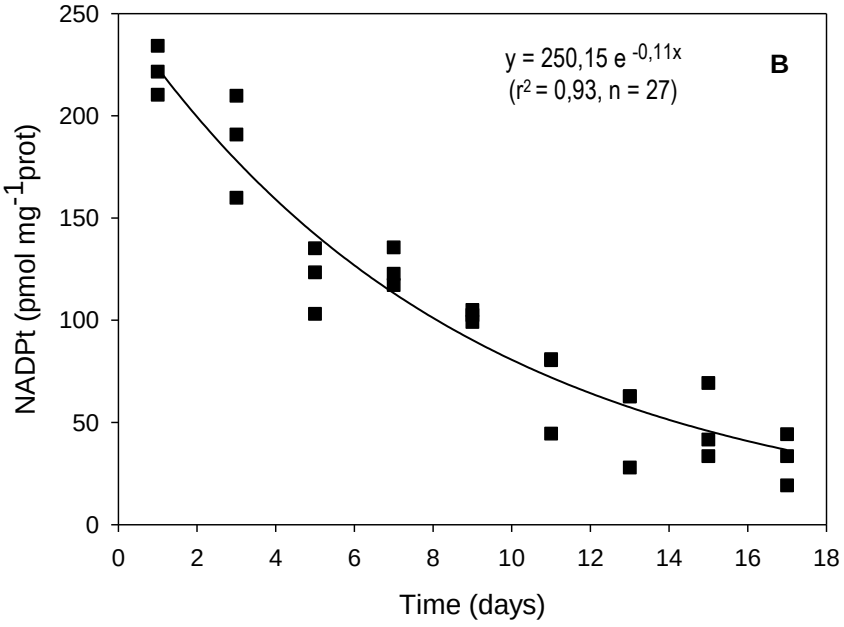
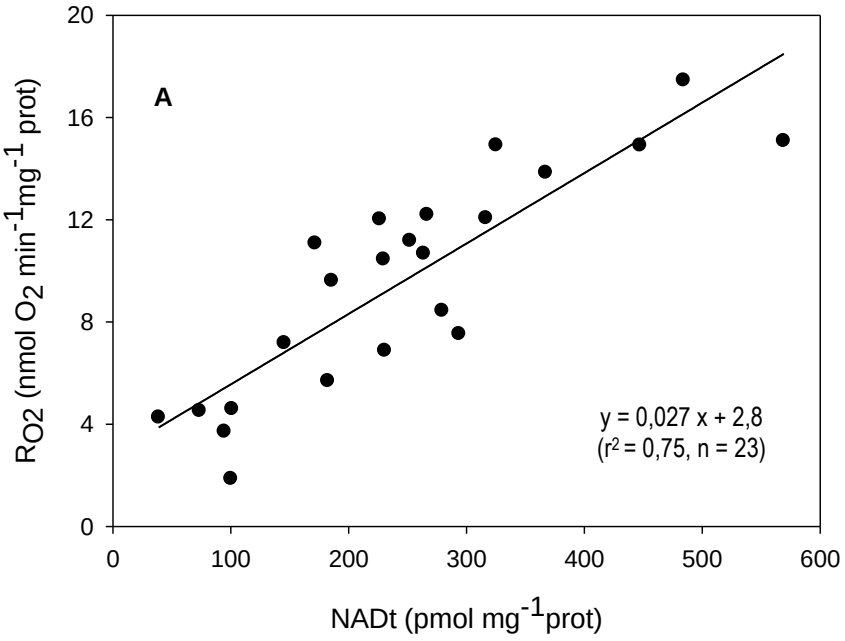

y = 250,15 e -0,11x
(r2 = 0,93, n = 27)
B
NADPt (pmol mg-1prot)
Time (days)
0
50
100
150
200
250
0
2
4
6
8
10
12
14
16
18

Figure 6.


A
y = 0,027 x + 2,8
(r² = 0,75, n = 23)
RO2 (nmol O2 min-1mg-1 prot)
NADt (pmol mg-1prot)
0
4
8
12
16
20
0
100
200
300
400
500
600

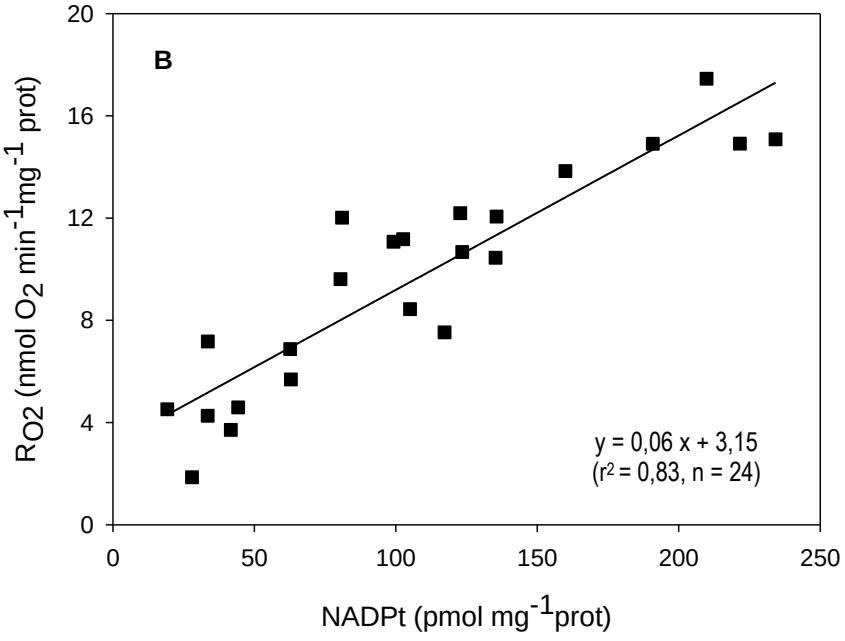

B
y = 0,06 x + 3,15
(r² = 0,83, n = 24)
RO2 (nmol O2 min-1mg-1 prot)
NADPt (pmol mg-1prot)
0
4
8
12
16
20
0
50
100
150
200
250

Figure 7.