Respiratory metabolism in the marine macroalga, *Ulva* spp.: Exploratory studies with the respiratory electron transport system (ETS)

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ABSTRACT

Respiration is an indicator of metabolism in individual organisms and in ecological populations, and along with other data can provide carbon flow rates through food webs. Here, we studied the metabolism of the green algae, *Ulva rotundata* Bliding and *Ulva rigida* C.Agardh that inhabit intertidal pools along the coast of Gran Canaria. To do this we explored the use of the respiratory electron transport system (ETS) in a pilot study of the variability of potential respiration in Ulva and its relationship with chlorophyll and with dry mass. We studied both field and culture samples. In the initial part of our exploration we learned that homogenization with a Potter-Elvehjen tissue grinder yielded much more enzyme activity per sample than grinding in liquid nitrogen with a mortar and pestle. Furthermore, we learned that NADPH dehydrogenase, not NADH dehydrogenase, was the dominant contributor to the overall ETS activity. The contribution of succinate dehydrogenase activity is negligible.

ETS activity as a metabolic proxy has been successfully applied to study many different organisms in the ocean including bacteria, phytoplankton and zooplankton, but it has not been used to study marine macroalgae. These neritic and littoral macrophytes have major ecological and industrial importance, yet little is known about their respiratory physiology. Such knowledge would strengthen our understanding of the resources of the coastal ocean and facilitate its development and best use. In this first phase of our research we have learned that potential respiration (φ) in Ulva ranges from 0.56 µmol O_2 min^{-1} (g dry mass)$^{-1}$ to 1.24 µmol^{-1} O_2 min^{-1} (g dry mass)$^{-1}$. We have learned that the relationship between changes in potential respiration and dry mass in Ulva follows the equation: φ =0,36*DM + 0,013, r^2 = 0,8363 (n = 29); changes in potential respiration and chlorophyll *a* in *Ulva* spp. follows the equation: φ= 1,19*Chl *a* -1,8, r^2 = 0,8556 (n =29); changes in potential respiration and chlorophyll *b* in *Ulva* spp. follows the equation: φ = 0,70*Chl b -1,93, r^2 = 0,8643 (n =29). Finally, in a pilot time-course experiment to determine the impact of nutrient-limitation on the metabolic capacity in Ulva, we learned that Ulva does not have sufficient reserves to maintain its potential respiration for a week. It would lose between 25 to 60% of its initial potential respiration over this time period.

In this first phase of our research we have developed the methodology for the homogenization of *Ulva* spp., and used a standard spectrophotometric based kinetic enzyme assay to describe the impact of nutrient limitation on the metabolic capacity in *Ulva* spp. samples collected in the wild and maintained in controlled cultures during a week, being not enough to bring the algae to total starvation conditions.

INDICE

1. INTRODUCTION

In the Canary Islands there are more than 600 macroalgae species, this high number of marine species is the result of its oceanography conditions, the geographical and geological features of the archipelago, and its paleoclimatology (Haroun et al, 2002, Prud' homme van Reine and van den Hoek, 1990). The cold waters of the Canary current flowing from NNE confer to the Archipelago a colder water temperature than the expected for its latitude. The average sea surface temperature varies 2ºC between the eastern and western islands (Barton et al. 1998; Davenport et al. 2002). Gran Canaria has a rocky coastline that consists mostly of weathered basalt and is bathed by persistent trade winds which induce strong water motion (swells). As a result, one can observe different hydrodynamic regimes between the exposed north and the more sheltered south of the island.

To quantify metabolic status and stress of marine algal communities it is essential to estimate rates of oxygen consumption (respiration including organic matter oxidation) within these communities. To do this, we use a sensitive enzymatic proxy for the algal respiratory capacity, the electron transport system (ETS) (Packard *et al.*, 1974) and we apply this proxy to detect different growing conditions in the Ulva communities around Gran Canaria. In this island one of the most common macroalgae inhabiting the upper intertidal is the green algae *Ulva* spp. (*Ulva rotundata* Bliding and *Ulva rigida* C.Agardh*).*We selected this algae because it is accessible all year round and it is easy to identify.

Normally the respiration (metabolic) rate (R) is closely related to body mass (M) and this relationship can be expressed as a function of biomass according to the power function: $R=aM^b$ (Brody and Procter, 1932; Kleiber, 1932; Benedict et al., 1938) and for years it has been accepted that the coefficient b, equaled 0.75 for all organisms. This is known as "Kleiber's law". Glazier (2005) in an extensive review concluded that Kleiber's law is not universal, either within or among animal species. These variations in the power scaling can be related to taxonomic, physiological, and environmental differences. Our study showed that environmental conditions and/or physiological state impacted the Kleiber relationship.

To detect and measure ETS in any biological community one could use the natural electron acceptor, ubiquinone, but it is easier and common practice to use an artificial electron acceptor. Here we use the water-soluble tetrazolium-salt, INT, often

called, tetrazolium violet. In addition, in order to measure the capacity, the maximum rate of the ETS (V_{max}) rather than some unknown fraction of this maximum, we add various substrates that naturally donate reducing equivalents to the ETS. Specifically, we add pyridine nucleotides, nicotine adenine dinucleotide (NADH), nicotine adenine dinucleotide phosphate (NADPH) and succinate. The addition of these substrates to the ETS reaction stimulates its activity to capacity and hence serves as a measure of potential respiration (φ) (Packard and Gómez, 2008). This practice of adding substrates to the maximal velocity of a biochemical reaction, *in vitro*, has been standard for nearly 100 years.

A characteristic of this reaction is that INT reduction is stoichiometrically related to oxygen consumption due to the action of cytochrome oxidase, a key enzyme in the ETS. This is because the reduction of 2 mol of INT by the ETS is equivalent to the natural reduction of 2 atoms of oxygen (or 1 molecule of O_2) at the end of the ETS. Thus the relation between the INT reduction in this *in vitro* enzyme assay, the ETS activity, and potential respiration is straight-forward.

The study of potential respiration by the ETS technique has been successfully applied to study many different organisms in the ocean including bacteria, phytoplankton and zooplankton, but it has not been used to study marine macroalgae. The present investigation is a preliminary study of respiratory metabolism in Ulva aims to:

1. Optimize the homogenization method to determine the activity of the electron transport system (ETS).
2. Study of the relative importance of the three enzymes, NADH-dh, NADPH-dh and Succinate-dh in determine ETS activity.
3. Determine the relationship between: a) dry mass and chlorophyll, b) dry mass and potential respiration and c) potential respiration and chlorophyll.
4. Study the variability of the potential respiration, dry mass and chlorophyll in three different locations around Gran Canaria.
5. Determine the time-course of metabolism in aquaria with filtered sea water over a week.
6. Determine the relation between biomass and respiratory activity in the three different locations.

2. MATERIAL AND METHODS

2.1. Sampling

The organisms, *Ulva* spp. (*Ulva rotundata* and *Ulva rigida*) were collected at random at low tide, between December 2012 and September 2013 for the experiments and in different locations from Gran Canaria (San Cristóbal, Taliarte and Bocabarranco, Fig. 1).

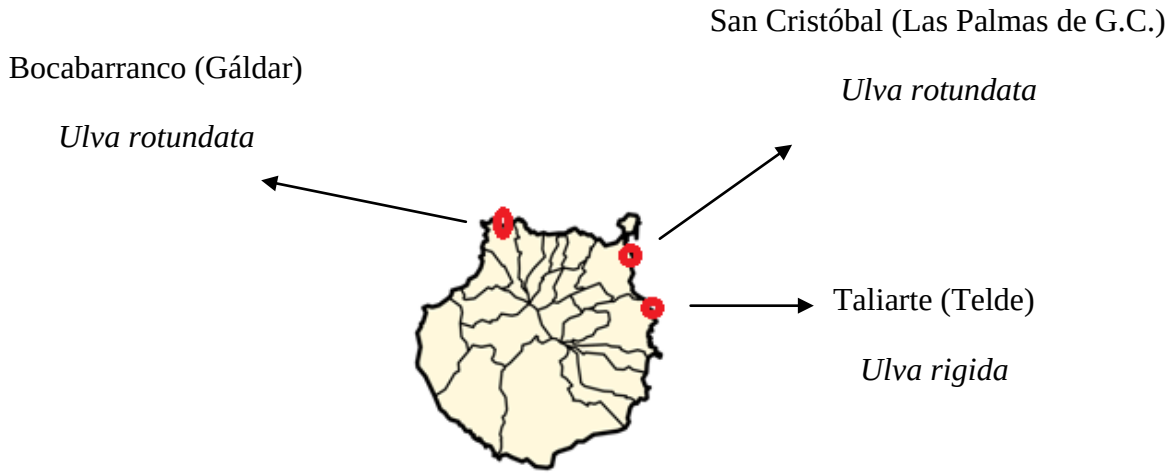


Figure 1: Map of Gran Canaria

The samples were transported in water bottles from the coast to the laboratory and processed for future analysis. The samples were then cut into circles with cork borer. Each measurement was based on identical algal circles (0, 9 cm diameter). The circles weighing on average 1, 9 mg was placed within a glass fiber filter (GF/C Circles 25 mm Cat NO 1822, Whatman.**)** for analysis.

2.2. Preparation of cell-free extracts. Optimization for activity measures of the respiratory electron transport system (ETS).

Tests were conducted to determine whether grinding or freezing in liquid nitrogen would be the better method of homogenization. These methods are as follows:

- **Grinder method:** ten replicated samples with 20 algae circles per sample, were homogenized with 2 ml of TRIS solution in a Teflon-glass tissue grinder for 4 minutes with a glass fiber filter (GF/C Circles 25mm Cat NO 1822, Whatman).Then the homogenate was centrifuged for 8 min at 2500

rpm and maintained in an ice-bath at 0–4 °C to preserve the enzymatic activity (Gómez et al., 1996).

- **Liquid Nitrogen method**: ten replicated samples with 20 algae circles per sample, were homogenized by first freezing in liquid nitrogen and then thawing between 2 and 5 times and second, macerating the in a marble morter at -195°C. After the cells were macerated and the liquid nitrogen had evaporated, TRIS buffer was added (Kenner and Ahmed, 1975).The homogenate was centrifuged for 8 minutes at 2500 rpm and maintain in an ice-bath at 0–4 °C to preserve the enzymatic activity (Gómez et al., 1996). The supernatant fluid was used for the analysis.

The tissue-grinder method was chosen as the best way to homogenize the samples. With these crude enzyme preparations the ETS activity was determined by the phytoplankton ETS assay of Kenner and Ahmed (1975) which was designed to saturate the electron flux by adding high levels of the natural substrates, succinate, NADH and NADPH. Triplicate test solutions of different concentrations of *Ulva* spp. (2 , 4, 6, 8, 10, 12, 14, 16, 18 and 20 circles) were homogenized in 0,05 M phosphate buffer at pH=8.5 (6,72 g Na_2HPO_4, 0.362 g KH_2PO_4, 18.5 mg $MgSO_4 \cdot 7H_2O$, 1.5 g/ml polyvinyl pyrrolidone and 2 ml/l Triton X-100). For each reaction, 1 ml of each test solution (Buffer substrate at pH 8.5, 0.133 M disodium succinate, 0.835 mM NADH, and 0. 24 mM NADPH) and 0.5 ml of 4 mM INT, Sigma Lab) was added in a 1 cm (light path) spectrophotometer cuvette.

Measurements were then made in duplicate with a Beckman DU® 650 spectrophotometer at 490 nm at 20±1 °C for 8 min in kinetic mode. For each sample a blank was performed without ETS substrates. Absorbance was read spectrophotometrically (Beckman DU 650, USA) at 490 nm and 750 nm (turbidity). Potential respiration was calculated from ETS activity according to Packard and Christensen (2004). Potential respiration rates (φ) were normalized by biomass (dry mass) resulting in units of μl O_2 h−1. mg dry mass−1.

Cell-free extracts for biochemistry studies are notoriously difficult to obtain from macroalgae. Consequently, algologists often employ freezing-thawing techniques-*cum*-grinding in a mortar and pestle to free and disrupt cells from these plants. ETS investigations in phytoplankton have largely relied on tissue grinding in a teflon-glass,

Potter–Elvehjem type. In the Methods section we compared the two techniques on Ulva spp. The difference in the Specific potential respiration from the two methods was significant; activity from grinding in the Teflon-glass tissue grinder was much greater. The pooled data set did not have a normal distribution but, when they were treated individually, the tissue-grinder and liquid nitrogen data sets did have normal distributions (Fig. 2). When we applied the STUDENT-† test the two techniques were significantly different from one another (p-value <0, 01). Consequently, we decided to use the tissue-grinding method because, in addition to its being more efficient, it was less expensive and easier.

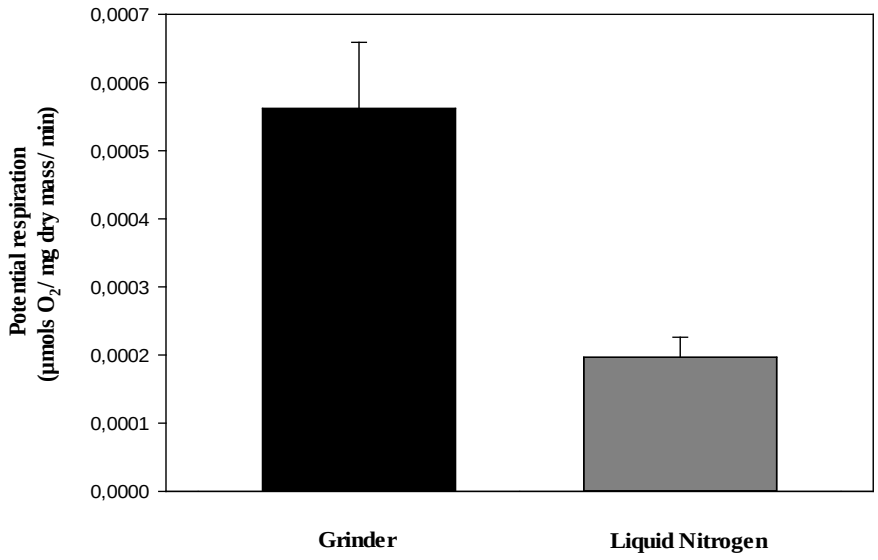


Figure 2. Comparison between specific potential respirations in the two different homogenization methods.

2.3. The relative importance of the three enzymes, NADH-dh, NADPH-dh and Succinate-dh in determine ETS activity.

The algae were collected from Taliarte and San Cristóbal, then were frozen in liquid nitrogen and later used in this study to determinate the activity of the three substrates.

- Case 1: ETS assay minus Succinate and minus NADPH (NADH dehydrogenase)
- Case 2: ETS assay minus Succinate and minus NADH (NADPH dehydrogenase)

- Case 3: ETS assay minus NADH and minus NADPH (Succinate dehydrogenase)

The Samples were homogenized by the tissue-grinder method. All procedures were realized as in the paragraph 2.2, but all reagents were added individually

2.4. Determination of Biomass, Dry mass, Chlorophyll and Optical Density.

Some of the samples were dried at 60ºC for 24 h in a drying oven and weighted on an analytic balance (COBOS model: XT–TOP) to determinate dry mass (Lovegrove, 1966). Different numbers of circles from 2 to 20 were measured by triplicate and compared with dry mass (mg) and chlorophyll (Fig. 3 and 4), showing a good relationship between them, with r^2 =0, 96 (dry mass), r^2= 0, 91(chl *a*) and r^2= 0, 92 (chl *b*) respectively. These linear equations were used to convert the number of circles to the other units of measure.

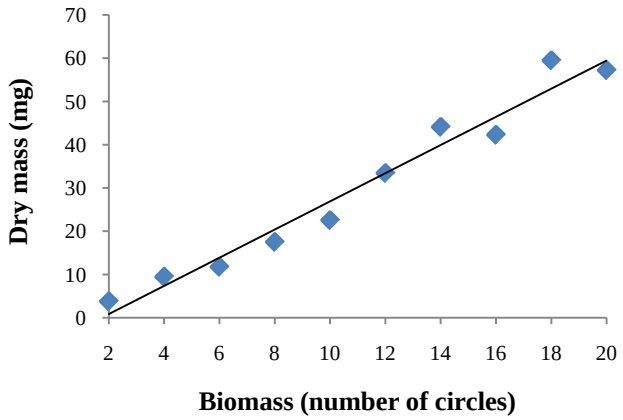


Figure 3. The linear relationship between circles cut from the sheet- like thallus of Ulva and their biomass in dry mass follows this equation: Dry mass = 3,25 * biomass - 5,63 and r^2= 0,9649.

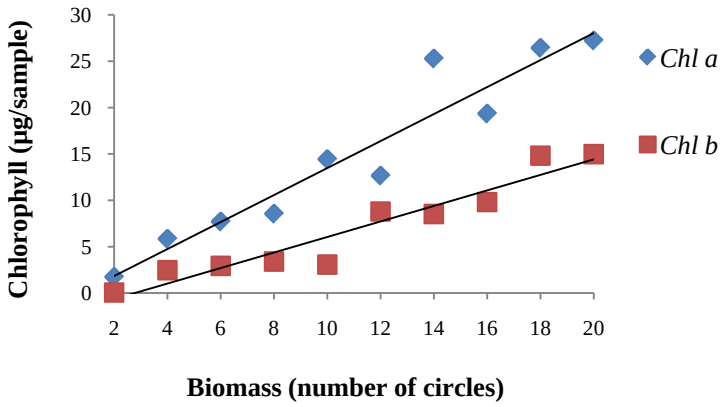


Figure 4. The linear relationship between circles cut from the sheet- like thallus of Ulva and their chlorophyll content. The equations for Chlorophyll are: Chlorophyll *a* = 1, 45 * biomass -1, 08; r^2=0, 9131 and Chlorophyll *b* = 0, 84 * biomass - 2, 32; r^2=0, 9185.

In order to determine the Chlorophyll maximum peak the absorbance was read spectrophotometrically with (a) and without filter (b). We used the sample with 22, 56 mg of *Ulva* spp. (10 circles). With filter the maximum was at 669 nm (a) and without filter 672nm (b). The mean absorbance was at 670, 5 nm and we decided to use at 670 nm (Fig. 5).

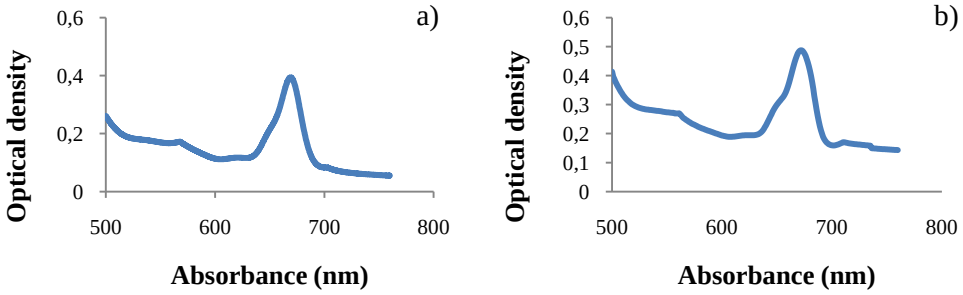


Figure 5. Absorbance measures with (a) and without filter (b) to detect the chlorophyll maximum peak.

Phytoplankton biomass can be estimated from photosynthetic pigments just as it is done for phytoplankton. One simply measures the chlorophyll *a*, which is found in all autotrophic algal cells and certainly in the Chlorophyta, the plant division that contains *Ulva* spp. To obtain a reproducible sample size one can use a cork borer to cut replicate circles of Ulva from the sheet. The samples are then stored for 23h at – 20ºC in 90% acetone to facilitate extraction (100 µl of each sample was mixed with 5 ml of the 90% acetone). Then, to obtain a cell-free extract of the algae, the samples (circles from *Ulva* spp.) were thawed for an hour at room temperature and then ground with a glass-fiber filter in a Teflon-glass tissue grinder containing 90% acetone. This is the same procedure that widely used for phytoplankton. The process liberates the chlorophyll from the chloroplasts and dissolves it at the same time. This technique has been shown to give the most efficient extraction without pigment alteration (Mantoura and Llewellyn, 1983).

For 30 samples we needed 150 ml acetone 90%. The extraction volume of acetone was 5 ml and this was mixed with 0.1 ml of sample. Thus the sample was diluted by a factor of 51. Extraction volume was**:** 5000 µl: 5000 µl acetone + 100 µl sample, dilution factor: 5100/ 100 = 51 and homogenized volume: 2 ml .

The spectrophotometer measures the absorption of light at a particular wavelength. Chlorophyll *a*, normally, has two absorption maxima, one at 432 nm and another at 664 nm. Since many accessory pigments absorb light in the 400's and only chlorophylls and phaeophytins absorb in the 600's, we use the red maximum (664 nm) for our optical density (OD) readings.

In all published equations for determining the concentration of chlorophyll *a*, an extinction coefficient is multiplied by the OD at 664 nm (for chl *a*), then to correct for interference from other pigments, the OD at 647 nm (for chl *b*) and at 630 nm (for chl *c*) are subtracted. If one is using a culture with no chl *b*, one can use an equation that doesn't correct for it. All ODs should have a reading at 750 nm subtracted from them to correct for background solvent absorption.

Common Spectrophotometric Equations for Chlorophyll Determinations, in higher plants and green algae are reported by Mitchell and Kiefer (1984) there are:

- Chlorophyll *a* = 11.93 E664 – 1.93 E647
- Chlorophyll *b* = 20.36 E647 – 5.50 E664

The chlorophyll optical density at 670 nm increases (upper panel, Fig. 6) with dry mass. The strong relationship in this figure (r^2= 0, 9668) is described by the equation, y = 0,0114x + 0, 0905. This equation will be used to convert the chlorophyll absorbance measurements at 670 nm into dry mass in mgs.

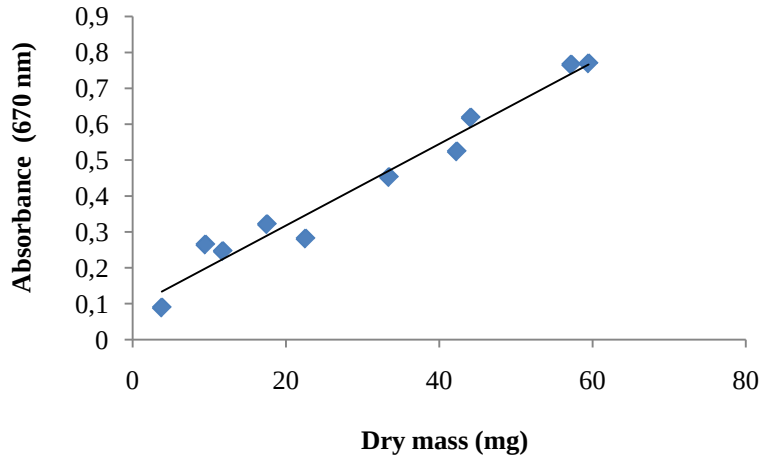


Figure 6. Correlation between dry mass (mg) and absorbance (nm).

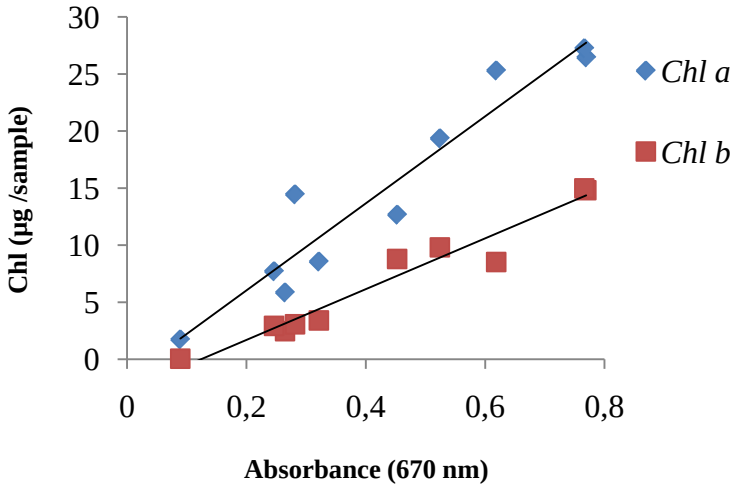


Figure 7. Correlation between chlorophyll and absorbance at 670 nm per sample. The equations for Chlorophyll are: chlorophyll *a* = 32, 12 * absorbance (670nm) - 1, 59; r^2=0, 9186 and Chlorophyll *b* = 22, 25 * absorbance (670nm) - 2, 76; r^2=0, 9517.

2.5. Study the variability of the potential respiration, dry mass and chlorophyll in three different locations around Gran Canaria.

The three locations were chosen because they experience municipal runoff. The sampling side at Bocabarranco (Gáldar) was predominantly rocky, precipitous, and highly exposed to the waves. Taliarte was also characterized by a steep rocky intertidal zone and highly exposed to wave action. There are important sources of contamination from The Technological Center of Marine Science in Taliarte, however, the large algal populations act as filters decreasing the quantity of the contamination.

The algae from San Cristóbal and Taliarte were collected on May 8th, 2013 and the algae from Bocabarranco on June 13th 2013. In San Cristóbal and Bocabarranco the specie was *Ulva rotundata* and in Taliarte it was *Ulva rigida.*

2.6. Time-course experiments of metabolism in aquaria with filtered sea water over a week.

After collecting the samples, the Ulva was analyzed on day one (the collection day). The samples were then maintained in an incubation chamber inside the aquaria with filtered sea water at 20ºC with 10-14h photoperiod. On 5 and day 8, ten replicates samples were taken and analyzed immediately. Each sample consisted of five identical circles (0, 9 cm diameter) of Ulva thallus.

2.7. Statistical analysis

The data were analyzed using the program R Development Core Team 2010 (R Foundation for Statistical Computing, Vienna, Austria). To confirm normality potential respiration data were analyzed by the Shapiro Wilk test and the homoscedasticity of the residuals was assessed graphically. Data were $\log_{10}$ x transformed prior to analyses. Differences in potential respiration were tested with 2-way ANOVA test, including the factor “Location” (fixed) with 3 levels (Bocabarranco, San Cristobal and Taliarte) and “Day” (fixed) with 3 levels (Day 1, Day 5 and Day 8). Pairwise comparisons resolved differences among days at each locality and among localities.

3. RESULTS

3.1. Relative importance of the three enzymes, NADH-dh, NADPH-dh and Succinate-dh in determining ETS activity.

The three different enzymes and their contribution to the total ETS activity in different Ulva species (*Ulva rigida* and *Ulva rotundata*) are shown in Fig. 8. The most important contribution was NADPH-dh (63,89% in the *Ulva rotundata* experiments and 88,59 % in *Ulva rigida* experiments) followed by NADH-dh (36,06 % in the *Ulva rotundata* experiments and 11,41 % in *Ulva rigida* experiments). Succinate-dh could not be measured because was below the limit of detection.

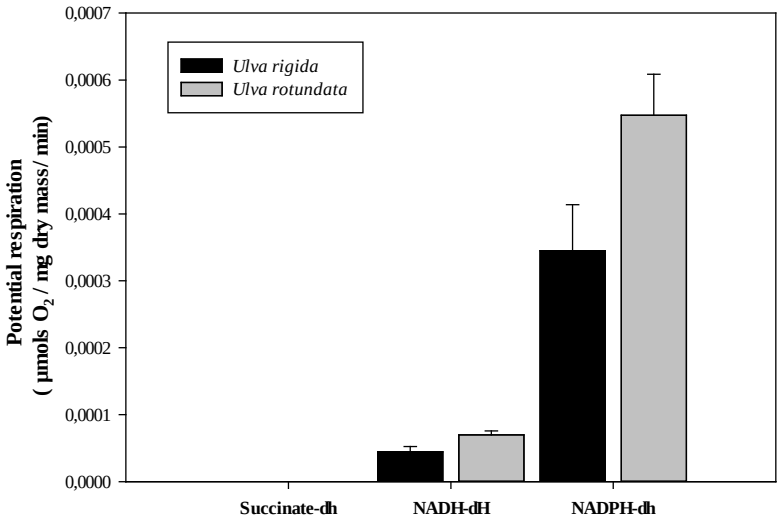


Figure 8: Relative contribution in the ETS activity from the three different enzymes in *Ulva rigida* and *Ulva rotundata.*

3.2. Determination of the relationship between: a) dry mass and chlorophyll, b) dry mass and potential respiration and c) potential respiration and chlorophyll.

These experiments were performed in *Ulva rotundata* and we found a good correlation between dry mass and Chlorophyll *a* and Chlorophyll *b* as it is showed in Fig. 9.

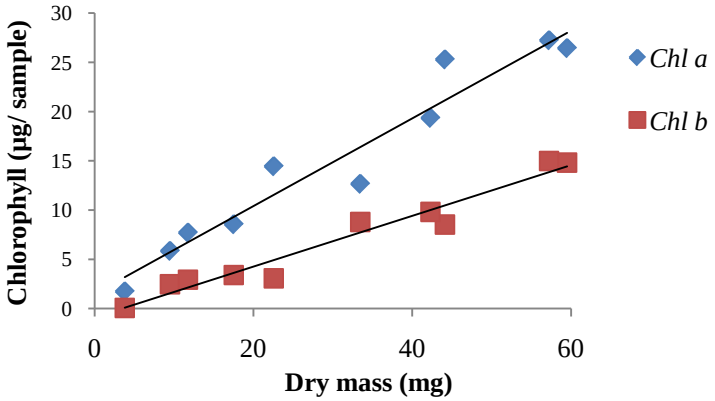


Figure 9. Relation between Dry mass and chlorophyll. The equations are: Chlorophyll *a* = 0, 44*dry mass + 1, 49; r^2= 0, 9391 and Chlorophyll *b* = 0, 26*dry mass + 0, 89; r^2= 0, 9551.

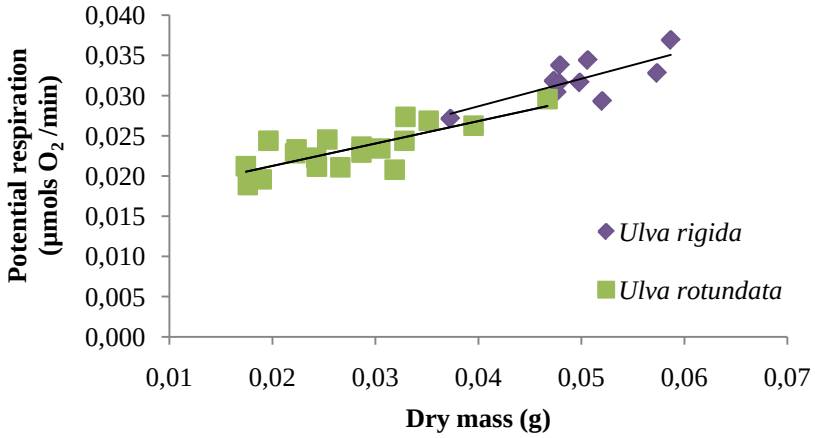
The good relationship that exists between biomass as dry mass, as the number of thallus circles, chlorophyll, and its absorbance peaks is shown in table 1 and these results are presented in paragraph 2.4 of Material and Method.

Nº circle/sample	Dry mass (mg/ sample)	Optical density at 647 nm	Optical density at 664 nm	Chl *a* (µg/sample)	Chl *b* (µg/sample)
2	3,80	0,0004	0,0015	1,74	0,06
4	9,49	0,0026	0,0052	5,86	2,46
6	11,76	0,0033	0,0067	7,71	2,93
8	17,52	0,0037	0,0077	8,56	3,40
10	22,56	0,0049	0,0127	14,45	3,07
12	33,44	0,0074	0,0116	12,67	8,79
14	44,11	0,0102	0,0224	25,30	8,53
16	42,29	0,0094	0,0174	19,36	9,81
18	59,49	0,0136	0,0239	26,45	14,82
20	57,22	0,0139	0,0246	27,25	14,98

Table 1: Dry mass, Optical density and Chlorophyll *a* and *b* measures *Ulva rotundata* from San Cristóbal sampling site.

Figures 10, 11 and 12, show the relationship between potential respiration, dry mass, chlorophyll *a*, and chlorophyll *b* data in *Ulva rigida* and *Ulva rotundata.* The coefficients of the regression equations are presented in table 2, 3 and 4.

a)



b)

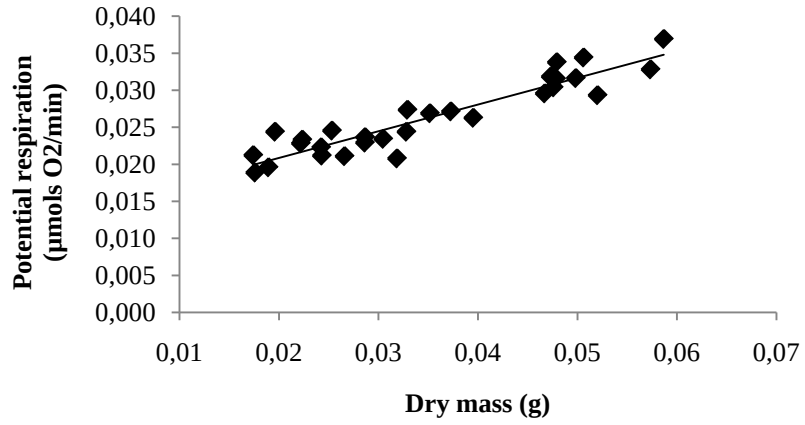
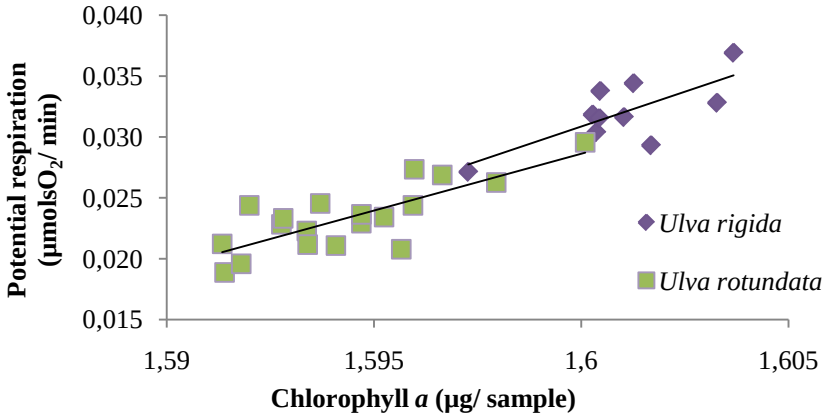


Figure 10. Relation between dry mass and potential respiration per sample, a) the equation for *Ulva rigida* is: Potential respiration = 0, 34*dry mass + 0, 015; r^2=0, 5468 and for *Ulva rotundata* is: Potential respiration = 0, 28*dry mass + 0, 015; r^2=0, 615. b) The pooled data equation is: Potential respiration = 0, 36*dry mass + 0, 013; r^2=0, 8363.

a)



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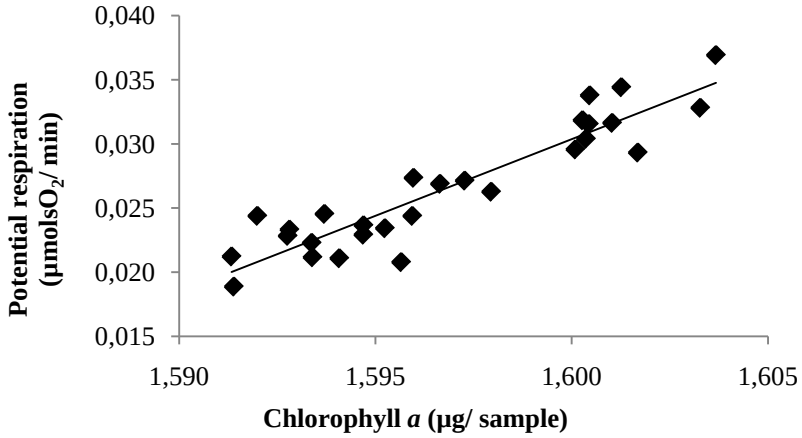
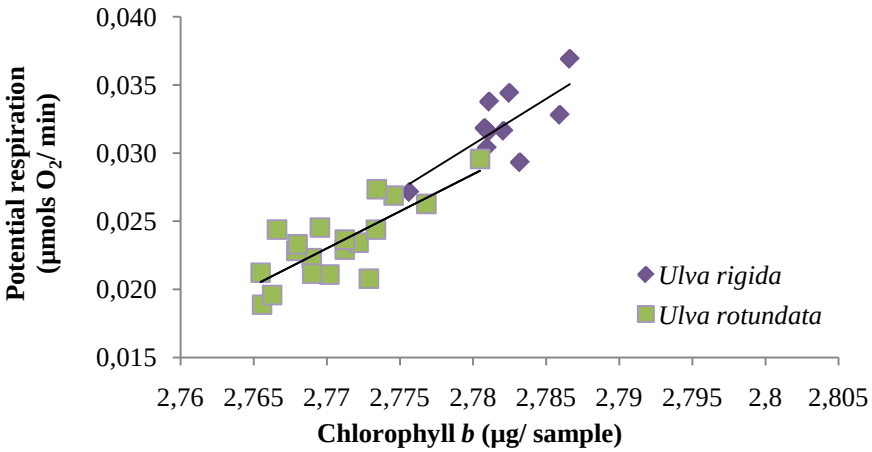


Figure 11. Relation between chlorophyll *a* and potential respiration per sample, a) the equations for *Ulva rigida* is: Potential respiration = 1, 14*chlorophyll *a* – 1, 80; r^2=0, 5468 and for *Ulva rotundata* is Potential respiration = 0, 93*chlorophyll *a* - 1, 5; r^2=0, 615. b) The pooled data equation is: Potential respiration = 1, 19 * Chlorophyll *a* – 1, 88; r^2=0, 8556.

a)



b)

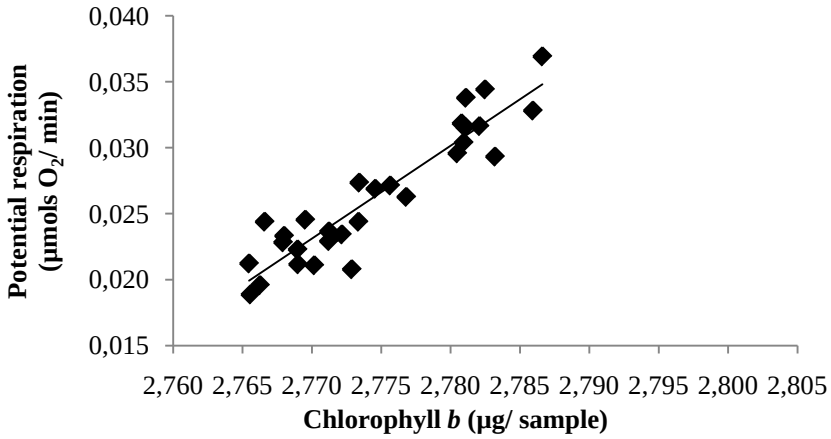


Figure 12. Relation between chlorophyll *b* and potential respiration per sample, a) the equation for *Ulva rigida* is: Potential respiration = 0, 67*chlorophyll *b* – 1, 82; r^2=0, 5468 and for *Ulva rotundata* is Potential respiration = 0, 54*chlorophyll *b* – 1, 49; r^2=0, 615. b) The pooled data equation is: Potential respiration = 0, 70 * Chlorophyll *b* - 1, 93; r^2=0, 8643.

Y	X	a	b	r^2	n
Pot. Resp.	DM day 1	0,015	0,342	0,5468	10
Pot. Resp.	DM day 5	0,0107	0,275	0,763	10
Pot. Resp.	DM day 8	0,00363	-0,1111	0,0174	8
Pot. Resp.	Chl *a* day 1	-1,7987	1,1434	0,5468	10
Pot. Resp.	Chl *a* day 5	-1,4476	0,9195	0,763	10
Pot. Resp.	Chl *a* day 8	0,6257	-0,3716	0,0174	8
Pot. Resp.	Chl *b* day 1	-1,8247	0,6674	0,5468	10
Pot. Resp.	Chl *b* day 5	-1,4686	0,5367	0,763	10
Pot. Resp.	Chl *b* day 8	0,6341	-0,2169	0,0174	8

Table 2*:* The regression analysis for *Ulva rigida* give a and b for the equation Y= bX + a; n is the number of determinations and r is the correlation coefficient. The units used are µmols O_2/ min. The dry mass is given in units of grams.

Y	X	a	b	r^2	n
Pot. Resp.	DM day 1	0,0157	0,2789	0,615	19
Pot. Resp.	DM day 5	0,0065	0,4082	0,6473	20
Pot. Resp.	DM day 8	0,0174	-0,1307	0,086	20
Pot. Resp.	Chl *a* day 1	-1,4638	0,9327	0,615	19
Pot. Resp.	Chl *a* day 5	-2,1583	1,3648	0,6473	20
Pot. Resp.	Chl *a* day 8	0,7108	-0,4372	0,086	20
Pot. Resp.	Chl *b* day 1	-1,485	0,5444	0,615	19
Pot. Resp.	Chl *b* day 5	-2,1894	0,7966	0,6473	20
Pot. Resp.	Chl *b* day 8	0,7207	-0,2552	0,086	20

Table 3. The regression analysis for *Ulva rotundata* give a and b for the equation Y= bX + a; n is the number of determinations and r is the correlation coefficient. The units used are µmols O_2/ min. The dry mass is given in units of grams.

Y	X	a	b	r^2	n
Pot. Resp.	DM day 1	0,0136	0,3606	0,8643	29
Pot. Resp.	DM day 5	0,0091	0,3018	0,8921	30
Pot. Resp.	DM day 8	0,0056	0,3016	0,7197	28
Pot. Resp.	Chl *a* day 1	-1,8798	1,01939	0,8556	29
Pot. Resp.	Chl *a* day 5	-1,5941	1,0108	0,8932	30
Pot. Resp.	Chl *a* day 8	-1,8341	1,1585	0,7197	28
Pot. Resp.	Chl *b* day 1	-1,9262	0,7037	0,8643	29
Pot. Resp.	Chl *b* day 5	-1,6171	0,5899	0,8932	30
Pot. Resp.	Chl *b* day 8	-1,8604	0,6762	0,7197	28

Table 4. The regression analysis of pooled data give a and b for the equation Y= bX + a; n is the number of determinations and r is the correlation coefficient. The units used are µmols O_2/ min. The dry mass is given in units of grams.

3.3. Variability of the Potential respiration, Dry mass and Chlorophyll in three different locations around Gran Canaria.

The variability of the different measurement is presented in table 5. *Ulva rotundata* from Bocabarranco and San Cristóbal exhibited the higher Potential respiration, consistent with the higher level of nutrients founded in the sea water there. *Ulva rigida* from Taliarte, where the lowest level of nutrients were found, has the lowest Potential respiration. However *Ulva rigida* presented the highest dry mass.

	Ulva rigida **(Taliarte)**	***Ulva rotundata*** **(Bocabarranco)**	***Ulva rotundata*** **(San Cristóbal)**
Specific potential respiration (nmols O_2/ dry mass/ min)	0,65 ± 0,05	0,98 ± 0,18	0,78 ± 0,12
Dry Mass (mg)	49,65 ± 5,93	23,44 ± 5,51	32,33 ± 7,31
Chlorophyll *a* (µg/ sample)	1,60 ± 0,0018	1,59 ± 0,0016	1,59 ± 0,0022
Ammonium (mg/l)	1,23	2,78	1,58
Nitrate (mg/l)	1,2	2,4	2,7

Table 5. Comparation of the Specific potential respiration, dry mass and chlorophyll measures at three locations.

3.4. Metabolism time-course in aquaria with filtered seawater.

The evolution of Specific potential respiration over a week (Table 6 and Fig. 13) clearly showed that the potential respiration, in units of nmols O_2/ dry mass/ min, was the highest on the first day in all locations and in the two species, and this value decreased with time. Very likely some essential nutrient was exhausted during this period

Time (days)	*Ulva rigida* (Taliarte)	*Ulva rotundata* (Bocabarranco)	*Ulva rotundata* (San Cristobal)
1	0,6481 ± 0,0526	0,9844 ± 0,1765	0,7805 ± 0,1171
5	0,4447 ± 0,0543	0,6220 ± 0,0533	0,7532 ± 0,1692
8	0,4159 ± 0,0735	0,3612 ± 0,0771	0,5688 ± 0,1256

Table 6. Specific potential respiration time-course in *Ulva* spp. from the three different locations.

Statistically we detected significant differences between locations and between the times (p<0, 05) too except in this cases:

a) There is not significant differences in *Ulva rotundata* from San Cristobal between the day 1 and day 5 (Pairwise comparation p>0, 05).
b) There is not significant differences in *Ulva rigida* fromTaliarte between the day 5 and day 8 (Pairwise comparation p>0, 05).
c) There is not significant differences in *Ulva rotundata* from Bocabarranco and *Ulva rigida* fromTaliarte the day 8 (Pairwise comparation p>0, 05).

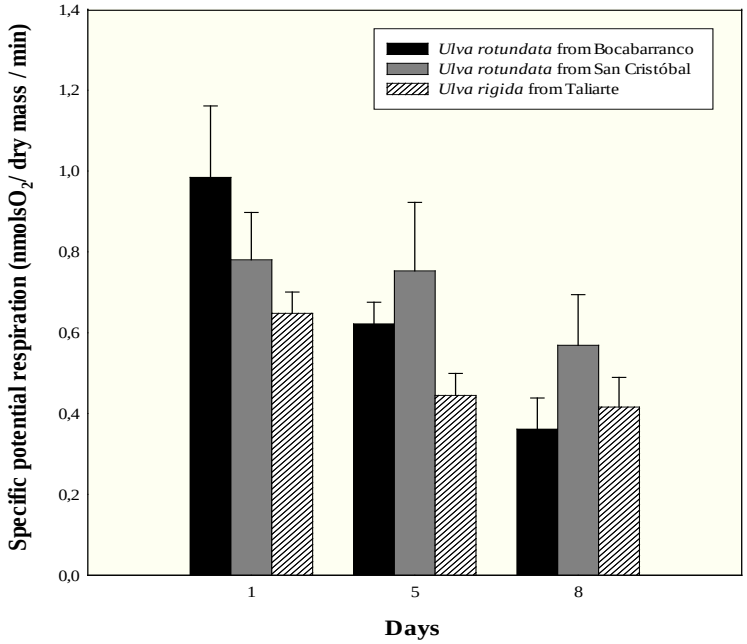


Figure 13: Specific potential respiration time-course in Ulva from the three different locations.

3.5 Relation between Biomass and Respiratory activity in the three different locations.

Table 7 presents time-course variability in the Kleiber equations between $\log_{10}$ (ETS) and $\log_{10}$ (dry mass) for the three different sampling locations around Gran Canaria. It shows the decrease in the Kleiber coefficient (b) from the first day to the last day. Taliarte has the highest slope on the first day and the smallest slope on the last day. After 8 days of incubation in aquaria, b has fallen more by than 100% from its original value.

Time (days)	*Ulva rigida* (Taliarte)	*Ulva rotundata* (Bocabarranco)	*Ulva rotundata* (San Cristóbal)
1	y = 0,5211x - 2,3324 r^2 = 0,5664	y = 0,2605x -2,0078 r^2 = 0,4303	y =0,4255x-2,25 r^2 = 0,547
5	y = 0,6156x - 2,658 r^2 = 0,7828	y = 1,0329x - 3,2564 r^2 = 0,8131	y = 0,1584x- 2,0469 r^2 = 0,1316
8	y = -0,1945x - 1,192 r^2 = 0,0077	y = -0,1421x - 1,7225 r^2 = 0,0186	y = 0,169x -2,064 r^2 = 0,0593

Table 7: Kleiber coefficient time-course during a period of a week in the three locations.

4. DISCUSSION

This research advances our knowledge of macroalgae metabolism. It was the first time that ETS activity was measured in a marine macroalgae. Furthermore, the relative contributions of the component dehydrogenases that feed the ETS were unknown. To begin this investigation, methodological studies of homogenization efficiency had to be made. Macroalgae are known to be difficult to homogenize because of their thick cell walls. For this reason, physiologists studying macroalgae historically have frozen their algal samples in liquid nitrogen and have manually ground, with a pestle, the solidified algal cells in a ceramic or marble mortar. We tried this technique, but found that homogenization in a Potter-Elvehjen type Teflon-glass tissue grinder works better.

After optimizing the homogenization step in the ETS assay we turned to verify the substrates. We did not intend to change this part of the ETS assay technique now, but we wanted to make a pilot study of substrate specificity with the intent of modifying the assay in the future. In Zooplankton studies, exemplified by Gómez et al. (1996), the most important contributor to the ETS activity was NADH. Here, the most important substrate for the two Ulva species was NADPH, a substrate known to be important in bacteria and archaea as well as in the microsomes of the eukaryotes (Strittmatter, 1968; Packard, 1985a and b). It would be interesting to investigate whether NADPH is as important in other chlorophytes and in red and brown macroalgae. According with Kenner and Ahmed (1975), more than half the ETS activity in the green single-celled alga, *Dunaliella tertiolecta*, is supported by NADPH. In another study with the green alga, *Scenodesmus quadricauda*, Rhee (1978) found that NADPH activity decreased in cultures with low protein content and in cells with low microsomal respiration rates. This suggests that future studies of ETS activity in Ulva and other macrophytes should investigate both the respiratory oxygen consumption rate as well as the protein content of the algal cells.

Knowing the mathematical relationships between the biological properties (size, mass, absorption spectra, etc.) and biological dynamic processes (enzyme activities and physiological rates) is important in making ecological calculations of biogeochemical cycles and food chain dynamics, as well as in building oceanographically realistic ecosystem models. Towards this end, we investigated the relationships between dry

mass, chlorophyll and ETS activity in Ulva. The resulting relationships were shown in Figs. 9, 10, 11 and 12; the equations were given in Table 2, 3, and 4.There showed that when the ulva data were pooled the relationships between all three variables were high and could be used in models of the Ulva's physiology and ecology.

This study showed variability between sampling location of the different measurement (potential respiration, nutrients and dry mass). The chlorophyll was similar in the three locations. We could see that *Ulva rotundata* from Bocabarranco exhibited the highest potential respiration, consistent with the high levels of nutrients founded in the sea water there. Instead, Taliarte present the lowest level of nutrients which coincided with the lowest potential respiration found in *Ulva rigida*. Another strong difference between species is the dry mass, *Ulva rigida* from Taliarte had the highest value. These findings of the higher metabolism in *Ulva rotundata* associated with elevated nutrients should be investigated in a more detailed future study.

Herrera et al. 2011 reported Kleiber coefficients were in the mysid *Leptomysis lingvura.* In that case, φ per unit of biomass was higher in mysids that were well fed for a long period of their growth. It was lower in starved mysids. Others authors, Gómez et al., 2008, and Martínez et al., 2010, found similar results in zooplankton samples from upwelling and oligotrophic areas, and also in well-fed and starved Artemia cultures. All of these results are similar to those obtained here. According to our results the Kleiber coefficient (b) decreased from the first to the last day due to the partial starvation. The φ values are higher in natural's conditions (day 1).

The measurements of potential respiration, after a few days of incubation showed that Ulva metabolism decreased with the time, probably due to a deficiency of some essential nutrient. However we conclude that one week is not enough to completely starve the algae. We suspect that they were using their internal resources and nutrients pools or perhaps were extracting the nutrients from the filtered sea water. More long-term experiments using artificial seawater should be done to verify our results.

5. CONCLUSIONS

1. Comparison between potential respiration in both homogenization methods demonstrated a significant difference. We used the tissue-grinding method because it was less expensive and easier.

2. The most important contribution to the ETS activity of *Ulva* spp. was NADPH, followed by NADH and succinate.

3. There was good correlation between the biomass parameters, dry mass, chlorophyll, and optical density at 670 nm. As a result, we used this optical as a measure of biomass.

4. *Ulva rotundata* from Bocabarranco had the highest potential respiration consistent with the high levels of nutrients and *Ulva rigida* from; Taliarte has the lowest potential respiration coinciding with the lowest level of nutrients in the area. However *Ulva rigida* from Taliarte has the highest dry mass. The differences in the 8-day potential respiration time courses for the three areas were statistically different. There was a loss between 25 to 60% of its initial potential respiration over this time period.

5. There was a decrease in the Kleiber coefficient (b) from the first day to the last day, suggesting a shift in the nutritional state.

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7. REFERENCES

Barton ED, Arístegui J, Tett, P., Cantón M. and 18 others, 1998. The transition zone of the Canary Current upwelling region. Prog Oceanogr 41:455–504.

Benedict, F.G., et al., 1938. Vital energetics. a study in comparative basal metabolism. Vital energetics. A study in comparative basal metabolism.

Brody, S., Procter, R., 1932. Relation between basal metabolism and mature body weight in di_erent species of mammals and birds. University of Missouri Agricultural Experiment Station Research Bulletin 116, 89-101.

Davenport R, Never S, Helmke P, Perez-Moreno J, Llinás O., 2002. Primary productivity in the northern Canary Islands region as inferred from Seawifs imagery. Deep-Sea Res. II 49:3481–3496.

Glazier, D., 2005. Beyond the '3/4-power law': variation in the intra- and interespecific scaling of metabolic rate in animals. Biological Reviews 80, 611-661.

Gómez, M., Torres, S., Hernández-León, S., 1996. Modification of the electron transport system (ETS) method for routine measurements of respiratory rates of zooplankton. S. Afr. J. Mar. Sci. 17 (1), 15–20.

Gómez, M., Martinez, I., Hernández-León, S.,Packard, T., 2008. Does ETS activity follow Kleiber's law?, International Symposium on Eastern boundary Upwelling Ecosystems(2-6 junio 2008).Las Palmas de Gran Canaria, Canary Island, Spain.

Haroun, R.J., Gil-Rodríguez, M.C., Díaz de Castro, J., Prud'homme van Reine, W.F., 2002. A Checklist of the Marine Plants from the Canary Islands (Central Eastern Atlantic Ocean). Botanica Marina Vol. 45, pp. 139-169.

Herrera, A., Packard, T.T., Santana, A., Gómez, M., 2011. Effect of starvation and feeding on respiratory metabolism in Leptomysis lingvura (G.O. Sars, 1866).Journal of Experimental Marine Biology and Ecology 409 (2011) 154–159.

Kenner, R., Ahmed, S., 1975. Measurements of electron transport activities in marine phytoplankton. Mar. Biol. 33, 119–127.

Kleiber, M., 1932. Body size and metabolism. Hilgardia 6, 315-353.

Lovegrove, T., 1966. The determination of the dry weight of plankton and effect of various factors on the values obtained. In: Barnes, H. (Ed.), Some contemporary studies in marine science. George Allen and Unwin Ltd., London, pp. 429- 467

Mantoura and Llewellyn, 1983. The rapid determination of algal chlorophyll and carotenoid pigments and their breakdown products in natural waters by reverse-phase high-performance liquid chromatography. Institute for Marine Environmental Research, Prospect Place, The Hoe, Plymouth PL1 3DH Gt. Britain. Volume 151, 1983, Pages 297-314.

Martínez, I., Gómez, M., Packard, T., 2010. Potential respiration is a better respiratory predictor than biomass in young Artemia salina. Journal of Experimental Marine Biology and Ecology 390, 78-83.

Mitchell, G and Kiefer, D. 1984. Determination of absorption and fluorescence excitation spectra for phytoplankton. Lecture notes on costal and estuarines studies, VOL. 8, PP. 157-169.

Packard, T.T., Harmon, D., Boucher, J., 1974. Respiratory electron transport activity in plankton from upwelled waters. Tethys 6 (1–2), 213–222.

Packard, T.T., Christensen, J.P., 2004. Respiration and vertical flux in the Gulf of Maine water column. J. Mar. Res. 62, 93–115.

Packard, T.T., Gómez, M., 2008. Exploring a first principle based model for zooplankton respiration. ICES J. Mar. Sci. 65 (3), 371–378.

Prud'homme van Reine, W.F., van den Hoek, C., 1990. Biogeography of Macaronesian seaweeds. Courier Forschungsinstitut Senckenberg 129: 55-73.

Rhee, G.Y., 1978. Effects of N: P atomic ratios and nitrate limitation on algae growth, cell composition, and nitrate uptake. *Limnol. Oceanogr.* 23 (1): 10- 25.