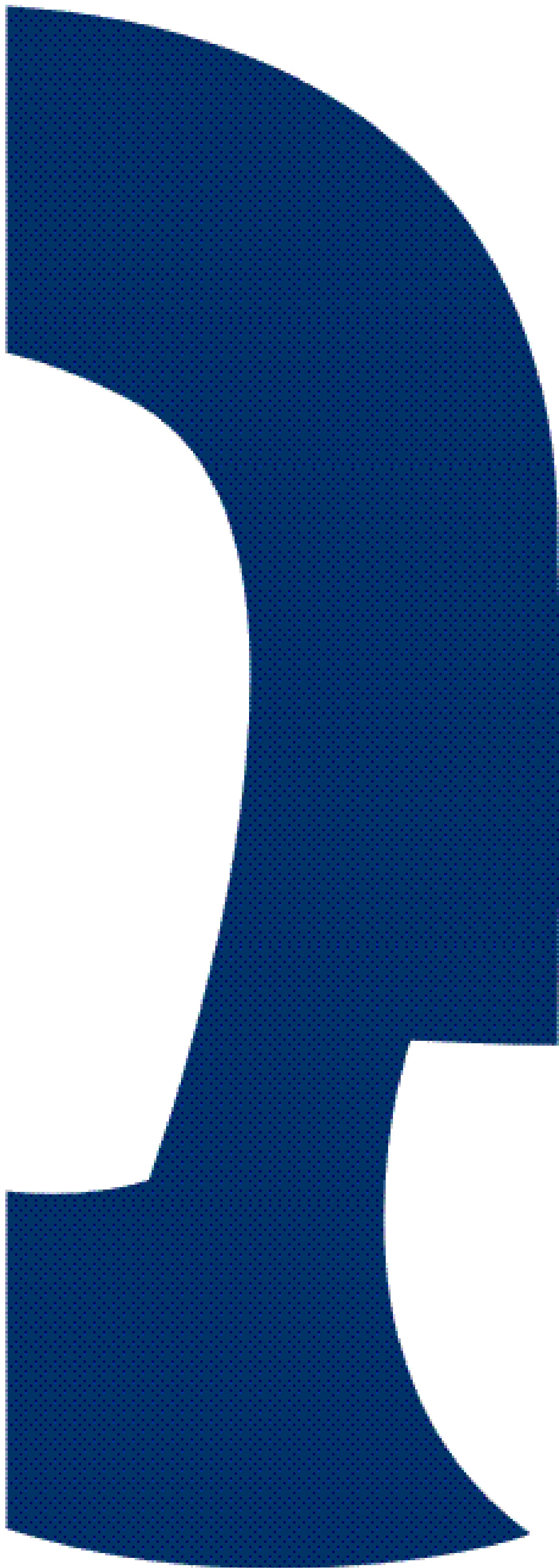

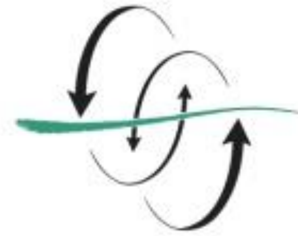

Respiration in an Autotrophic and a Heterotrophic Organism: Comparison of four Different Methods

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Curso 2015/2016

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Trabajo Fin de Título para la obtención del título de Máster en Oceanografía

TÍTULO

Respiration in an Autotrophic and a Heterotrophic Organism: Comparison of four Different Methods (Estudio comparativo de la respiración en organismos autotróficos y heterotróficos a partir de diferentes métodos.)

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INTRODUCTION

In the following research we studied the differences between the respiratory behavior of an autotrophic and a heterotrophic organism while at the same time comparing different respiration measurement techniques. The respiration methodologies were the Winkler method, oxygen electrodes, oxygen optodes and the kinetic assay for the respiratory electron transport system (ETS), methods that allow the determination of both physiological and enzymatic respiration. The organisms chosen for these experiments were a coastal crustacean, the mysid *Leptomysis lingvura,* and a coastal macroalgae, the chlorophyte, *Ulva rigida*.

This work will briefly introduce the ecology of *L. lingvura* and *U. rigida* as well as explain the interest and importance of respiration and its measurement. It will continue with the description of each method used for a better understanding of their strengths and weaknesses. Then the results will be discussed and finally used to draw conclusions on the comparability of these methods and on the differences between the mysid and Ulva respiration and their possible physiological and biochemical causes.

Mysid: *Leptomysis lingvura*

Mysids are pericardian crustaceans distributed globally in both neritic and pelagic ecosystems (Fig. 1). They are omnivorous feeders, eating several planktonic components such as copepods, tinntinids, diatoms and particulate organic matter (*Tattersall & Tattersall, 1951; Mauchline, 1980; Murano, 1999; Jumars, 2007*). In nature they form swarms (Fig. 1) and exhibit circadian rhythms (*Dauby, 1995, Herrera et al. 2011*). They are also important prey for both coastal and pelagic plankton feeders (*Murano, 1999*).

The mysid species used in this work, *Leptomysis lingvura*, is found near sandy and rocky bottoms in the coastal waters around Gran Canaria. It is not exclusive to this island and, as in other areas, it is a component of the regional food chain for coastal fish (*Castro, 1998*). It is an important and abundant member of the seagrass meadows where it plays a vital role in this ecosystem's productivity (*Herrera et al., 2014*). The interest in this species has increased with investigation of its potential as live prey for aquaculture purposes (*Wittmann, 1981; Domínguez et al., 2000; Herrera et al., 2011[a]*).
It has been documented to survive well in captivity even completing its life cycle, which makes it desirable experimental organism for marine research (*Herrera et al., 2011[a]*). The mysids cultured here were captured while scuba diving in the northeast coast of Gran Canaria. It is not the only mysid species in the area so care was taken to taxonomically differentiate it from other species such as *Siriella armata*.

Fig. 1. Leptomysis lingvura

Left: one individual under a stereoscopic microscope.

Right: Swarm of individuals in the their medium near an anemone (www.biologia marina.org)

Macroalgae: *Ulva rigida*

Ulva is the genus of the green macroalgae commonly known as sea lettuce. Ulva species are very common worldwide and form part of rocky shore ecosystems around the world, cohabiting areas with other seaweed (Fig. 2). This genus is also commonly known for its ease in culturing, culinary uses and abundance in eutrophic waters near oceanic outfalls (*Haroun et al., 2009*) which sometimes makes it an important component of green tides (*Smetacek & Zingone, 2013*).

In particular, *Ulva rigida,* along with *Ulva rotundata,* is one of the most abundant Ulva species in the eulittoral and upper sublittoral zone of Gran Canaria. These two species are quite similar, both are foliaceous and rigid with a flat thallus (*Haroun et al., 2002; Haroun et al. 2009*). The *Ulva rigida* used in these experiments was collected from the Northeast of the island near the mouth of Las Palmas harbour. In the upper intertidal area it cohabites with Cystosseira and Gelidium species from which it is easily differentiated. To distinguish it from *Ulva rotundata,* with which it also cohabites, microscopic examination is required (*Afonso & Sanson, 1999*). Like most seaweeds, it is important for coastal productivity because it provides shelter, nourishment and water purification for fish and invertebrates, such as amphibious isopods, that share this ecosystem. Easing our experimental research, *Ulva rigida* was abundant and accessible most of the year and it was easy to identify, collect and culture as noted by *Asensio (2013).*

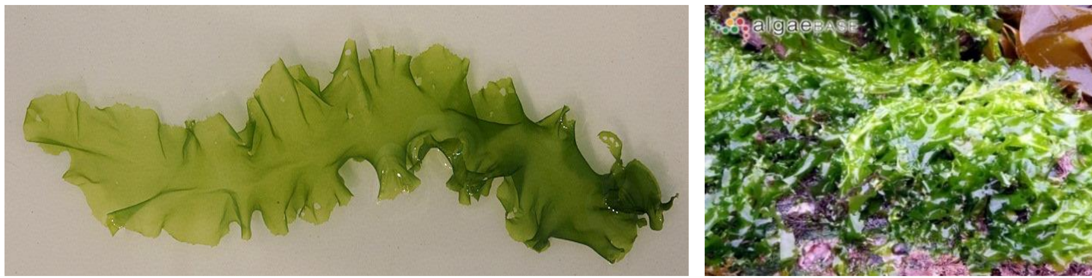


Fig. 2. Ulva rigida. Left: Individual of U. rigida in the laboratory Right: Group of U. rigida in its medium with other seaweeds. (www.nature22.com (algues verts), www.algaebase.org (Ulva rigida C. Agardh))

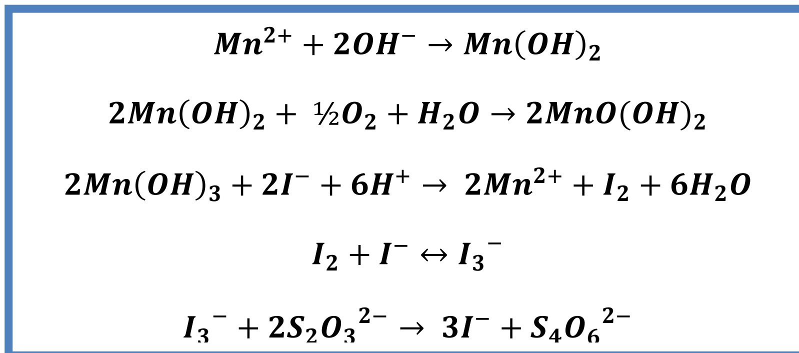
Respiration

Respiration is a catabolic process by which all organisms, except obligated fermenters, obtain vital energy as a result of a series of reactions that end with the reduction of a final electron acceptor. Respiration has been extensively studied, but the anabolic/productive process, photosynthesis, has attracted more attention within the ocean research community. The explanation for this difference stems from considering respiration as a small fraction of primary production. This is a questionable assumption when studying oceanic anabolism and catabolism due to their complex coupling. (*Williams, 1993; Aristegui & Harrison, 2002; Williams & Del Giorgio, 2005; Duarte et al., 2013*)

The following discussion is focused on one type of respiration, namely, aerobic respiration. For this type of respiration, organic material is used as an electron donor to produce energy using oxygen as final electron acceptor. In the process carbon dioxide and water are the resulting products from the organic matter oxidation and oxygen reduction. Eukaryote organisms need to be able to obtain organic matter and oxygen to produce energy. Heterotrophic organisms obtain both of them externally through feeding, food processing, and respiration. Autotrophic organisms are able to produce both of them during photosynthesis using light, water and CO_2. When light is not available they respire external oxygen and use up their reserves of organic matter. Therefore, the rate of oxygen consumption in darkness in both types of organisms can be used as a measure of respiration. This is physiological respiration (R). The reduction of free oxygen found in the surrounding water, in itself, does not produce energy. But it is stoichiometrically related to the organic carbon consumed in respiration and related, through the process of oxidative phosphorylation, to energy (adenosine triphosphate [ATP]) production.

To measure respiration via oxygen consumption, the organism of interest should be incubated in a sealed container which does not allow oxygen exchange between the interior and exterior. After some time there should be a decline on the oxygen concentration. To measure this decrease several techniques have been developed. The first, and the most common, is the chemical detection of oxygen concentration in water, the Winkler Method. It was first used to measure plankton respiration in a dark bottle to correct photosynthesis measurements (*Gaarder and Gran, 1927*). Later it was used by *Marshall et al., (1935)* to measure respiratory O_2 consumption in the planktonic copepod, *Calanus finmarchicus*. Respiration measured for its own sake was uncommon in the literature compared to its association with photosynthesis until the end of the 1950s (*Ikeda et al., 2000; Williams & Del Giorgio, 2005*).

The Winkler technique is based on the measurement of oxygen (O_2) at the start and end of an incubation. The difference (ΔO_2) is interpreted as the amount of oxygen consumed by the organism in the time interval (Δt) and is used to calculate a rate of O_2 consumption ($\Delta O_2/\Delta t$). This calculation assumes that respiration was constant throughout the experiment. With time and the application of new technology this technique was improved by using smaller water volumes and by measuring oxygen multiple times throughout the incubation period (*Ikeda et al., 2000*). These improvements made the measurement much more reliable than the previous two-point analysis technique. The basis of this chemical method is using manganese and iodine compounds to fix the dissolved oxygen and then performing a titration with a known concentration of thiosulphate (Fig. 3) (*Omori & Ikeda, 1984*).



$$Mn^{2+} + 2OH^- \rightarrow Mn(OH)_2$$

$$2Mn(OH)_2 + ½O_2 + H_2O \rightarrow 2MnO(OH)_2$$

$$2Mn(OH)_3 + 2I^- + 6H^+ \rightarrow 2Mn^{2+} + I_2 + 6H_2O$$

$$I_2 + I^- \leftrightarrow {I_3}^-$$

$${I_3}^- + 2{S_2O_3}^{2-} \rightarrow 3I^- + {S_4O_6}^{2-}$$

Fig. 3. Simplified chemical equations of the reactions of the Winkler Method.

(Hansen, 1999)

After the Winkler technique was introduced other techniques and instruments were developed to measure oxygen. One of the first of the new instruments was the O_2 electrode. Its used was based on measuring oxygen concentration through an electrochemical reaction. The most common electrode was the Clark- type (*Clark, 1956*) (Fig.4) where the anode and cathode were made of silver-silver chloride and platinum respectively. A flux of electrons would migrate from the Ag-AgCl anode to the Pt cathode where the O_2 would be reduced (Fig. 4). With adequate calibration the electrons flux is related to the partial pressure of oxygen and hence, the O_2 concentration. Electrode results of O_2 concentration are not as accurate as Winkler measurements, but they can be obtained easily, and continuously in even smaller volumes of water (*Ikeda et al., 2000*).

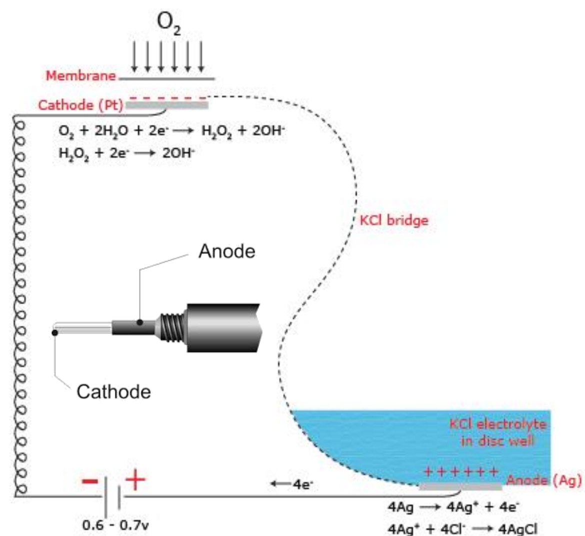


Fig. 4. Diagram of the functioning of a Clark type electrode. Hansatech instrument website (Walker, 1987)

Both the electrodes and the Winkler method depend on a reaction that consumes the dissolved oxygen (*Ikeda et al., 2000*). The most recent methodology for O_2 detection, the oxygen optode does not.

It measures oxygen through fluorescence quenching and is based on oxygen's ability to alter fluorescence emitted by certain dyes (oxygen quenchable flourophores). Exploiting this property, an apparatus was designed to project a certain wave length light through an optic fiber on to a fluorophore exposed to ambient oxygen (*Lubbers & Opitz, 1983; Lubber & Opitz, 1985, Opitz, 1986*). The timing or wave length of the fluorescence emitted by the fluorophore would be altered in relation to the O_2 concentration. So with proper calibration the O_2 can be determined continuously without stirring and without consuming O_2 (*Klimant et al., 1995; Gatti et al., 2002; Warkentin et al., 2007*).

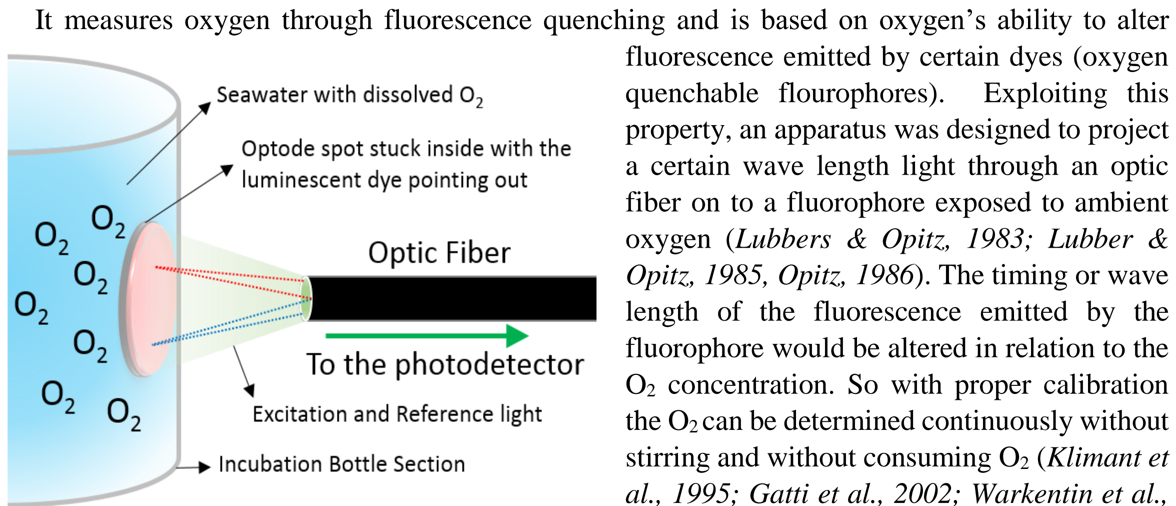


Fig. 5. Diagram of the functioning of an O_2 optode.

In the 1980s and 90s other methodologies, including mass spectrometry (*Bender et al., 1987; Bender, 1990*) and the Micro-Oximax (*Berdalet et al., 1995*), were explored to determine respiration. However, in the following work will focus on the O_2 optode and electrode.

These instruments, as well as the Winkler technique, normally require prolonged incubation. The underlying assumption is that the respiration of an organism inside a container is the same as it is in situ. Unfortunately, for all organisms, this assumption is tenuous. Sampling, undoubtably, and likely, entrepement, will may cause sufficient stress to alter respiratory behavior. In addition, during the incubation period starvation will depress the organism's physiology, reducing its respiration (*Ikeda et al., 2000*).

Indirect methods, designed to eliminate incubation and based on the enzymology behind respiration, have been under development since the 1960s. Instead of observing how dissolved oxygen is removed from the water these methods measure the maximum reaction rate of the enzymes involved in cellular respiration. The assay for succinate dehydrogenase (SDH), electron transport system (ETS), citrate synthase (CS), and isocitrate dehydrogenase (IDH) are prime examples (*Curl & Sandberg, 1963; Packard et al., 1971, Berges et al., 1990, Thuesen & Childress, 1993, Berdalet et al., 1995; Packard et al., 1996; Maldonado et al 2012, Romero-Kutzner et al, 2015*). However, here the focus is on the ETS (*Packard & King, 1975; Kenner & Ahmed, 1975; Owens & King, 1975; Packard et al., 1996a; Packard & Williams, 1981, Gómez et al., 1996*) and its measurement by kinetic analysis (*Packard & Christensen, 2004*). Inside the cell the oxygen is reduced to water at the mitochondrial membrane. The oxygen acts as the final electron acceptor in a series of enzyme-catalyzed reactions that develop a proton gradient responsible for ATP production (Fig. 6) (*Hill et al., 2006; Nelson & Cox, 2009*). This ETS kinetic analysis is based on adding the appropriate substrates to activate the enzymes of the electron transport system and on using an indicator, a redox dye, to observe and quantify the reaction taking place. In the technique designed by *Packard et al. (1971)* the indicator is a tetrazolium salt (INT) which acts as an artificial electron acceptor (*Smith & McFeters, 1997*). When INT is reduced it forms a red formazan that, in solution, can be stoichiometrically quantified spectrophotometrically via the Beer-Lambert Law. The rate of formazan production can be used to estimate the rate of oxygen consumption at this cellular/enzymatic level. The result is the theoretical maximum respiration of the organism, the potential respiration. This potential respiration (Φ) is a rate higher than physiological respiration, but one that is not susceptible to sampling stress or incubation errors. From this Φ one calculates the actual respiration using first-principles models based on the internal concentration of the substrates of the enzymes and the enzyme kinetics (*Packard et al, 1995, Packard & Gómez, 2008; Osma et al., 2016*) or by using a model based on a statistical relationship to respiration (*King & Packard*, 1975; *Packard & Williams, 1981; Hernández-León & Gómez, 1996; Ikeda et al., 2000; Packard & Christensen, 2004; Hernández-León & Ikeda, 2005*).

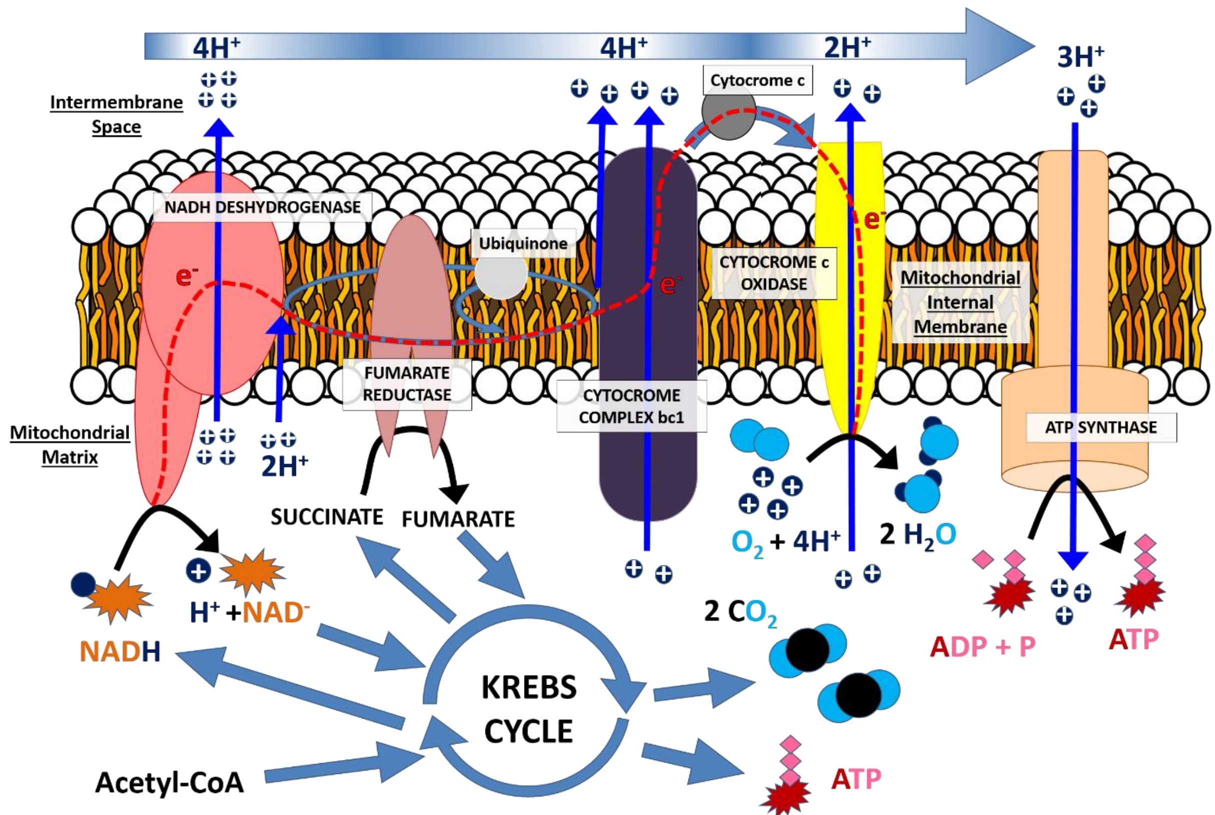


Fig. 6. Simple diagram of the electron transport system in the mitochondrial membrane and its connections to the Krebs cycle and oxygen reduction.

The aim of the following investigation is to compare these methods and then to identify and to understand their similarities, relationships and differences.

Better understanding of respiration, the factors that affect it, and the way we measure it could improve our knowledge about multiple oceanic processes. Respiration values are employed for numerous purposes: investigating physiology, quantifying the carbon cycle, determining primary and secondary production, estimating carbon flux, studying the biological pump, calculating carbon dioxide emission and oxygen depletion rates and understanding oceanic metabolic balance, amongst others. Basically, aerobic respiration is a key metabolic reaction for generating energy as ATP in mitochondria, for each cell and thus for the organism as a whole. But, it is also a process in which oxygen and carbon dioxide participate and therefore the planetary homeorhesis of these two gases is partially regulated by respiration. It is especially significant in its role in the carbon cycle affecting the biosphere, hydrosphere and atmosphere. The organic matter that is respired is turned partially into CO_2 and so respiration controls the intensity of carbon flux and the biological pump. Determining if particular oceanic regions act as sinks or as a sources for CO_2 (autotrophic or heterotrophic) is a recent polemic (*Del Giorgio & Williams, 2005; Duarte et al., 2013; Ducklow & Doney, 2013; Williams et al., 2013; Packard et al., 2015*).

This study also tries to delve into the respiratory behavioral differences between organisms that produce and consume O_2 (autotrophs) and ones that only consume O_2 (heterotrophs). Both organisms in this work are coastal, but Ulva is autotrophic, having both catabolic respiration and anabolic photosynthesis, while the mysids are heterotrophic, having only catabolic respiration. Their different metabolism could impact their normalized physiological respiration (R), their enzymological respiration (Φ) or their physiological to potential respiration relationship (R/Φ). The measurement of ETS and the comparison between the physiological and cellular respiration has rarely been studied in macroalgae (*Asensio, 2013*). This study is a step towards better understanding respiration and the techniques to measure it in organisms.

METHODOLOGY

Sampling and Culturing Techniques

The organisms used in this experiment inhabit two different areas of the coast. The mysid *Leptomysis lingvura* live near sandy and rocky bottoms at 5 to 15m depth while the green macroalgae *Ulva rigida* inhabited the intertidal zone of the rocky coastline. The crustacean is mobile, swimming in the water column, and the macrochlorophyte is sessile, attached to the rocky surface. Accordingly, the sampling techniques were different for each organism.

Fig. 7. Location of sampling sites marked on the map of Gran Canaria. Orange spot: Mysid sampling site (Sardina del Norte). Green spot: Ulva sampling site (Castillo de San Cristobal). Left: Diving map of Sardina del Norte. Right: photograph of Castillo de San Cristobal. (www.buceopandora.es, Google maps, Juan Ramón Rodriguez Sosa)

Leptomysis lingvura

Mysid sampling took place the 14th of October of 2015 at 13:00 near the shore of Sardina del Norte, at the northwest of Gran Canaria (Canary Islands, Spain) by scuba diving (Fig. 7 & Fig. 8). Once in the subaquatic sampling site, mysids were captured with a 500 µm mesh hand net. The dense clouds of mysids eased the sampling near the rocks or at a distance from the sandy bottom. After capture the mysids were transported in a closed container that was kept cool and open to air exchange. Once in the laboratory the organisms were separated accordingly to their species. A stereomicroscope was used to differentiate taxonomical characteristics of the telson and head of the mysid species (*Tattersall & Tattersall, 1951; Wittmann, 1983; Gomez, 2000; Cebrian et al., 2001; Herrera-Ulibarri, 2013, Meland et al., 2015*). There were only two species identified, *Leptomysis lingvura* and *Siriella armata.* The first one was chosen for the experiments because it was more numerous, was easier to culture, well-known in our laboratory, and had a solid literature base.

The species were first separated in glass tanks of 35 litres and later cultured at 18ºC±0.5ºC in aquaria (Fig. 8) with a recirculation system based on *Lussier et al. (1988)* under conditions described by *Herrera-Ulibarri (2013).* The mysids were fed 48h artemia nauplii twice a day and their tanks were frequently cleaned according to *Herrera-Ulibarri (2010)*. Mysids were left 10 days to acclimate to the new culture environment before they were used in experiments.

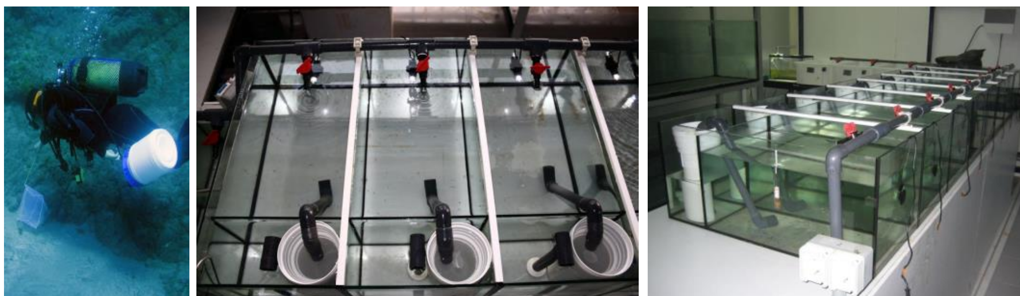


Fig. 8. Left: L. lingvura sampling by hand net on a scuba dive. Centre & Right: Aquaria for L. lingvura (Herrera-Ulibarri, 2013).

Ulva rigida

Ulva samples were collected on October 20, 2015 at the rocky shore of Castillo de San Cristobal on the northeast of Gran Canaria (Canary Islands, Spain) (Fig. 7) during a low tide at 8:30. The organisms were carefully extracted from the rock making sure that they kept their basal disc. They were stored in a container filled with local sea water and transported to the laboratory. There they were cleaned, removing dirt and other organisms, identified using a microscope (*Afonso & Sanson, 1999*) and separated into three groups of approximately 10 grams. These groups were cultured in 3 different 2L Florence flasks containing 1L filtered seawater enriched with 10µM NO_3^- and PO_4^{-3} (Fig. 9). The seawater and nutrients were renewed every morning. They were cultured in an incubator chamber at 18ºC (Fig. 9). The photosynthetic photon flux density in the chamber was 100 µmoles · m^{-2} · s^{-1} from cool white TL-D 36W/840 fluorescent lamps with a photoperiod of 12 hours (Fig. 9). The conditions were similar to the ones described by *Cabello-Pasini et al. (2011).* The Ulva samples were cultured for 7 days before using them in an experiment.

Fig. 9. Right: Culturing chamber with Florence flask for U. rigida culture. Left: Florence flask with U. rigida samples.

Biomass Determination

The macroalgae and the mysid individuals were not the same size, thus, their biomass was determined and used to normalize the respiratory results. The biomass, as protein, was measured using the protein method described by *Lowry et al. (1951)* and modified by *Rutter (1967).* Analyses were made on homogenates used for the ETS analysis described below. In brief, this method consists of a chemical reaction, a spectrophotometric analysis, and calculation based on a bovine serum albumin standard-curve with solutions of different protein concentration (Fig. 10). The spectrophotometer used in this study was a Cary100 UV-Vis Spectrometer from Agilent Technologies. As a backup measure wet mass was determined for *Ulva rigida.*

Fig. 10. Examples of cuvettes for protein measurement of different protein concentration during Lowry's method.

Respiration Measurements

The Winkler reagents were standardized and the electrodes and the optodes were calibrated before any measurement of physiological respiration.

After transfer from the aquaria to the lab for experimentation, the mysids were kept in the dark at 18ºC to acclimate to a new smaller space for one hour before the experiment. This acclimatization process was an attempt to minimized stress and its associated increase in oxygen consumption.

Before measuring their respiration, Ulva samples were blotted to remove excess seawater, weighed for their wet biomass, and then preincubated in darkness for one hour. This dark treatment prevented residual photosynthesis that could alter the subsequent measurement consumption of oxygen.

Finally, Eppendorf tubes were labelled and readied to store the ETS samples at -80ºC.

Bottle/Physiological Methods

The three following methods required sealed bottle incubations to measure respiration by detecting the depletion of oxygen (oxygen consumption). The bottles had to be kept in the dark to stop any photosynthetic reaction from generating oxygen. If the bottles were not kept in darkness during the incubation, photosynthesis would reduce the oxygen decrement inside the bottle and cause an underestimation of oxygen consumption.

The bottles used were 50ml BOD bottles. Water inside them was kept at 18ºC±0.5ºC with a thermostatic bath and constantly magnetically stirred. This was necessary for the proper functioning of the electrode, but it also precluded stratification which would distort the measurements (*Ikeda et al., 2000*). This stirring is not aggressive and the stirring-bar is separated from the organism by a net to prevent injury.

Winkler Method

This method is based on *Winkler's (1888)* method to measure dissolved oxygen in water. The technique described for this study comes from *Ikeda et al. (2000)* which largely follows *Oomori & Ikeda (1984).* As mentioned previously this is an end-point chemical analysis in contrast to a kinetic (time-course) measurement.

The procedure took place as follows for 10ml flasks (Fig. 11):

1. The water of the culture bottles was transferred to the three smaller 10ml flasks where the oxygen fixation took place.
2. The appropriate volumes of $MnCl_2$ (3M) and NaI (4M) were added and the mix was vigorously shaken to fix the dissolved oxygen.
3. After an iodine precipitate, proportional to the dissolved oxygen, had formed it was redissolved by adding sulphuric acid (50%) and transferred to an Erlenmeyer for titration.
4. A $Na_2S_2O_3$ (0.018M) solution was used as the titrant until the yellow solution turned pale.
5. Then starch indicator was added, which turned the solution blue.
6. Finally the titration continued until the colour blue had disappeared and the solution was transparent.

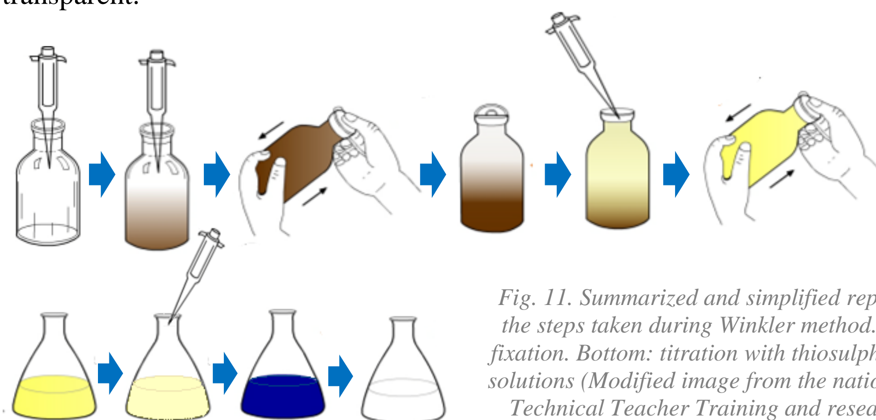


Fig. 11. Summarized and simplified representation of the steps taken during Winkler method. Top: Oxygen fixation. Bottom: titration with thiosulphate and starch solutions (Modified image from the national Institute of Technical Teacher Training and research of India)

The volume of the titrant solution employed was used to calculate dissolved oxygen concentration in the incubation bottle as described in *Ikeda et al. (2000).* Also, every day that the Winkler analysis took place the reagents were standardized and blanks were determined using a KIO_3 solution (0.00167M). Triplicates of the Winkler test were performed for each incubation bottle.

Electrodes

It was already explained in the introduction how these instruments work. The model used in this study was a Strathkelvin 928 6-Channel of Oxygen System (Fig. 12). Three electrodes measured

oxygen decrement in bottles with organisms and one electrode was used to measure the decrement in a bottle without any organisms. This bottle served as a blank.

The electrodes were properly maintained and cleaned between experiments, and stored in distilled water. Before their use they were always calibrated. The calibration procedure was done according to the instruction manual. It was a two point calibration with a maximum concentration of dissolved oxygen, achieved by stirring filtered seawater at a high speed for 20 min, and a zero, for which a sodium sulphite solution was employed.

The electrodes were sealed in the top part of the bottle and required stirring to prevent oxygen depletion at the electrode surfaces as a result of the electrochemical reaction at the cathode. The electrodes measured oxygen concentration continuously and simultaneously in the four bottles.

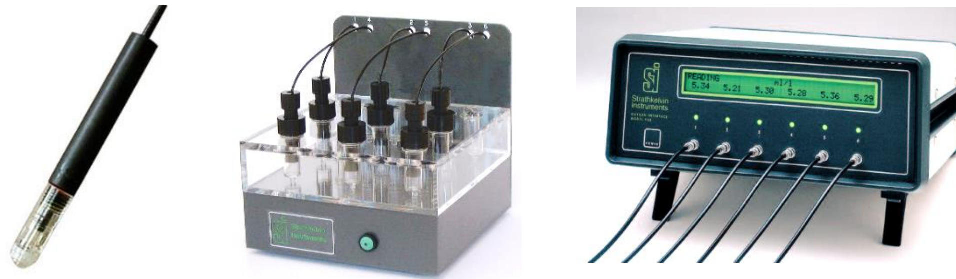


Fig. 12. Strathkelvin 928 6-Channel of Oxygen System. Left: Sensor. Centre: Sensors and bath. Right: Signal processor (From Strathkelvin & warneronline websites)

Optodes

In this study the optode was a 1-Channel Fibox 4 from Presens. The sensor consisted of a circular oxygen-sensing spot containing the photoluminiscent dye. It had to be stuck inside the incubation bottle to be affected by the oxygen present in the media. This spot was comprised of an insoluble inert metal porphyrine complex immobilized in an oxygen-permeable matrix. An optic fiber connected to the control-box shines a beam of light, at a precise wave-length, on the optode spot from the outside. The fluorescence of the spot is processed by the apparatus and converted to oxygen concentration.

The optode was calibrated manually to match the calibration conditions of the electrode using the same two-point calibration solutions, calibrating them both simultaneously.

This optode system was limited to one only measurement at a time, so it was used to measure oxygen concentration periodically moving from bottle to bottle. Each bottle had its own spot installed inside and the apparatus maintained a profile, a separate file, for each spot to save the data in an organized manner.

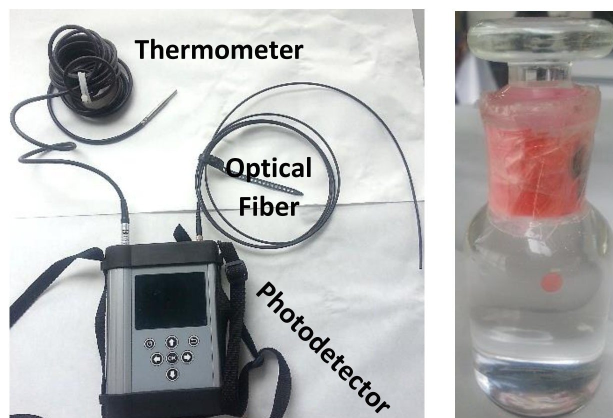


Fig. 13. Left: One channel Fibox 4 from the company Presens. Right: BOD incubation bottle with a pink optode spot.

Enzymatic/Biochemical Method

Physiological respiration is a macroscopic mechanism resulting from the biochemical reactions that sustain life in an organisms' cells (*Hill et al., 2006*). Accordingly, the activity of the respiratory system inside the cell should also be an index, or measure, for respiration. This approach can be achieved through the activity of several enzymes as discussed in the introduction.

To measure an enzymatic reaction, the enzymes must first be freed and second, the substrate should be adjusted to an optimum concentration. To achieve this, the sample must be homogenated, centrifuged and then enriched with the substrates involved in the reaction. The concentration of one of the products or substrates has to be measured to determine the rate of the reaction (formation or consumption).

ETS Analysis

The ETS analysis centres its attention on measuring the activity of the enzymes of the respiratory electron transport system. This system, in the mitochondrial membrane, has oxygen as its final electron acceptor (Fig. 6). The methodology to detect it will give the potential respiration of the organism, the oxygen consumption rate resultant if all the responsible enzymes in its cells are reducing oxygen.

Based on Packard et al, 1971, this method specifically employs an organic substance, a tetrazolium salt known as INT (2- (4-iodophenyl)-3-(4-nitrophenyl)-5-phenyltetrazolium chloride) which acts as an artificial electron acceptor (*Smith & McFeters, 1997*). When reduced the INT changes to formazan which has an intense red colour that can be measured spectrometrically (Fig. 14). Using a spectrophotometer to measure the colour absorbance of formazan at 750nm during 8 minutes, the rate of formation can be determined. To carry out this kinetic analysis the substrate must saturate the ETS enzymes thus NADH, succinate and NADPH are added in the appropriate concentrations.

The methods used for mysids and Ulva are slightly different and follow different authors. The mysid analysis was conducted according to the method of *Owens & King (1975)* for zooplankton; while the analysis of Ulva followed the directions of *Kenner & Ahmed (1975[a])* for phytoplankton with the modifications of *Gómez et al., 1996*. They differ in the substrates and the buffered solutions used to homogenate the samples and to free the enzymes. The mysid samples were homogenized using sonication. The Ulva samples were homogenized with a motor-driven teflon-glass tissue grinder using a disintegrated glass microfibre filter (GF/C Circle, 25mm) as an abrasive. Each in their respective buffered solutions. Both solutions were centrifuged for 10 minutes at 4000 revolutions per minute to only keep the enzymes of interest free in the medium and to reduce the turbidity caused by other organic material. Then the sample extract from the supernatant fluid, substrates and INT were mixed in a cuvette that was then used for the spectrophotometric kinetic analysis.

The kinetic analysis was run at 18ºC. A Cary100 UV-Vis Spectrophotometer from Agilent Technologies was used (Fig. 14).

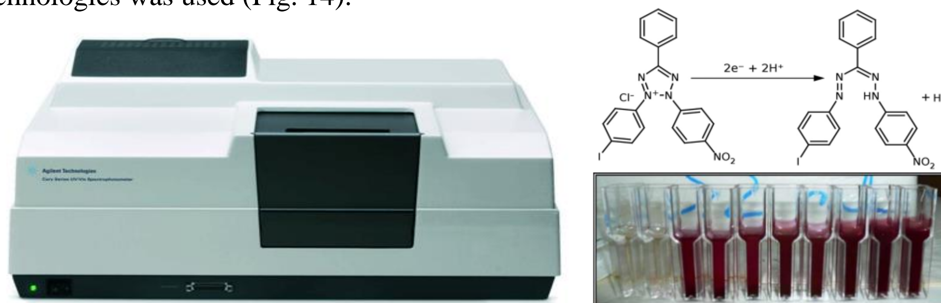


Fig. 14. Left: Cary 100 UV-Vis Spectrometer. (Agilent Technologies). Right: Reaction of tetrazolium reduction and formazan production in INT. Modified from Rogers (1953) and Packard (1971) and an example of cuvettes used for ETS analysis with different concentrations of formazan.

Combined Routine

Fig. 15 should help visualize the different parts of this combined routine. The measurements employing the three physiological methods were made simultaneously and then the sample was used for the ETS analysis.

The Winkler method required a chemical reaction with the water, so it was only used to measure start and end point concentrations. This way the sensor-based measurements monitoring the oxygen in continuum, were not impacted. Both electrodes and optodes measured oxygen concentration simultaneously during the incubation. Electrodes measured continuously, the optodes periodically. Each experiment consisted of respiration measurements in three bottles with samples and one bottle without a sample that was used as blank.

Before adding organisms in their respective bottles, the time was noted and a blank start point sample was extracted from another bottle. The reagents to fix the dissolved oxygen were added and later, this initial concentration was measured by the Winkler method. Then the organisms were inserted in their corresponding bottles and sealed by the electrodes. After this, the electrodes started to autonomously register oxygen concentration every 2 seconds. The measurements were displayed as a function of time on a monitor in the form of a graph. At the same time, every few minutes, the optodes were used to measure oxygen concentration manually and individually in each bottle. After a few oxygen concentration points were determined by the physical methods, the Winkler analysis was continued with the water sampled at the beginning serving as the starting point concentration. When this first titration was finished, the periodic measurements with the optodes continued; always verifying how the experiment was unfolding according to the electrodes' display.

This routine continued for 3 hours after which the oxygen consumption was large enough to allow a considerable volume of titrant used during Winkler analysis. It was a way to reduce the error caused by air mixing. After this time one electrode was taken out of the bottle and the water was carefully siphoned into three 10ml flasks until it displaced some of the volume. These water samples were used for Winkler titration. After fixing the oxygen in these flasks, the organisms left in the bottles were filtered from the remaining water and stored in a labelled Eppendorf tube in a -80ºC freezer for ETS and protein analyses. Once the organism sample was properly stored, the Winkler analysis of the water continued with the extracted triplicates in the flasks. The volume of titrant used was recorded. When the titration was finished some optode measurements were performed with the remaining bottles and then this last part of the procedure was repeated until all the organisms were stored in the freezer. Finally, the blank-bottles oxygen-concentration was siphoned and analyzed. After finishing all the physiological respiration measurements, the ETS activity was analyzed as described above. The homogenate remaining from the ETS analysis was then used to measure the protein content that will be used to normalize respiration results.

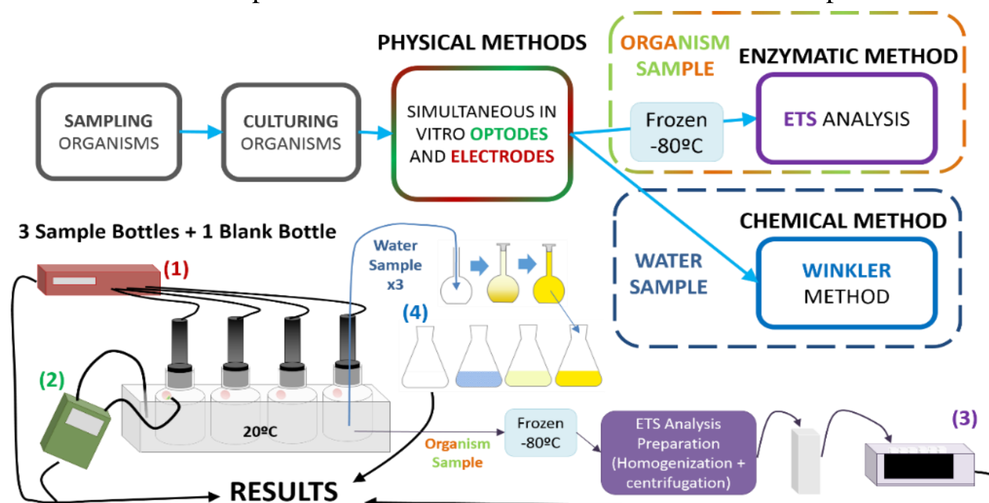


Fig. 15. Diagram and scheme of the steps taken during the experiment when using multiple methodologies. (1) Electrode, (2) Optode, (3) Spectrophotometer for ETS analysis and protein measurement, (4) Winkler

Data Analysis

Microsoft excel was used to perform the calculations, conversion and for graphic design.

The R software was used for the statistical paired comparison between the results obtain by each methodology and to contrast between the respiratory behaviour of the heterotrophic and the autotrophic samples. The procedure will be described further in the results and discussion.

RESULTS

In total, 72 respiration measurements were made, 18 per each of the four methods, 9 with mysids and 9 with Ulva, doing 3 measurements with each method each day.

The output from the optode and electrodes were already in units of oxygen concentration and time, therefore the rate calculations were easily made. However, some results had to be filtered to select an adequate time range to correctly estimate the respiration.

Winkler results were in sodium thiosulphate volume units which had to be converted to oxygen concentration. These results consisted of triplicate of initial and end point concentrations.

ETS activity had to be converted from the formazan-formation rate to O_2 consumption. This resulted in potential respiration.

All results had to be normalized by biomass in milligrams of protein to be able to compare them. Ulva data could also be normalized by wet biomass. Additionally, the dry biomass could be determined from wet biomass and proteins with suitable conversions.

Here, in Fig. 16 are some examples of the evolution of dissolve oxygen concentration inside the incubation bottle measured by the three methods:

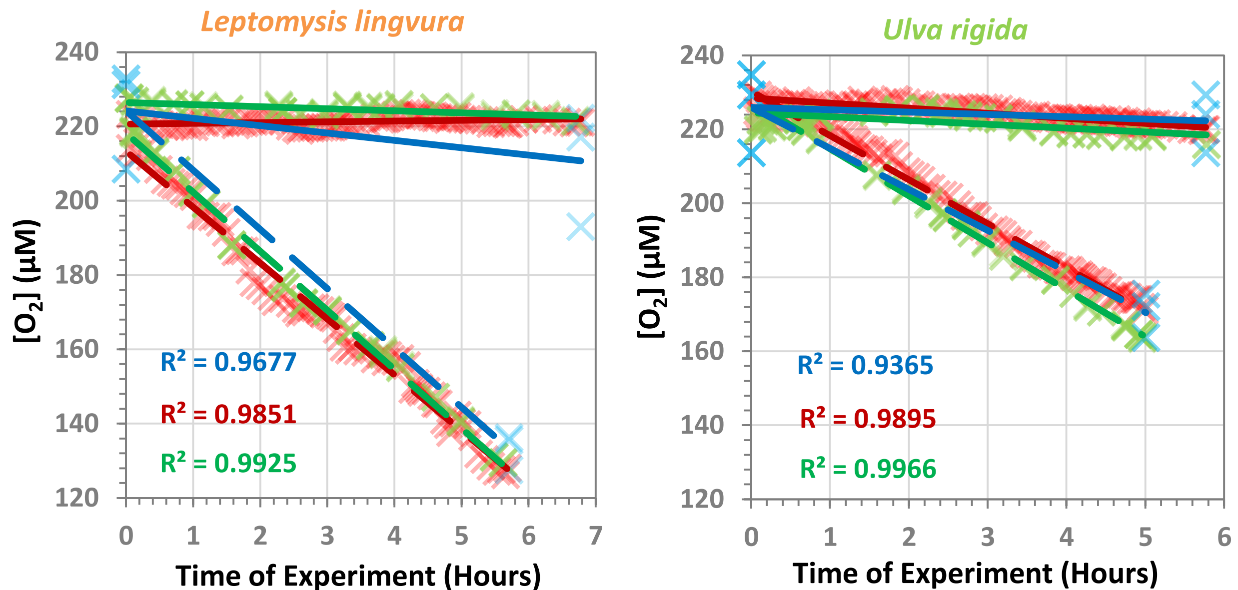


Fig. 16. Graphs showing an example of oxygen concentration inside two incubation bottles measure by the three oxygen related methods. The left graph is for a Leptomysis lingvura experiment and right graph for an Ulva rigida experiment. Colour code: blue (Winkler), red (Electrodes), green (Optodes). Crosses represent punctual measurements of [O2]. Full lines are trend lines for blank bottles. Dashed lines are trend lines for bottles with organism incubation. The coefficients of determination for the dashed line of each method are displayed. The equations of the dashed trend lines are: (Left) y = -15.944x + 224.14, y = -15.062x + 213.35, y = -15.895x + 218.15; (Right) y = -11.083x + 225.9, y = -11.931x + 230.25, y = -12.697x + 227.43.

The physiological oxygen consumption is calculated from the slope of oxygen concentration inside the incubation bottle subtracting the blank slope for the corresponding method as shown in Fig. 16. The R^2 for each respiration slope were always above 0.9.

Plotting line graphs where the x and y components are the results of the different physiological methods reveals the correlation between them (Fig. 17).

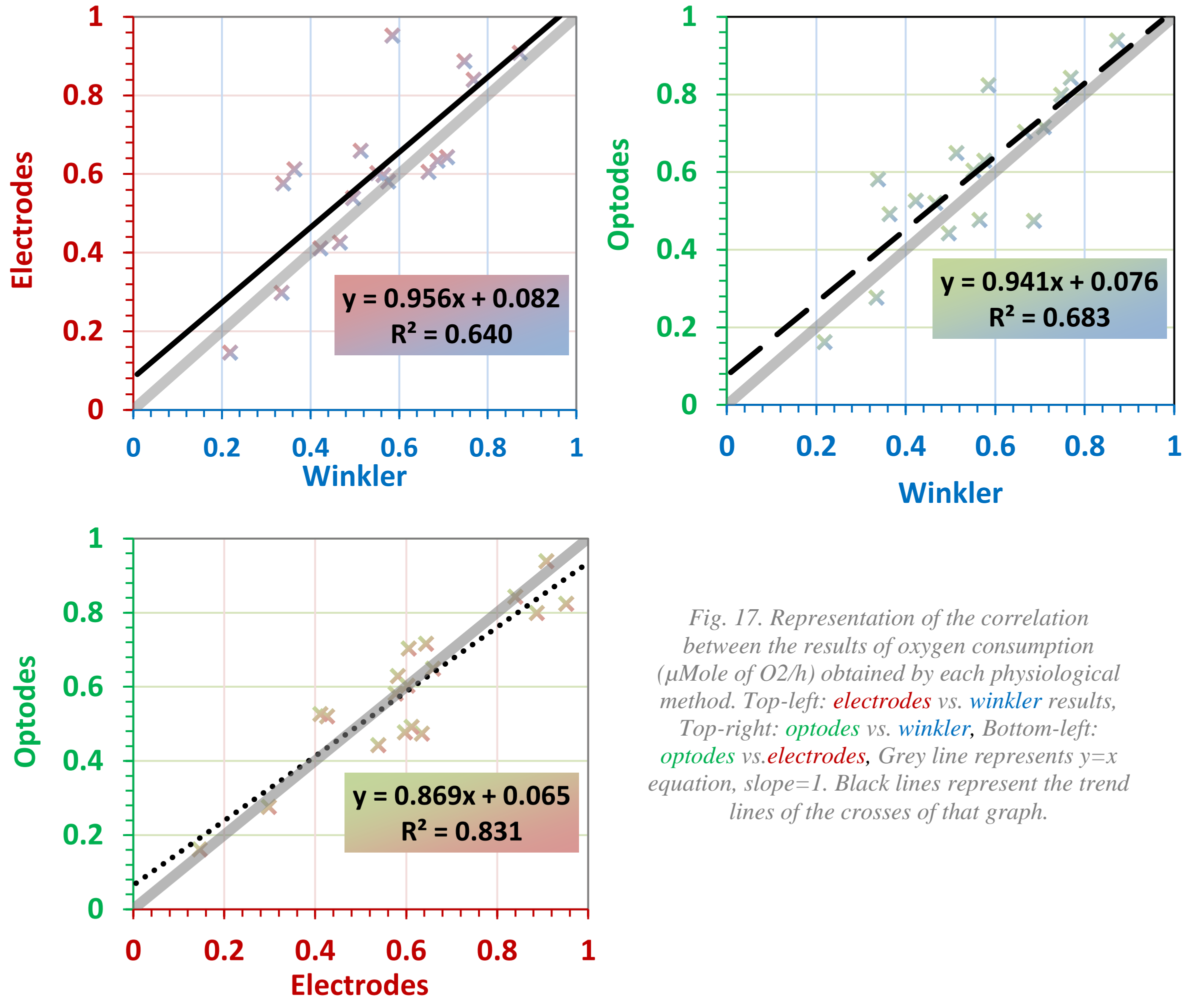


Fig. 17. Representation of the correlation between the results of oxygen consumption (μMole of O2/h) obtained by each physiological method. Top-left: electrodes vs. winkler results, Top-right: optodes vs. winkler, Bottom-left: optodes vs.electrodes, Grey line represents y=x equation, slope=1. Black lines represent the trend lines of the crosses of that graph.

A statistical analysis of the results of the physiological methods was performed (Table 2). Specifically, a T-student test for paired results, using a null hypothesis that the results were the same, served to compare the methods. This test is adequate for these results because the three methods measured oxygen depletion in the same bottle and therefore one method's result was not independent of other method's results in one bottle. The normality of the results of each method was confirmed using a Shapiro-Wilk test.

Another research objective was to compare the oxygen consumption of an autotroph (*Ulva rigida*) and a heterotroph (*Leptomysis lingvura*). The difference can be displayed in a box plot of the respiration by each method of the two organisms (Fig. 18). It can also be seen in a table displaying the averages and standard deviations (Table 1). This not only reveals the differences between organisms but also shows the similarity between the respiratory measurements from the three different methods. This graph is accompanied by a statistical analysis showing the significant difference between the Ulva's and the mysid's respiration.

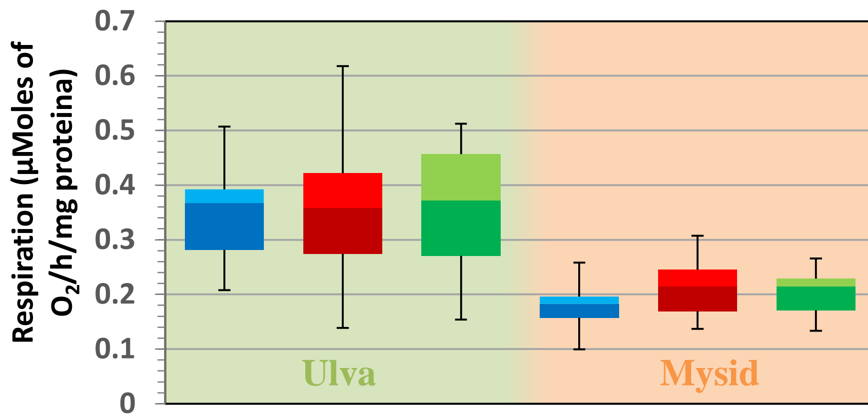


Fig. 18. Boxplot of protein specific physiological respiration of Ulva rigida (Left) and Leptomysis lingvura (right) determined by each method. Colour code: blue (Winkler), red (Electrodes), green (Optodes).

The physiological data was also compared to the enzymological data. It was interesting to see the physiological respiration results (R) juxtaposed with the results of the ETS kinetic analysis (Φ) (Fig. 19). Hence, representing a boxplot with the ETS results next to the R data and also the R/ETS was a good way to reflect on this relationship (Fig. 20). Table 1 displays the averages and standard deviations of all this different parameters.

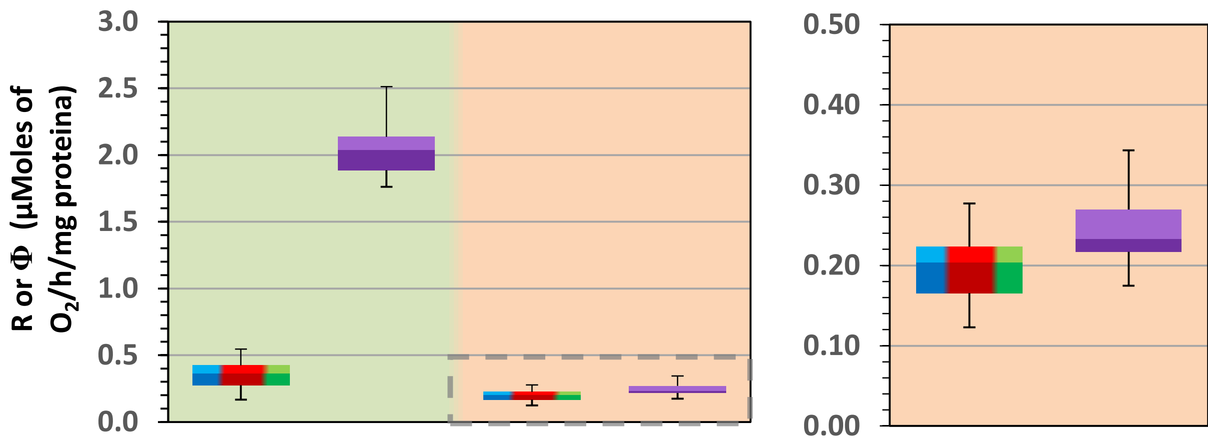


Fig. 19. Boxplot of the average protein specific physiological respiration (blue (Winkler), red (Electrodes), green (Optodes)) and potential respiration (Φ) from the ETS analysis (purple) of Ulva rigida and Leptomysis lingvura samples. Right: ampliation of the mysid side.

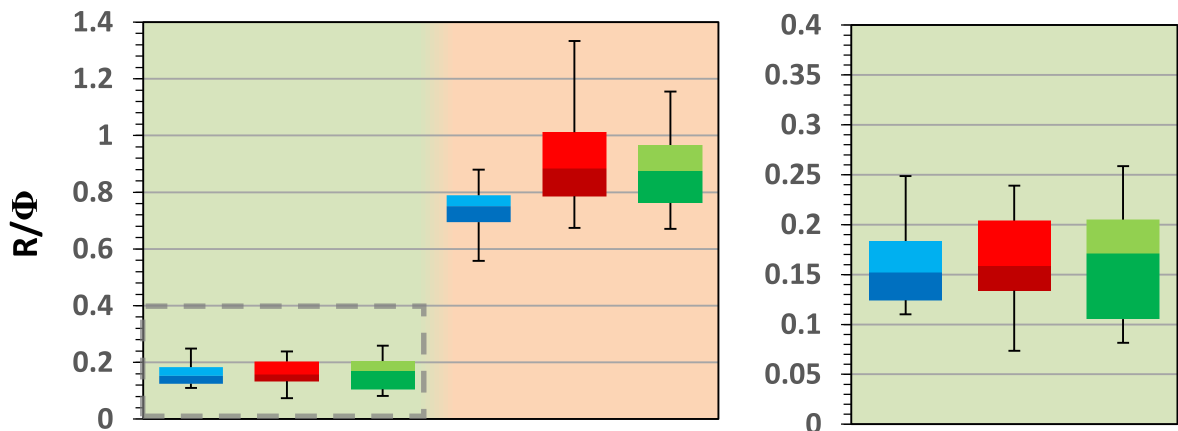


Fig. 20. Boxplot of physiological respiration (R) normalized by potential respiration (Φ) of Ulva rigida and Leptomysis lingvura. Right: ampliation of the Ulva side. Colour code: blue (Winkler), red (Electrodes), green (Optodes).

	Method	*Ulva rigida*	*Leptomysis lingvura*
Respiration (µMoles of O_2/h/mg protein)	Winkler	0.35 ± 0.10	0.18 ± 0.05
	Electrodes	0.35 ± 0.14	0.21 ± 0.06
	Optodes	0.36 ± 0.13	0.20 ± 0.04
	Mean	0.353 ± 0.123	0.197 ± 0.05
	Φ	2.14 ± 0.41	0.23 ± 0.07
R/Φ	Winkler	0.17 ± 0.05	0.73 ± 0.10
	Electrodes	0.16 ± 0.05	0.92 ± 0.21
	Optodes	0.17 ± 0.06	0.87 ± 0.17
	Mean	0.167 ± 0.053	0.84 ± 0.16

Table 1. Display of the respiration results obtained by each method normalized by mg of protein and potential respiration (Φ).

DISCUSSION

Comparing the Methods

One aim of this study was to compare the 3 physiological respiration methods. To do so, a statistical analysis of the results was required to verify if the oxygen consumption rate determined by each method was significantly different from the others. The software R facilitated this comparison using a null hypothesis and multiple student T test for paired data. This test was chosen because all three methods measured, simultaneously, the oxygen consumption in the same bottle. Consequently, instead of comparing average results, paired data were compared (*Mcdonald, 2009*). This way, we investigated how divergent each result was from its homologue measured at the same time with a different method. Before this, the results' normality was tested using a Shapiro-Wilk test (*Shapiro & Wilk, 1965*). Table 2 shows the p-values of each analysis.

			Winkler	Electrodes	Optodes
Normality	Shapiro-Wilk test p-value		0.9911	0.29	0.912
Comparison between methods	T-test paired data p-value	Winkler	-	-	-
		Electrodes	0.0656	-	-
		Optodes	0.1179	0.4878	-

Table 2. Statistical comparison of the oxygen consumption rates (µMole of O_2/h).

All results seem to follow a normal distribution as supported by $p > 0.05$, so the described multiple paired test could be performed. The results of the test showed no significant difference between any of the methods. However, when the Winkler method was involved p approached 0.05. This could be attributed to the entrance of air while transferring and filtering the water into the flask where oxygen was fixed. This mix with oxygen while transferring through the tube would not increase the initial oxygen concentration, which is maximum, but would increase the final oxygen concentration which would lead to a slight underestimation of the oxygen consumed and the oxygen consumption rate as we observe in Fig. 18 and Fig. 17. This deviation is greater in the experiments involving mysids because their movement, caused by their stress from the introduction of the transferring tube, promotes mixing with air.

When comparing the applicability of each method used in this work lots of different factors have to be taken into account. The oxygen related methods previously compared show to have comparable results. However, even if the measurements are similar the procedure's steps and simplicity to apply them vary. In the following paragraphs some of the methods characteristics will be compared (Table 3).

The Winkler method needed periodic standardizations and blanks to check the state of the reagents. Likewise, electrodes had frequent calibrations, precisely, before each use. Optodes instead were more stable after being calibrated.

Even though it could have been very accurate the respiration results obtained by the Winkler method are based on a start and end point concentration assuming a lineal trend. Instead optodes and electrodes provided of continuum evolution of oxygen concentration during incubation to calculate the oxygen consumption rate. Theoretically, between both of these, only the electrodes consume oxygen during the measurement altering slightly the results (*Liley & Lombard, 2015*). But in this case optodes and electrodes were measured in the same bottle and this consumption by the electrodes is difficult to address.

METHODS' CHARACTERISTICS COMPARISON				
DISADVANTAGES	**W**	**E**	**O**	**ETS**
Needs Bottle Incubations	✓	✓	✓	×
Needs living samples	✓	✓	✓	×
Requires adapting incubation bottles	×	×	✓	-
Requires killing the sample	×	×	×	✓
Chemical reagent preparation needed for reactions	✓	×	×	✓
Requires siphoning water	✓	×	×	-
The results obtain show potential respiration	×	×	×	✓
Disturbs incubation conditions	✓	✓	×	-
ADVANTAGES	**W**	**E**	**O**	**ETS**
Easy to use in unconventional incubation bottles	×	×	✓	-
Easily portable	×	×	✓	×
Allows autonomous measuring	×	✓	✓	×
Allows continuous measuring of oxygen variation	×	✓	✓	-
Free from electronic sources of error	✓	×	×	×
Easy and fewer calibrations needed	×	×	✓	✓
Higher precision in respiration measurements	×	×	✓	✓
Measurements are not affected by physiological state	×	×	×	✓
The results obtain are actual physiological respiration	✓	✓	✓	×
Simple to use/fewer steps	×	✓	✓	×
Weakly affected by changes in temperature and pressure	×	×	✓	✓
Higher data acquisition rate	×	×	×	✓
Most accurate oxygen measurement	✓	×	×	-
Can measure multiple samples at once	×	✓	×	✓

Table 3. Advantages and disadvantages of this work's methods. ETS does not require incubation nor oxygen consumption so some characteristics cannot be compared. This table refers to these methods with the cited instrumentation; new or different instruments or techniques may have improvements or defects not considered here.

On the other hand, optodes and electrodes are vulnerable to electronic problems or errors that would not affect the Winkler method that does not use a computerized intermediary.

From our point of view the two physical methods were easily employed while the chemical method had more steps and the requirement water transfer was an important source of error.

Other properties that differed between the techniques are their portability and their ability to measure in more than one bottle at once. The optode was the most portable but the electrodes were the only one measuring in continuum in 4 bottles simultaneously.

All these oxygen related methods required live organisms and time for the incubation. As mentioned before, the respiration during incubation may be affected by the physiological state of the organism that particular day. The alternative enzymatic methods may require several steps to prepare reagents, process the sample and prepare the kinetic analysis but even with this is the method with the higher data acquisition rate of the four. To use the method the organisms have to be stored adequately without needing them alive. However to apply this method the organism has to be killed. Also the resulting output is potential respiration which would need to be adequately modelled to physiological respiration as previously mentioned.

Therefore each method has its advantages and disadvantages (Table 3) but all seem to give valid measurements to work with and compare.

Comparing the Organisms

Respiration

Another aim of this study was to compare the respiratory behaviour of an autotrophic and a heterotrophic organism. At first sight, glancing over the figures and tables, it already seems that the organisms show differences and similarities worthy of discussion. Fig. 18 shows that respiration normalized by milligrams of protein looks slightly greater in the Ulva specimens than in the mysids. This seems somewhat peculiar because you would expect a sessile organism like the Ulva to require a smaller use of energy than the planktonic mysid which requires swimming activity to survive. The statistical analysis comparing the average oxygen consumption of the three methods showed that the respiration of *L. lingvura* was significantly less than that of *U. rigida* with $p = 0.00197$. However, it must be considered that the normalization using milligrams of protein may be causing this difference. The average protein concentrations in the Ulva samples was relatively low compared to their size. These being 1.41 ± 0.30 mg in the Ulva and 3.57 ± 1.32 mg in the mysids, and Ulva samples were larger than the mysid in size and mass. It is possible that if the respiratory data were normalized by another parameter, this difference would change as discussed below.

Potential Respiration and R/Φ Ratios

In contrast with protein-specific physiological respiration, physiological respiration normalized by Φ (Fig. 20) seems to show a completely different scenario. In this case R/Φ of the mysid is significantly higher than R/Φ of the Ulva. Similarly to the previous normalized R this is caused mainly because the normalizing parameter is very different for each organism. Φ is much greater in Ulva than it is in mysids. Moreover, the relationship between physiological and enzymological respiration is very different in each organism. While mysids have slightly greater, but similar Φ and physiological respiration, Ulva displays a much higher Φ compared to its own physiological respiration. This was not expected, taking into account the life style of each organism. One would think that Ulva only needs a little extra capacity to respond chemically to environmental stress when, for example, it must chemically fight a fungus or try to warn other Ulva, via pheromone production, of imminent predation. A mysid needs to escape predation by active swimming, it needs to respond to successful feeding by enhancing its digestive and anabolic metabolism. This requires heightened respiration and ATP production. This is why, finding that protein-specific Φ in Ulva is an order of magnitude higher than it is in mysids was completely unexpected.

Φ is normally expected to be greater than physiological respiration because external stress of all types forces respiratory enzymes to work harder, higher than when stimulated by normal metabolic demands of the organism's intermediary metabolism. In mysids, the R/Φ ratio ranges between 0.7 and 0.3 (Herrera et al, 2011[b], Osma et al, 2016). An R/Φ value close to 1 indicates a well fed organism or one with a high activity level. It has been described before by *Herrera et al. (2011[b])*. Φ can be used to calculate in vivo respiration using models based on the nucleotide concentration in the sample (*Packard & Gomez, 2008; Osma et al., 2016[a]; Osma et al., 2016[b]*). Macroalgae Φ has not been investigated as much. The low R/Φ will be described further when compared with the literature results in other autotrophic organisms.

Comparing with the Literature

Most of the results found in the literature for the respiration of these organisms have slightly different units therefore the units of this study had to be transformed to compare with the literature.

Leptomysis lingvura

The physiological respiration of the mysid *L. lingvura* in this study was normalized by mg of protein because dry weight was incompatible with the enzymatic analysis. In the literature many of the results for mysid respiration are normalized by dry mass. Hence, to compare, the protein specific respiration results were converted into dry mass specific respiration using the relationship established by *Herrera et al. (2011[a])* between dry mass and protein mg 74.19% ± 5.22%.

Also not every author worked at the same temperature so the respiration results that were at a different temperature were transformed using the Arrhenius equation (*Newell & Northcroft, 1967*; *Hirche, 1984*).

Ikeda, 2013 described a relationship between the respiration rate of mysid and their capture depth therefore this coastal specie was only compared with samples that lived at a similar depth.

With these criteria the results shown were compared with respiration measurements of *Leptomysis lingvura* and other members of the Mysidae Family found in the literature (*Szalontai & Muskó, 2003; Ikeda, 2013*) as it can be seen in Fig. 21.

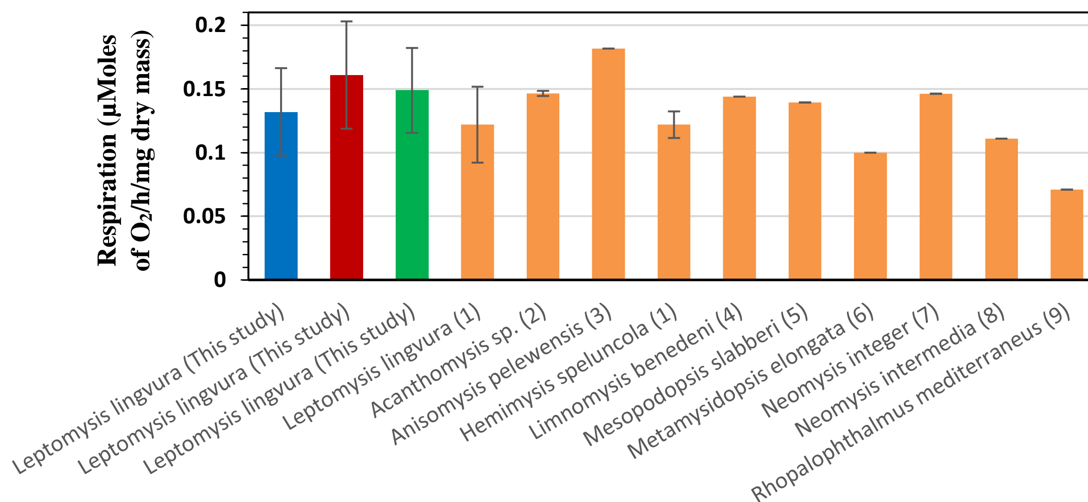


Fig. 21. Comparative barchart of this study's results of respiration with other coastal mysid species of the Mysidae family in the literature. Most were obtained from Ikeda, 2013. (1): Gaudy et al., 1980; (2): Ikeda, 1974; (3): Morioka et al., 1987; (4): Szalontai et al., 2003; (5): Vilas et al., 2006; (6): Clutter & Theilacker, 1971; (7): Weisse & Rudstam, 1989; (8): Ogonowski et al., 2012; (9): Omori, 1969. Orange bars: literature results, blue: winkler method, red: electrodes, green: optodes.

The measurements of this study seem to fit between the results in the literature. The respiration of the organisms of this study could seem slightly higher because they were cultured for a number of days with artificial feeding conditions. This, as mentioned, can not only increase physiological respiration compared to worse nourished specimens, but also causes the R to be closer to the Φ (*Hernandez-León & Gómez, 1996*).

Ulva rigida

The transformations for the *U. rigida* results were more intricate and the respiration results for this genus were less abundant. Results on respiration in the literature were normalized sometimes by area, chlorophyll content or by wet or dry mass; when the ones presented in this study were protein specific. Conversion numbers found in the literature were used to transform these to the same unit. The percentage of protein content per dry mass in *Ulva rigida* measured by Lowry's method was 6.64% as determined by *Satpati & Pal (2011).* The ratio of dry to wet mass, for Ulva, used to convert literature data was 0.12 ± 0.02 from *Beer & Israel (1986)*. Needless to say that the results were only compared with those of articles with the adequate data to convert their results into dry mass specific respiration (Fig. 22).

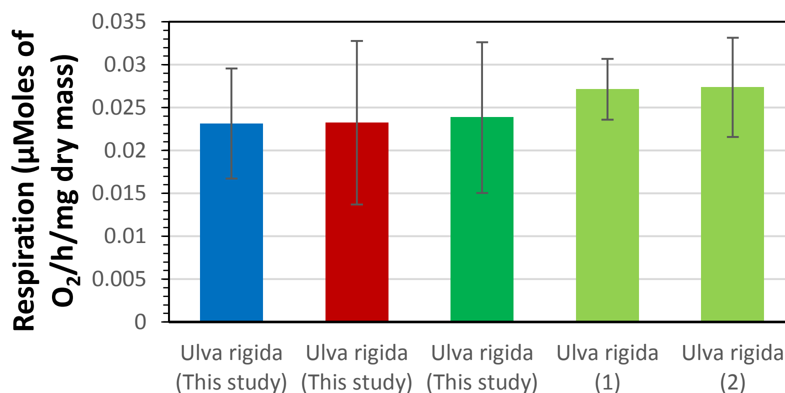


Fig. 22. Comparative barchart of this study's results of respiration with other studies with Ulva rigida: (1) Cabello-Pasini & Figueroa, 2005; (2): Cabello-Pasini et al, 2011. Light green bars: literature results, blue: winkler method, red: electrodes, green: optodes.

The results seem slightly lower than the ones found in the literature however they are not that much off considering the error bars. The reason for this difference comes from the number of transformation needed. In this study's case changing results from protein to dry weight specific and in the others transforming from area to dry weight specific. This operations create distance between the final results. Yet, this gives an estimation to compare once again the mysid respiration with the Ulva respiration.

Now that the respiratory data is normalized by dry biomass it shows that the dry mass specific respiration of *L. lingvura* (0.15±0.04 µMoles of O_2/h/mg DM) is an order of magnitude greater than the dry mass specific *U. rigida* respiration (0.02±0.01 µMoles of O_2/h/mg DM). This oxygen consumption better matches with the level of activity of these two organisms. The oxygen consumption per milligram of dry mass of the sessile Ulva is lower than this consumption for the free swimming mysid. The comparison between the organisms' Φ when transformed from protein specific to dry mass specific also swapped positions. After the transformations the Φ in µMoles of O_2/h/mg DM were more similar being 0.14±0.03 in the Ulva and 0.17±0.05 in the mysid. However, these operations did not change the large difference between the R/Φ of each organism.

There is a lack of measurements of Φ for Ulva in the literature. But Φ and R/Φ of marine plants and phytoplankton were available and these results were very revealing. Both potential and physiological respiration results, separately, were different to those presented here; but the resulting R/Φ were not. A comparison between these R/Φ ratios was useful to verify if this large difference between physiological and enzymatic respiration was consistent for other autotrophic organisms. Fig. 23 is represented to compare the results of this macroalgae's R/Φ with the ones present in the literature regarding other autotrophic aquatic organisms.

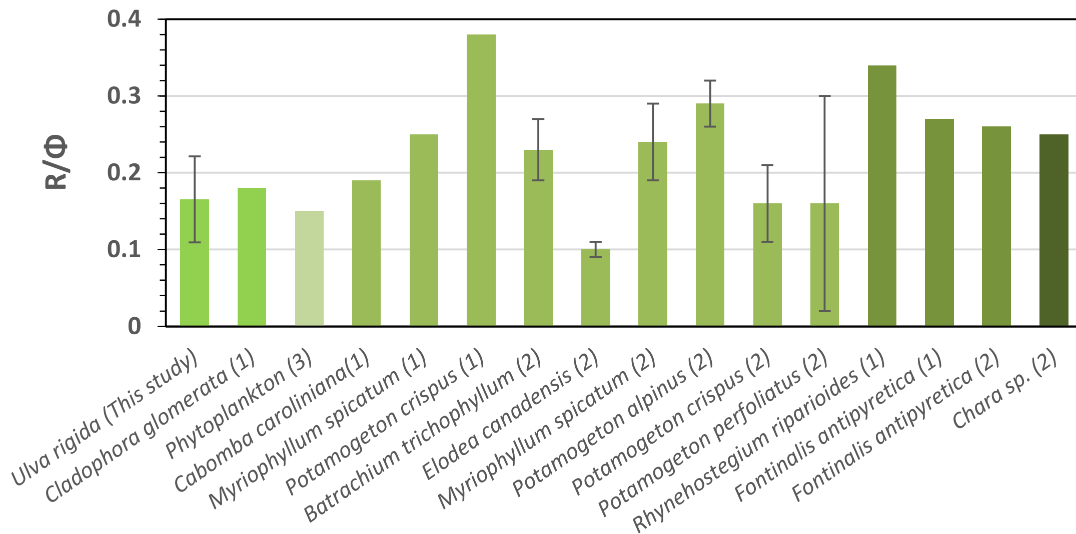


Fig. 23. Comparative barchart of this study's U. rigida with other autotrophic R/Φ ratio in the literature. (1): Peñuelas et al., 1988; (2): Mazej & Gaberscik, 1999; (3): Kenner & Ahmed, 1975[b]. Light green: Macrochlorophytes; very light green: Phytoplankton; green: Angiosperms; dark green: Bryophyte; very dark green: Charophyte.

It seems like autotrophic life has a biochemical respiratory system very different from the one describe for heterotrophic organisms. While zooplankton tend, in general, to have relatively high R:ETS ratios (*Hernandez-León & Gómez, 1996*), all the different autotrophic aquatic organism represented in Fig. 23 show an R/ETS between 0.1 and 0.4. Investigating on the enzymatic composition of autotrophic organisms it is clear that there is a high presence of enzymes completely absent in heterotrophs, those involved in photosynthesis found in the chloroplasts. The chloroplast's thylakoids have their own electron transport system with the enzymes needed for photosynthetisis (*Nelson & Cox, 2009; Falkowski & Raven, 2013*). *Raven & Beardall, (2005)* described 6 different oxygen consuming pathways in algaes, including mitochondrial respiration. Therefore there are several other processes which consume oxygen in the Ulva samples. Some of these require light such as the Mehler-peroxidase reaction and the rubisco oxygenase but others like chlororespiration can occur in darkness. This last process, chlororespiration, is of particular interest in explaining the high autotrophic ETS activity. The evidence for the phenomenon now termed chlororespiration were first described by *Kok (1949).* Goedheer *et al. (1963)* was the first to postulate the presence of a chloroplastic respiratory system in *Chlorella*, a chlorophyte like *U. rigida*. *Bennoun (1994)* proposed that the thylakoid's photosynthetic electron transport system was connected in its membrane to a respiratory chain. The existence of said process has been intensively questioned in the past (*Peltier & Cournac, 2002*). But sequencing the plastid's genome of higher plants have triggered the discovery of homologous genes to those encoding subunits of mitochondrial NADH dehydrogenase complex (*Guedeney et al., 1996; Burrows et al., 1998*) and, also, plastid terminal oxidase (*Wu et al., 1999; Cournac et al., 2000*); which have provided molecular support to the concept of chlororespiration. The purpose of this remains a mystery and there have been suggestions like for example (*Beardall et al., 2003*): supplying ATP for maintenance and synthethic processes in chloroplasts in the dark (*Raven & Beardall, 2002*), energizing the membrane to facilitate ATP synthesis upon illumination (*Peltier et al., 1987; Jakob et al., 1999*), reducing NADPH to attenuate damage generated by superoxides preventing photoinhibition (*Peltier & Schmidt, 1991*), participating in regulation with photosystem 1 and light (*Büchel & Wilhelm, 1990*), etc

In the case of chlorophytes, the division of *U. rigida*, chlororespiration would take place with NAD(P)H hydrogenase like enzymes, which transfer the electrons through the reduction of plastoquinone, which will then transfer these electrons to terminal oxidase that will reduce oxygen to water (*Casano et al., 2000*; *Peltier & Cournac, 2002*) in a very similar dynamic to that of the mitochondria (Fig. 6, Fig. 24).

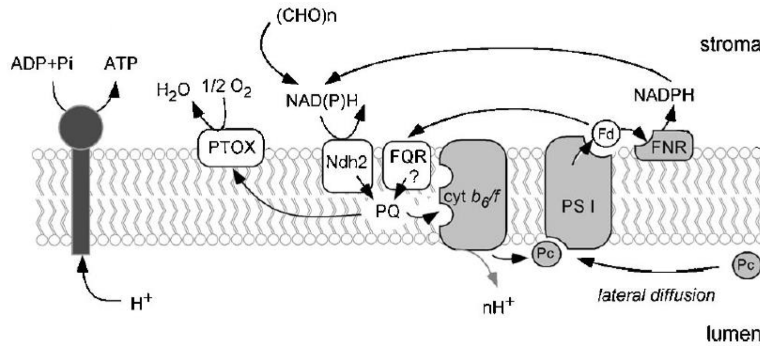


Fig. 24. Current model of Chlororespiration for most Green algae.

(Peltier & Cournac, 2002)

For this reason we suspect that the presence of these enzymes in the medium during the ETS analysis could maybe increase the formation of formazan and produce an overestimation of potential respiration. It is difficult to distinguish between these oxygen consumption reactions (*Büchel & Garab, 1996*) but the ETS analysis provides a new method to demonstrate this enzymatic activity and it could be used to study this enigmatic mechanism. This process may have been also taking place during the incubation measurements but it was weaker than when performing the ETS analysis similar to what happens with traditional respiration. This meaning that the result of the ETS analyses in autotrophic organisms would have been the sum of potential respiration, potential chlororespiration and other INT reducing phenomena.

Furthermore, chlororespiration may be intensified due to the presence of polysaccharide storage in the plastid that would be liberated during homogenation. *Raven et al. (2002)* suggest that this enrichment of polysaccharides may stimulate chlororespiration rates.

All the results displayed in Fig. 23 are primarily from either higher plants or chlorophytes which have been confirmed to show chlororespiratory mechanisms and all have this low R/ETS. Further ETS activity measurements with other autotrophic species such as rhodophytes or heterokontophytes could show something different and shine some light on the chlororespiration of these other divisions that are so poorly researched (*Büchel & Garab, 1996*). Nevertheless, the phytoplankton data of *Kenner & Ahmed (1975*[b]) had a diverse composition and still showed this low R/ETS. These results for phytoplankton could probably indicate chlororespiration or other electron transports taking place in phylums not considered before such as heterokontophytes (Bacillariophyceae (diatoms)), dinoflagelates or haptophytes.

Peñuelas et al., 1988 also suggested that contamination of mitochondrial respiration with chloroplast fragments could indeed cause the overestimation of ETS activity. The author related this increase to the effect of the photosynthetic ETS enzyme ferredoxin NADP reductase on the INT reduction.

Other factors that could be a source of error in the estimation of ETS activity could be the use of *Kenner & Ahmed (1975*[a]) method which was design for phytoplankton. Tests and calculations to upgrade the precision of the method for this macroalgae could improve the results. Still, *Kenner & Ahmed (1975*[b]) R/ETS for phytoplankton were not that different from this study's results (Fig. 23), which indicates that there could be another cause for this as discussed before.

Nevertheless it must also be contemplated the possibility that this R/ETS ratio is variable and certain conditions or stressors may trigger the physiological respiration of the autotrophs to be closer or further from Φ. Similar to what it has been described for zooplankton in relation to stress and feeding conditions (*Hernandez-León & Gómez, 1996*).

CONCLUSIONS

1. There was no significant difference between the oxygen consumption measured by the different oxygen based methods. The Winkler method showed a non-significant, but appreciable underestimation caused by the water transferring procedure. This suggests that improvements in the combined methodology are needed for a better comparison.

2. Both protein specific physiological and enzymological-based respiration (Φ) were greater in *U. rigida* than in *L. lingvura.* However, dry mass specific physiological and potential respiration was higher in *L. lingvura* showcasing the importance of the normalizing parameter when interpreting metabolic rates.

3. *U. rigida* and *L. lingvura* had completely different respiratory behaviours both at a physiological and enzymological level, even the R/Φ ratio was different in both organisms.

4. The physiological respiratory results obtained in this investigation do not differ considerably from those in the literature for both organisms, neither do the R/Φ ratios. That R/Φ ≈1.0 can be related to proper nourishment which could also be responsible for the slightly higher physiological respiration compared to the literature. The relatively low R/Φ of *U. rigida* has been observed in several other autotrophic organisms.

5. Different life styles could be the cause of the difference between the physiological respiration of sessile autotrophic *U. rigida* and free-swimming heterotrophic *L. lingvura*.

6. The different enzymatic composition could cause the difference between the Φ and R/Φ results. This is especially true considering the presence in autotrophic organisms of chloroplastic electron transport systems. Not only can certain photosynthetic enzymes consume oxygen, but genetical code for enzymes similar to those in the mitochondria's ETS have been identified in the chloroplast confirming their similar ETS structures. These are considered responsible for chlororespiration which could cause an undesirable reduction of INT.

7. Further investigation is needed to confirm if these different relationships between physiological and biochemical respiration are due to different enzymatic composition or due to a dependence on a yet undescribed condition that could alter physiological respiration in autotrophs as has been described for heterotrophs. Also more autotrophic species should be tested to verify if these observations apply to other algae groups.

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TFT REPORT

Detailed description of the activities performed for TFT

This TFT revolved around respiration of marine organisms therefore the activities to achieve this can be divided into those concerning the organisms and those concerning the methodologies.

The organisms used in the TFT have previously been described as well as the sampling techniques. I only assisted in the sampling of the mysids because I do not possess the diving formation needed to have a more active role during sampling. However, I went with my colleagues, who captured the mysids, to the sampling area. This way I could see the sampling procedure and also the climatology and the environment of the mysids I used.

On the other hand, one of my colleagues and I sampled manually the Ulva which was accessible without diving, just checking the tides.

After sampling the organisms their species had to be determined in the laboratory. The colleague with whom I sampled the Ulva identified the species using a microscope. We both learn this procedure and then I accompanied him during this process. One of my colleagues also started the mysid classification using a stereoscopic microscope to show me the procedure then he relegated this activity to me after some practice.

Previous to the sampling and classification everything had to be ready to culture these two organisms. Hence, with the help of lab companions I prepared the culture flasks for the algae and the tanks for the mysids. This also included setting up the culture chamber to have the desired light periodicity and preparing the nutrient solution for the algae, as well as, learning how to culture artemia to feed the mysids.

To maintain the culture conditions as explained above the mysids had to be fed twice a day and the tanks had to be regularly cleaned; activities which I performed alternating with one of my laboratory companions until I had practice. In the case of Ulva, another master student working with Ulva and me performed the water changes daily until he had to stop for personal reasons and I continued to use these samples. I tried to be as rigorous as I could keeping these conditions so that they were not a factor modifying respiration.

Apart from all the activities related to the organisms I had to familiarize myself with the respiration techniques. I documented myself to prepare the reagents for the Winkler method and also on the procedures to standardize and measure blanks with this technique. I performed some tests with different salinities and temperatures to see if there was a variation on the volume used to titrate. This way I rehearsed with this method before the real experiments which required speed and precision.

Likewise, I read the manuals and tested the other instrumentation. The optode spots were installed in the bottles later used in the experiments. I performed numerous measurement with the optodes to check there stability and sensibility measuring oxygen concentration in water with different salinities and temperatures. I also spent some time to understand the apparatus configuration and how to use it properly.

Lab personnel had worked previously using the electrodes so I had some help to learn how to use them. Nevertheless, these had to be cleaned and the electrolyte solution changed because they had been without use for some time. I also perform some tests to verify their stability and to check the precision of each electrode after being calibrated.

I also did combined tests to compare the results of oxygen concentration determined by the different methods. However the precision to give the same oxygen concentration could have been different to the precision to measure oxygen variation.

Some results not shown in this work came from testing the combined oxygen related methods to measure respiration. The first attempts with this combined technique had some errors that were corrected for the next measurements that gave the results present in this essay.

Apart from these oxygen related methods I had to learn and use the enzymatic methods and Lowry's method to measure proteins.

Companions from the laboratory assisted and supervised me during the realization of this analysis. This included: reagent preparation, sample homogenation and centrifugation, mixing the sample and reagents in the cuvette and using the spectrophotometer for the kinetic analysis.

The organisms used had to be correctly stored in the -80ºC freezer.

All this apparatus and laboratory material had to be carefully cleaned and maintained.

The combined routine to measure with all the methods was designed by myself once I understood the requirements and limitation of each technique.

All these were the more practical activities in which I actively participated during my TFT. Furthermore, other components needed to create this TFT were the processing of the data and an extensive reading of the literature. These were reflected in this work with the graphical displays, statistical analysis, data interpretation, comparisons with other author's results and with the multiple citations.

Additionally, I participated in several activities which allowed me to learn, structure my ideas and train abilities I would use in the TFT. These activities were:

- Participating in divulgation events like "La Noche de los Investigadores" during which I measured, live, oxygen concentration inside a bottle with an Ulva sample. At the same time I explain the relationship of respiration and photosynthesis with dissolved oxygen concentration, the organelles in the cell and the presence and absence of light in photosynthetic aquatic organisms.
- Prepared posters for two international congresses (CECOMA 2016, ICES-PICES 6th Zooplankton Production Symposium). In these I showed the comparison between these two organisms' respiratory behaviour or/and between the 4 methodologies used. These allowed me to write and structure ideas that are present on the TFT, have useful exchanges with other scientists working on metabolism and use the literature to support the concepts being displayed in the posters which are also present in this work.

Received training

The ecophysiology group EOMAR has worked in several occasions with the mysid species of the coasts of Gran Canaria. Therefore I receive training and literature on the technique and apparatus in the lab to sample and culture the mysids. This training included culturing *Artemia franciscana* to feed the mysids captured.

The other organism cultured was *Ulva rigida*. For this the training came from another Master student working with Ulva that had planned culturing the macroalgae in Florence flasks in a culture chamber.

I received training to identify mysid species using a stereoscopic microscope form colleagues who had worked previously with the mysids in Gran Canaria.

I was taught on the procedure to perform the electron transport system analysis which included several steps such as the ones mention in the previous section. It was different for mysids and Ulva. I was also taught how to measure protein with Lowry's method.

I was explained how to work with the electrodes and the optodes which I then prepared for the experiments.

All these activities included learning to work in this laboratory's environment with its distribution, rules and dynamics. I was explained where to find the material, reagent and tools and how they should be stored and maintained for its reuse or disposed of them if needed.

Integration and involvement within de department and relationship with the staff

All my time working with EOMAR's research group has been really interesting and fun. I have established good relationship with other students working on their projects even helping each other to perform tasks jointly. The more experienced members of the group have not only been patient with my greenness but have provided very useful insights and have been very supportive and friendly. My tutors managed to attend me consistently and have been key not only to guide me through this project but also to orient me for my future.

I feel I have been sufficiently autonomous on my work during which I got actively involved in EOMAR's participation in "La Noche de los Investigadores", where we exhibit the purpose and line of research of the group and the complexity and importance of metabolical processes in the ocean. I also prepared posters for two international congresses which I attended. Other members of the group also attended to these events which helped strengthen the bonds between us.

My TFT was supported by the project BIOMBA. I felt really integrated when my research appeared in a common project with the other member of the group. In fact my efforts were considered by another companion in the lab worthy of being co-author in the poster she submitted to a congress.

Most significant negative and positive aspects of the TFT development

The most significant negative aspect is that I personally failed to organise myself to really exploit to its maximum all the learning possibilities I had during my time with this team. I also failed to deliver the TFT on time due to personal issues but also due to some preventable problems related to the availability of the amounts of Ulva and mysids for the experiment. I had some troubles working in a lab where the mysids and Ulva were being cultured for which I had to constantly ask permission to enter. This is not EOMAR's fault is just a consequence of location of the lab and the policy of the department regarding said lab, but it really was troublesome in several occasions. Also accessing the faculty was quite intricate due to some technical problems with the pedestrian exterior door. This

made working during weekends or holidays more complicated and this was necessary to maintain the conditions of the living organisms.

I also think my performance culturing the Ulva could have been improved with some assistance of someone with previous experience in the matter. Because even though the literature to learn was perfectly accessible some mistakes could have been prevented.

The significant positive aspects were that I was always supported by all the colleagues that worked in the EOMAR group. Any question, doubt, insecurity and proposal was listened with attention and concern. I feel that in the lab there was an equilibrium between autonomy and supervision. I was provided of plenty of space and material to feel comfortable in the laboratory. I will gladly keep working in that laboratory.

Personal assessment of the learning achievement throughout the TFT fulfillment

Not only have I felt really integrated, able to observe and learn about the different fronts of investigation of this group, but now I have a new view on the enzymatic and biochemical research. I have realized all its potential and applicability and I am now confident enough to tackle, with adequate research, any challenges that require an enzymatic or physiological parameter determination or approach. My interest for respiration has grown and I feel I have expanded upon the theory taught and explored in several subjects during the master.

Apart from this, the attendance to the two congress has been pretty fulfilling and determinant to understand the dynamic of the oceanographical research community.