Anexo I

D. José Manuel Vergara Martín, SECRETARIO DEL DEPARTAMENTO DE BIOLOGÍA DE LA UNIVERSIDAD DE LAS PALMAS DE GRAN CANARIA,

CERTIFICA,

Que el Consejo de Doctores del Departamento en sesión permanente tomó el acuerdo de dar el consentimiento para su tramitación, a la tesis doctoral titulada "**Respiratory Electron Transport Activity In Plankton: Determinants and Detection**" presentada por el doctorando D. Federico Maldonado Uribe y dirigida por los Doctores María M. Gómez Cabrera y Theodore T. Packard.

Y para que así conste, y a efectos de lo previsto en el Artº 6 del Reglamento para la elaboración, defensa, tribunal y evaluación de tesis doctorales de la Universidad de Las Palmas de Gran Canaria, firmo la presente en Las Palmas de Gran Canaria, a 9 de Noviembre de 2015.

Tesis Doctoral
Programa de Doctorado en Oceanografía
Departamento de Biología

Respiratory Electron Transport Activity In Plankton: Determinants and Detection

(Actividad del Sistema de Transporte de Electrones Respiratorio en el Plancton: Determinantes y Detección)

Memoria presentada por D. Federico Maldonado Uribe para la obtención del Doctorado en Oceanografía en la Universidad de Las Palmas de Gran Canaria y dirigida por los Doctores D.ª María M. Gómez Cabrera y D. D. Theodore T. Packard.

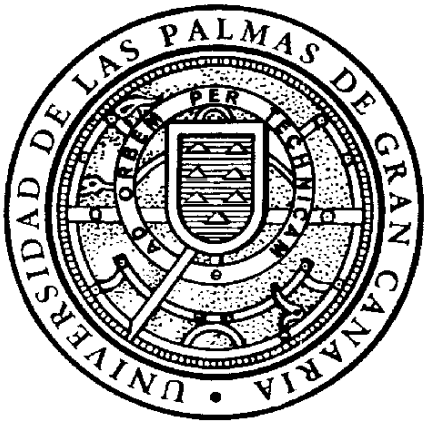
Universidad de Las Palmas de Gran Canaria
En Las Palmas a 09 de noviembre de 2015

El Doctorando
Fdo: D. Federico Maldonado Uribe

La Directora
Fdo: Dra. María M. Gómez Cabrera

El Codirector
Fdo:Dr.Theodore T. Packard

Respiratory Electron Transport Activity In Plankton: Determinants and Detection



TESIS DOCTORAL

Federico Maldonado Uribe

Doctorado en Oceanografía

Departamento de Biología

Universidad de las Palmas de Gran Canaria

Noviembre 2015

A los de que aquí y a los de allá

What is a scientist after all? It is a curious man looking through a keyhole, the keyhole of nature, trying to know what's going on.

- Jacques—Yves Cousteau

We live in a society exquisitely dependent on science and technology, in which hardly anyone knows anything about science and technology. This is a clear prescription for disaster.

-Carl Sagan

Agradecimientos

Sin lugar a dudas a mis padres. Ellos lo han dado todo lo que tenían y lo que no para que nunca me faltara nada, siempre estaré agradecido y se le quiere infinitamente.

A mi hermana por darme esa alegría que tanto necesitaba y que durará para siempre, mi sobrina Olivia.

A mis directores, gracias por la oportunidad. A May por siempre estar pendiente de nosotros, de codearse con cualquiera para que en lo posible no nos faltara nada. A Ted, esa persona que todos quisiéramos ser. Y jamás creí que se pudiera aprender tanto de una sola persona.

A todos los profesores, por la paciencia y enseñanzas.

A todos los tuve la oportunidad de conocer y nunca olvidare: Mioko, Zebenzui, Gabriela, Raül, Désirée, Cynthia, Sandra, Mireya, Sheila, Sara, Avelina, Ricardo, Jaramillo, Sarah, Laia, Inma, Borja, Anna, Lola, Rayco, Aridane, Tania, Guille & Migue, Fabio, Fabiola Bassi, Las Croquetas Mecánicas, Jose y la peña madridista de Tomas Morales con las que pude compartir la Decima.

A mis compañeros de viaje de ese barco llamado B201: Ico, Mar, Isa, Valeria, Iván, Vero, Fede, Marta, Mine, Mayiya, Claire, Juanca, Alejandro, y Acorayda, que me tuvieron que soportar y que con mucho esfuerzo y paciencia se aguantaron para no tener que reírse con mis comentarios y chistes buenísimos

A mi nueva familia: Alicia, Gara, Maite, Manuela, Migue, Pau, Cachai, Almudena, Suso, Pedro, Yeray, Carlos, Laura, sin duda sin uds no hubieran existido tantas chucurumbambiadas exitosas.

A Igor y Natalia, por aguantarme tanto, por ayudarme más de lo que podían y debían. Por demostrar que en raras ocasiones hay amistades más fuertes que algunos vínculos familiares

A la familia que ya tenia en España: Cami, Isabella, Alessandro, Eugenio y Mafe, que siempre tuvieron sus casas y la nevera con las puertas abiertas.

A los que a pesar de la distancia estuvieron pendientes: el grupo de Las Deportivas, Karla, Jennifer, Jaime, Sandra Velásquez, Claudia Lorena, Isa Calderón, Cristina Muños Grau (gracias por que aprendí que la ilusión es un gran motor), Lucas (por su incondicional apoyo y consejos)

Siempre estaré agradecido porque de cada uno de Uds. aprendí algo y nunca se les olvidará.

.

Resumen

La presente tesis, se centra en el estudio del Sistema de Transporte de Electrones (ETS) en organismos del plancton marino, los factores que lo influencian la interpretación de estas mediciones y su detección mediante espectrofotometría y espectrofluorometría, en muestras oceánicas naturales y en cultivos de organismos marinos. Se pudo establecer, la biomasa, la respiración (R) y la respiración potencial (ϕ), en tres transectos en los océanos Índico y Atlántico Norte Sur. A su vez, se determino el estado fisiológico, en tres tamaños del zooplancton, midiendo la relación R/ϕ.

Se exploró los efectos de la inanición sobre la R y la variación con respecto a la ϕ en el zooplancton, Los resultados mostraron que, después de 24 horas no se detectaron cambios en R o ϕ.

Con respecto a la medición de la actividad ETS, este trabajo trata de demostrar que los procesos enzimáticos deben cumplir unas condiciones esenciales, y el no cumplimiento de estos preceptos llevará inexorablemente a mediciones falsas y engañosas.

Después de hacer mediciones de ETS con el método espectrofotométrico hemos desarrollado un método espectrofluorométrico usando por primera vez en el campo de la

oceanografía y desde la perspectiva de la bioquímica que controla la respiración, el resazurin, un receptor artificial de electrones que al reducirse presenta como principal característica la fluorescencia. En teoría, debería ofrecer una mayor sensibilidad y especificidad para análisis del ETS en aguas oligotróficas y en aguas profundas, bajas en biomasa. Entre otras bondades del nuevo método, se encuentra la reducción de costes en reactivos y de ser más respetuoso con el medio ambiente ya que no es tóxico.

La aplicación de este avance en la mejora del método del ETS, se pudo probar en diferentes muestras de zooplancton, así como en diversos organismos marinos de distintos phyla y diferentes muestras de agua de mar, que da resultados fiables a pesar de que las biomasas en muchos casos eran muy bajas.

Abstract

This thesis is about measuring respiratory electron transport system (ETS) activity, using spectrophotometry and spectrofluorometry, in the open ocean and in laboratory cultures of marine plankton organisms, and the interpretation of these measurements and experiments. Biomass (B), respiration (R) and potential respiration ($\boldsymbol{\phi}$) were measured on three transects in the southern Indian and North Atlantic oceans. In turn, the physiological state, in three sizes of the zooplankton, was determined by measuring the ratio R/$\boldsymbol{\phi}$.

The effects of starvation, on R and its variation with respect to ϕ, in zooplankton from the above transects, were investigated. The results showed that, after 24 hours no changes were detected in either the R or ϕ.

With regards to measuring ETS activity, this work tries to demonstrate that the measurement of enzymatic processes requires the fulfillment of certain essential conditions. Non-compliance inevitably leads to false and misleading results.

After making ETS measurements from the field with the spectrophotometric method we developed a spectrofluorometric ETS method using the fluorometric phenoxazine electron acceptor,

resazurin. Upon reduction this compound is fluorescent in solution and thus can be used, literally, to count the electrons flow down the respiratory ETS. Currently it is not being used in oceanography as a respiration proxy and yet, theoretically, it should offer greater sensitivity and specificity to ETS analysis oligotrophic surface waters and sparsely-populated deep-ocean waters. Among other benefits of this new method is, through its sensitivity, its promise to reduce reagent costs and to be environmentally friendly since it is not toxic.

Applying this methodological breakthrough, we assayed ETS activity in different zooplankton samples, in various marine organisms from different phyla and different seawater samples, giving reliable results even though the biomass in many cases was low.

Index

	Agradecimientos	VIII
	Resumen	XI
	Abstract	XIII
1.	**Introducción general**	**1**
1.1.	Antecedentes	1
1.2	Objetivos	18
1.2.1.	Capítulo 2	18
1.2.2.	Capítulo 3	19
1.2.3.	Capítulo 4	20
1.2.4.	Capítulo 5	21
1.3.	Principales resultados obtenidos	22
1.3.1.	Capítulo 2: Actividad Metabólica del Zooplancton (respiración y respiración potencial) en los Océanos Índico Sur y Atlántico	22
1.3.2.	Capítulo 3: Reducción del INT y la importancia de los sustratos en la medición del sistema de transporte de electrones	27
1.3.3.	Capítulo 4: Optimización de un método fluorescente para la determinación del ETS	29
1.3.4.	Capítulo 5: Actividad ETS en diferentes organismos, así como en muestras de plancton: comparación de los métodos e influencia de los sustratos	34

2. Zooplankton metabolic activity (respiration and ETS) from the Southern Indian and Atlantic Ocean **37**

2.1. Introduction 38

2.2. Methods 40

2.2.1 Statistical analysis 43

2.3. Results 43

2.3.1. Specific activities 44

2.3.2. R/Φ 45

2.3.3. Kleiber's law 50

2.3.4. Food limitation (starvation experiments) 53

2.4. Discussion 54

2.4.1 Zooplankton physiology state 54

2.4.2. Food limitation (starvation) 59

2.5. Summary 60

3. Understanding tetrazolium reduction and the importance of substrates in measuring respiratory electron transport activity **63**

3.1 Introduction 65

3.2 Materials and methods 76

3.2.1 The assays 76

3.2.2 INT reduction in cell-free homogenates 79

3.3 Results 82

3.3.1 Comparison of methods 82

3.3.2 Non enzymatic formazan production 83

3.4. Discussion 83

3.5.	Summary	90
4.	**A fluorescent method for determination of the respiratory electron transport system activity**	93
4.1.	Introduction	94
4.2.	Materials, Methods, and procedures	101
4.2.1.	Instruments	102
4.2.2.	Reagents	103
4.2.3.	Fluorescence characteristics of resorufin	110
4.2.4.	Influence of oxygen on the reaction	111
4.2.5.	Effect of ionic strength	111
4.2.6.	Optimum substrates, diaphorase, and resazurin concentrations	112
4.2.7.	Resorufin calibration curve	112
4.2.8.	Resorufin reproducibility	113
4.2.9.	Limits of Detection and Quantification	114
4.2.10.	Diaphorase kinetics	114
4.2.11.	Biological samples (Methods comparison)	115
4.2.12.	Statistical analysis	117
4.3.	Results	118
4.3.1.	Fluorescence characteristics of resorufin	118
4.3.2.	Influence of oxygen and the effect of ionic strength	119
4.3.3.	Substrates conditions	121
4.3.4.	Diaphorase concentration	124
4.3.5.	Optimum resazurin – resorufin concentrations	125

4.3.6.	Repeatability and, Limit of detection (LOD) and Limit of quantification (LOQ)	127
4.3.7.	Diaphorase kinetics	128
4.3.8.	Biological Samples	130
4.4.	Discussion	136
4.4.1	Components assembly	136
4.4.2	Biological samples	138
4.5.	Summary	142
5.	**Respiratory Electron Transport Activities in oceanic samples and in different marine organisms: Comparison of methods and the influence of different substrates**	**143**
5.1.	Introduction	144
5.2.	Materials and methods	147
5.2.1.	Samples acquisition	147
5.2.1.1	Marine organsms	147
5.2.1.2	Seawater samples	147
5.2.2.	Electron Transport System analysis	148
5.2.3	Statistical analysis	151
5.3.	Results	15
5.4.	Discussion	157
5.5.	Summary	172
6.	**Discusión general**	175
7.	**Futuras líneas de investigación**	**189**
	Conclusiones generales	**193**
	References	**197**

Figure index

1.1. Diagrama esquemático del sistema de trasporte de electrones mitocondrial (tomado de Abou-Sleiman et al., 2006). 4

1.2. Desarrollo de la reacción del INT, que puede parecer incoloro, en su forma reducida, mientras que el formazan, es de color rojo o violeta, dependiendo de la concentración. 16

1.3. Desarrollo de la reacción del resazurin, de color azul-violáceo, en su forma reducida y el resazurin, de tonalidades rosáceas, y con caractristicas fluorescente. 17

2.1. Transects from which the zooplankton samples were acquired. Vertical hauls using a WP2 net down to 200m were made. Indian Ocean samples were taken between February 12 to March 13, and 17 to 30 March 2011. Atlantic samples were taken from June 20 to July 14, 2011. 41

2.2. Relation between R and ϕ, by ocean region, where the highest rates were found on the Cape Town-Perth transect ($R=0.84\phi - 0.07$, $r^2 =0.67$). The next highest rates were found on the Perth-Sidney transect ($R=0.37\phi + 0.5$, $r^2 =0.12$), similar values are present in the Atlantic region ($R = 0.37\phi + 0.4$, $r^2 =0.43$). Ranked according to size class, the higher rates were found in >1000 µm size-class ($R=0.86\phi - 0.04$, $r^2 =0.83$), followed by those in the 100-500 µm size-class ($R=0.41\phi + 0.5$, $r^2 =0.27$), and finally by those in the 500-1000 µm size-class ($R=0.30\phi + 0.5$, $r^2 =0.21$). It is important to note that low R/ϕ in the Atlantic region was influenced by the large number of measurements in the small size-class. No significant difference ($p >0.05$) was found between the three ocean regions nor between the three size classes. 49

2.3. Relationships, between either log Φ or log R against log B, of the different sections were determined to establish the scaling ratios (slope) or *b* (the correlation inside the parenthesis). The higher ratio for the LogR/Log B was 1.33 (r^2 =0.71) in the Atlantic section, followed by 0.98 (r^2 =0.83) during Perth-Sidney and 0.71 (r^2 =0.55) in the Cape Town–Perth transect. For the Logϕ/Log B is, 1.33 (r^2 =0.71) for Cartagena-Cartagena, followed by 1.07 (r^2 =0.87) inside the Great Australian Bight and finally 0.85 (r^2 =0.71) in the Indian Ocean. In general, R is more dispersed due to the rapid changes that can occur in these measurements due to manipulation or other environmental factors. However, for the different slopes, no statistical differences (p >0.05), neither with Log ϕ and Log R, could be found. 51

2.4. Relationships between either log Φ or log R versus log B, in the different size classes were made to establish the scaling ratios (slopes) or *b* (the correlation inside the parenthesis). The higher ratio for the LogR/Log B was 1.12 (r^2 =0.48) for >1000 µm, followed by 0.92 (r^2 =0.54) for 500-1000 µm and 0.78 (r^2 =0.75) in 100-500 µm size. For the Logϕ/Log B it was 1.12 (r^2 =0.42) for the >1000 µm size, followed by 1.09 (r^2 =0.87) in the 100-500 µm size and finally 0.80 (r^2 =0.46) in 500-1000 µm size class. The dispersion in both measurements was similar due to the variability of the zones. However, for the different plots no statistical differences (p >0.05) were found 52

2.5. Relationships between either log Φ or log R versus log B in recently caught organisms (0h) and in starved organisms (24h) were calculated in order to establish the scaling ratios (slope) or *b* (the correlation inside the parenthesis). The ratio for the Log R/Log B at 0h was 0.89 (r^2 =0.62) whereas the ratio for 24h was 1.40 (r^2 =0.69). These ratios were statistical different (p <0.05). The ratio, Log ϕ/Log B was 0.95 (r^2 =0.68) and 1.13, (r^2 =0.72) respectively, for the two different nutrient conditions, no statistical differences (p >0.05) between these ratios was found. 55

2.6. Relationship between R and Φ from the pooled data for the two treatments. The 0h give the lower ratio (R= 0.48 Φ +0.4; r^2 =0.40) compared to the 24h ratio (R= 0.64 Φ +0.2; r^2 =0.67). Even so, there was no significant differences (p >0,05) between the two treatments 57

3.1. Reaction of tetrazolium reduction and formazan production in INT. Modified from Rogers (1953) and Packard (1971). 71

3.2 Respiration–ETS activity relationship from the data of Arístegui and Montero (1995). Each data point represents the average data for each of the 7 different ocean expeditions. The regression equation is: R=0.5949(ETS) + 16.223 (r^2 =0.8967). The R/ETS ratio is 0.6, close to a prediction from enzyme kinetics of 0.5 if ETS is potential respiration (V_{max}). 74

3.3 The glutathione reaction with INT, as a function of time, for eight different glutathione concentrations. Only the first 5 min of the formazan production is shown. The velocity of the reaction is proportional to concentration. Note that no enzyme is involved in this reaction. 81

3.4. Potential respiration comparison between the Owens and King (●) assay, the Owens and King blank (○) (n =39), and the Båmstedt assay (▲) (n =53). The Båmstedt assay was done at 40 °C and the Owens and King assay and blank were corrected to *in situ* temperature using the Arrhenius equation. There is a clear difference in the biomass relationship between the two assays. The regression equation for the biomass dependence assay is: $y=9\times10^{-4}x - 0.06$ (n=49, r^2=0.62 (p>0.001)). The equivalent equation for the Båmstedt essay is $y=5\times10^{-6}$ x −0.003 (n=55, r^2=0.022 (p >0.001)). (Inset) Note the coherence between the Owens and King blank ($y =9\times10^{-6}x$ - 0.006) (n =49, r^2 =0.03 (p >0.001)) and the Båmstedt assay (equation above). Note also, that the Båmstedt essay and the Owens and King blank are only weakly sensitive to biomass variations. 84

3.5 Formazan production as a function of concentration for ascorbic acid (AA), cysteine, glutathione, glucose and phenol. Ascorbic acid (●) was the most powerful reducing agent, two orders of magnitude higher (Y axis right) than cysteine (□) and the glutathione (▽) (Y axis left). Both of which had a similar capability to reduce INT to its formazan form. Phenol (○) and the glucose (◊) did not show any reaction with INT. Note that for ascorbic acid the reaction becomes saturated above 1 mM. 85

4.1. One electron enzymatic reduction of the non-fluorescent blue-colored resazurin as catalysed by diaphorase. The pyridine nucleotides, NADH and NADPH were tested as substrates in generating the fluorescent pink-colored resorufin. (Modified from O'Brien et al., 2000; Catomeris and Thibert, 1988). 101

4.2. Comparison of the emission and excitation spectra of resorufin (gray lines) with, a. resazurin (blue and light green line) and, b. Tris buffer (light blue and red line). Note that in both cases the peaks of emission and excitation do not coincide with the resorufin peaks and that the resazurin and Tris Buffer fluorescence are scaled on the secondary ordinate axis (right) where the magnitude is smaller than it is on the primary ordinate (left). 119

4.3. Effect of cyanide and indirectly, oxygen, on the enzymatic reduction of resazurin to resorufin. The addition of NaCN (blocking cytochrome oxidase) generated a decrease in the velocity of the reaction, arguing that O_2 does not compete with resazurin for electrons from the ETS. So cyanide was omitted from the reaction. 120

4.4. Fluorescence intensity in counts per second (CPS) over time (slope), for different succinate concentrations. Note the generally low fluorescent signal. The net slope (reaction-mixture slope minus the buffer-blank slope) showed some negative values, this means that there was no reaction. The spectrofluorometer was measuring background noise. 121

4.5. Influence of succinate on the velocity of the enzymatic reaction. There was no evidence of the contribution of succinate to the overall reaction. 122

4.6. Fluorescence intensity production with time (slope), at different NADH and NADPH concentrations. With diaphorase catalyzing the oxidation of the substrates, NADH yielded higher activities than did NADPH. In the range tested there was no inhibition of the reaction by either pyridine nucleotide at high concentrations. 133

4.7. Fluorescence intensity production with time, at different diaphorase concentrations. Linearity without limitation or interference was observed (y= $2.37e^7$ + 4.08 e^3; r^2 =0.993) between zero and 0.016 U (mg prot.)$^{-1}$ ml^{-1}. 125

4.8. Rate of reduction of resazurin to resorufin. In the range tested, in the box is shown the self-quenching by the inner cell effect (y= $2.01e^4$x + 1.02e4; r^2 = 0.978) starts to show at some point after 5µM. Until 2 µM there is a good linearity ($2.88e^4$ + 16.3; r^2 = 0.995). 126

4.9. Example of a calibration plot of pure resorufin. Linearity was maintained to 5 µM (y= $7.18e^5$x + $2.32e^4$; r^2 = 0.999). Even though, in box, the fluorescence starts to be quenched at 10 µM (y= $5.50e^5$x + $1.30e^5$; r^2 = 0.974), this concentration was selected, for subsequent tests. No opportunity to produce this amount of resorufin, so no inner cell effect could be present. 127

4.10. Attempt to establish the K_m for diaphorase. The NADH concentrations are maintained while the NADPH changes. The graphics on the left show the entire range of NADH concentrations, while the right one only shows the four lower concentrations of this substrate. It shows that the rate of production of resorufin is proportional to the concentration of NADH. This is clearly evident because as the NADPH concentrations increase, the speed of resorufin production decreases. Notice that the scale on the *y* axis is the same for all plots. 131

4.11. Attempt to establish the K_m for diaphorase. The NADPH concentrations are maintained while the NADH changes. The graphics on the left shows the entire range of NADPH concentrations, while the right one only shows the four lower concentrations of this substrate. In the absence of NADPH the resorufin production is proportional to the NADH concentration. But once the NADPH is added, production rates stagnate independently of the concentrations of NADH. Notice that the scale in the *y* axis is the same for all the plots. 132

4.12. Φ comparison using INT (black circles) or resazurin (open circles) as electron acceptors in ETS assays. In **a.**, Φ as a function of different diaphorase concentrations. In both methods linearity with the increasing concentration of diaphorase was observed; r^2 =0.94 and 0.98 for the low concentrations and r^2 =0.997 and 0.995 for the whole range respectively. The resazurin method yields higher Φ and a higher sensibility at all concentrations, in both ranges the ratio between slopes was 1.68. In **b**, as in a, but with zooplankton homogenate dilutions providing the range of enzymatic catalysis instead of diaphorase. Here both methods shared the same correlation with biomass (r^2 =0.987), with slopes of 0.060 for INT and 0.068 for resazurin, this is a ratio Resazurin:INT of 1.14. 133

4.13. Effect of NADPH on the ETS signal from diaphorase-catalyzed reactions and in reactions based on a mysid homogenate. The spectrophotometric assay using, INT (white bars) and the spectrofluorometric assay using resazurin (gray bars) were compared. 135

5.1. ϕ (µmols O_2 h^{-1}) and specific ϕ (µmols O_2 µg protein^{-1} h^{-1}) of four male mysids. Even when there was no statistical differences ($p > 0.05$), the ϕ values were slightly higher with NADH in both methods. The F_m values for ϕ were 1.5 higher ($p < 0.05$).than those from the S_m in both substrate conditions. 153

5.2. ϕ (µmols O_2 h^{-1}) and specific ϕ (µmols O_2 µg protein^{-1} h^{-1}) of *A. salina*. There was no difference in both methods in relation to the substrate scenarios ($p > 0.05$). Indicating no contribution of NADPH or succinate to the reaction. The F_m signals were approximately 1.1 higher than those from the S_m ($p < 0.05$). 153

5.3. ϕ (µmols O_2 h^{-1}) and specific ϕ (µmols O_2 µg protein^{-1} h^{-1}) of the fish larvae *S. auratus*. Within the F_m and S_m there was a statistical difference ($p < 0.05$) between substrate conditions. With F_m there were higher ϕ rates with NADH, 1.55 times higher than SB ($p > 0.05$). The F_m signals were approximately 1.74 higher with NADH and 1.28 with SB respect S_m ($p < 0.05$). 154

5.4. ϕ (µmols O_2 h^{-1}) and specific ϕ (µmols O_2 µg protein^{-1} h^{-1}) of the seahorse *H. gutulatus*. With S_m the difference was lower than with ($p > 0.05$). With F_m the signal was 1.39 times higher with SB than with NADH alone ($p < 0.05$). The F_m signals were approximately 2.93 higher with NADH and 3.8 higher with SB as compared to the S_m results ($p < 0.05$). 154

5.5. ϕ (µmoles O_2 h^{-1}) and specific ϕ (µmols O_2 µg protein^{-1} h^{-1}) of the abalone *H. tuberculata*. In the S_m there is a small difference ($p < 0.05$). Between substrates however, with the Fm, the values for ϕ with NADH were 3 times higher than with SB ($p < 0.05$). There is a considerable difference between methods and substrate conditions ($p < 0.05$). 155

5.6. ϕ (µmols O_2 h^{-1}) and specific ϕ (µmols O_2 µg protein^{-1} h^{-1}) of the mussel, *Mytilus* sp. The F_m values for ϕ were approximately 1.19 higher than with S_m, under both substrate conditions. Even so, there was no statistical difference between substrate scenarios inside each method and between methods ($p > 0.05$). 155

5.7. Profile of the specific ϕ with the spectrofluorometric method (S_m). The specific ϕ followed the same pattern in both substrate scenarios. With NADH, ϕ was always higher ($p < 0.05$) at all depths except at 25 m. 162

5.8. Comparison of the profile of ϕ between the spectrophotometric method (triangles) and spectrofluorometric (circles). There was a difference between both methods of one order of magnitude ($p<0.05$). With the F_m, ϕ with NADH, as with SB, followed the same pattern until 200 m ($p> 0.05$), from there ϕ with SB stayed stable while ϕ with NADH declined untill 600m. All differences were statistical significant ($p < 0.05$) between substrate conditions, except at 400 m. 162

5.9. Activity ratio between NADH-dh and ETS. Depth profiles based on F_m (●) or S_m (○). Organism metabolism detected by F_m (▲) or by S_m (△). A ratio of one signifies that NADPH or succinate does not contribute to the ETS. Ratios slightly higher than one mean that there is some kind of inhibition of the reaction by NADPH or succinate. Ratios slightly lower mean that NADPH or succinate contribute to the reaction. Ratios much higher than one mean that NADH-dh dominates the reaction; lower than one, mean that NADPH and/or succinate are contributing to the reaction. 167

Table index

2.1. Mean biomass (B, in mg prot.), R, and Φ on each transect. Standard deviations are given in parenthesis. R/Φ ratio may signal the metabolic state of the zooplankters. It can be calculated two ways: (1) by direct division or from the slope of a regression (plot). Note that the difference is due to the correlation of the data given in the parenthesis (r^2). 47

2.2. Mean B (in mg protein), R and Φ, grouped by size class. Standard deviations are given in parenthesis. Since the starvation data for 24h was sparse, they are pooled, regardless of size or area. The R/Φ ratio signifies the metabolic state of the organisms; there are two ways to calculate it. One is by direct division of the values (division); the other is by the slope of a regression (plot). Note that the difference is due to the correlation of the data in the parenthesis (r^2). 48

3.1. Comparison of reagents and conditions used in the ETS and zero-substrate assays. Abbreviations: PVP (polyvinyl pyrrolidone); Tris (Tris (hydroxymethyl) aminomethane); NADH (nicotinamide adenine dinucleotide); NADPH (nicotinamide adenine dinucleotide phosphate (reduced)); INT (2-(ρ-iodophenyl)-3-(ρ-nitrophenyl)-5phenyl tetrazolium chloride). Note that the zero-substrate assay does not incorporate a substrate buffer and the Owens and King kinetic assay does not incorporate a quench or an extraction buffer. 80

3.2. Additional applications of the tetrazolium salt INT in enzymology and cytology. 87

4.1. Some uses of resazurin-resorufin are not related to measurements of mitochondrial respiration. Regardless of the field in which they are used, most of them are employed to determine the viability of the cells. Here are some examples. 99

4.2. List of reagents employed in these experiments. The manufacturer, the corresponding Chemical Abstracts Service (CAS) Registry Number, and the concentration used are included. 104

4.3. An example of the extinction coefficient determination for INT. Ascorbic acid (AA) was used to reduce INT from its tetrazolium-form to its formazan-form. $[AA]_I$ is the initial concentration of ascorbic acid and, $[AA]_f$ is the final concentration of (AA) in the reaction and, ABS is the Absorbance. 108

4.4. Reagents used in the Owens and King (1975) spectrophotometric ETS method (S_m) and the new resazurin-based spectrofluorometric ETS assay (F_m). The values are the initial concentrations, not the final concentrations in the cuvette. 110

4.5. Concentrations of the reagents used to determine their optimum conditions. All concentrations are initials. 113

4.6. Pyridine nucleotide concentrations used to determine Michaelis-Menten constants (K_m) for diaphorase. 115

4.7. Statistical variables to determine the reproducibility of the measurements of resorufin under our laboratory conditions. Where $\bar{x}$, is the mean; ***V***, the variance; σ, standard deviation; and **RSD**, the Relative Standard Deviation. 128

5.1. Comparison of reagents used in the spectrophotometric method (S_m) and the fluorometric method (F_m) method. Succinate is present in both methods. 150

5.2. ϕ and specific ϕ comparisson between substrate conditions with fluorometric (F_m) and spectrophotometric (S_m) method. 158

5.3. ϕ specific ratios, between substrate conditions and between methods. The NADH:SB substrate condition ratio indicates the degree of dependence of NADH on the reaction. Values higher than one indicate that the maximum velocity is reached only with NADH, lower than one with the SB and close to one indicate that there is no contribution or inhibition to the reaction due NADPH or succinate. 161

5.4. ETS substrates used in the analysis of different organisms and samples. 170

Capítulo 1

Introducción General

1.1. Antecedentes

En 1948, Lehninger y Kennedy descubren que la mitocondria es el lugar donde se desarrolla la fosforilación oxidativa (Nelson y Cox, 2008). Desde entonces un gran número de estudios y descubrimientos se han venido desarrollando. En las membranas internas de este orgánulo se encuentra embebido, lo que originalmente se llamó la cadena de transporte de electrones (Keilin, 1966), más adelante en los años setenta se determinó que era más complejo que una cadena, por lo que se le denominó el sistema de transporte de electrones. Este sistema está presente en los tres dominios de la vida (Eucaria, Bacteria y Archea) estando en la membrana mitocondrial de los organismos eucariotas y en la membrana celular de los procariotas, compartiendo en términos generales el mecanismo del flujo de electrones para producir ATP (Tzagoloff, 1982; Packard, 1985a, b; Nelson y Cox, 2008). Básicamente consiste en una serie de transportadores de electrones que genera flujo de protones que produce a su vez un potencial electroquímico a través de la membrana mitocondrial (positivo en la parte interna y negativo en

la matriz mitocondrial), que posteriormente impulsará la síntesis de ATP que es el fin último de este sistema (Figura 1.1).

El sistema está compuesto principalmente por el Complejo I o NADH deshidrogenasa. Este es el punto de control de todo el sistema. A este, el NADH le cede dos electrones ($NADH + H^+ \rightarrow NAD^+ + 2e^- + 2H^+$) y estos junto a los del Complejo II o succinato deshidrogenasa, son trasportados por la ubiquinona (CoQ) ($2e^- + 2H^+ + Q \rightarrow QH_2$) al complejo III o citocromo c (Cyt c) que toma un electrón, que es llevado al complejo IV o citocromo oxidasa, donde dos electrones y dos protones reaccionan con un átomo de oxígeno, para producir dos moléculas de agua (Nelson y Cox 2008; Packard et al., 1983; Packard, 1985). Es precisamente el consumo de este oxigeno junto a la producción de CO_2 y el consumo de carbono orgánico, lo que compone la respiración celular (Lane, 2010).

La respiración junto a la productividad primaria, componen dos de los principales procesos fisiológicos en los océanos y son fundamentales para evaluar su equilibrio metabólico (del Giorgio y Duarte, 2002). Este proceso puede ser utilizado para calcular la producción primaria, los flujos de carbono en el océano (Packard et al., 1988; Packard y Gómez, 2013), la eficiencia de la retención de nutrientes (Osma et al., 2014), y la producción de energía heterotrófica (Packard et al., 2015). Sin embargo, a pesar de su utilidad, la respiración raramente es medida en un sentido oceanográfico, es decir concordantes en escalas de tiempo y

espacio con las mediciones de nutrientes y de datos hidrográficos para la generación de secciones del océano.

La respiración de los organismos más grandes tiene un impacto insignificante sobre las tasas de consumo de O_2 y producción de CO_2. El plancton es el que impacta la química de los océanos y es su pequeño tamaño, fragilidad y baja concentración lo que hace que la respiración del océano sea tan difícil de medir. Esto se hace más evidente en el océano profundo, que es ≥80% de este ecosistema oceánico (Garrison, 2012). La señal de la respiración es generalmente sutil y débil, la propia medida es difícil de lograr, excepto en las aguas superficiales eutróficas, uno de los pocos lugares donde la señal de la respiración es fuerte. Sin embargo, incluso en estas aguas, recolectar la suficiente muestra o agua para las mediciones resulta difícil y costoso. En el agua de mar los organismos tienden a estar dispersos por lo que para obtener material biológico suficiente para realizar las mediciones fisiológicas, deben concentrarse ya sea por medio de redes (eg: Bongo, WP2), filtración u otros métodos. Durante estos procedimientos se suelen lesionar a los organismos, lo que genera una disyuntiva adicional ya que los organismos planctónicos capturados deben de estar sanos y completos, de lo contrario, cualquier medición no será representativa de su verdadera respiración *in situ*. Cumplir con este requisito es difícil, lento, laborioso y costoso.

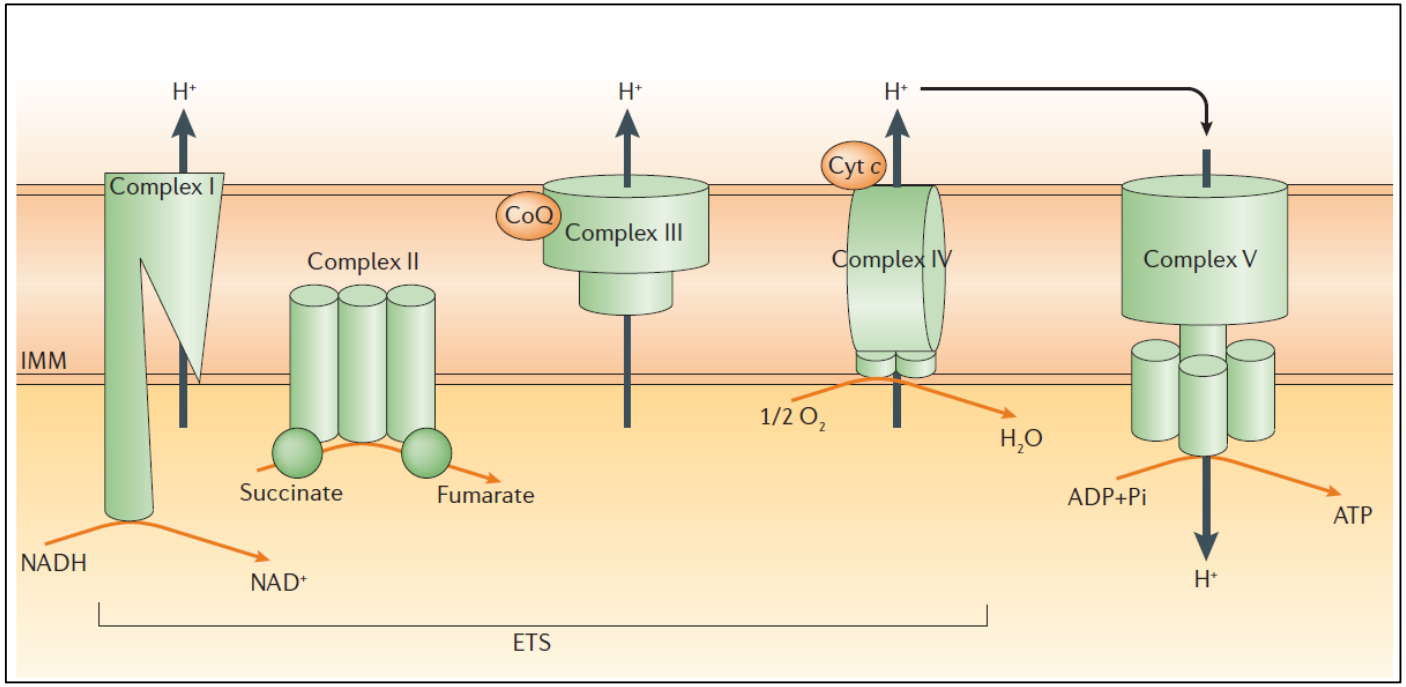


Figura 1.1. Diagrama esquemático del sistema de trasporte de electrones mitocondrial (tomado de Abou-Sleiman et al., 2006).

Debido a que la actividad enzimática es una propiedad característica de las células vivas, Aleem (1955) propone utilizar cloruro de trifeniltetrazolio para cuantificar la biomasa viva de las poblaciones de plancton en el Mar Báltico. Más tarde, teniendo en cuenta que la actividad enzimática es la base de la fisiología celular, Davey (1964) utilizó NAD^+ oxidoreductasa para evaluar la respiración en el fitoplancton y Curl y Sandberg (1961) utilizan succinato deshidrogenasa (SDH) para medir la respiración del zooplancton. En ambos casos, las enzimas eran parte del ciclo del ácido cítrico y del ciclo de Krebs. Sobre esta base, Pearre (1964) probó que a través de la SDH se podía realizar una aproximación de la respiración del zooplancton, más no de la respiración del fitoplancton. Packard y Taylor (1968) encontraron una correlación entre SDH y la respiración en *A. salina*, pero en el proceso de esta investigación se percataron que un mejor índice de consumo de oxígeno sería el sistema de transporte de electrones. La investigación de Azar y Williams (1955, 1956) mostró que este sistema de citocromos-enzima, y sobre todo en el complejo NADH deshidrogenasa controla la tasa de consumo de oxígeno. Basándose en esta idea, el método (ETS) fue desarrollado a principios de los años setenta por Ted Packard (Packard, 1969; Packard et al, 1971; Packard et al., 1974). En sus trabajos demostró, que las medidas de la actividad ETS en muestras de agua de mar podrían usarse para calcular las tasas de consumo de oxígeno en la columna de agua del océano. Esto condujo a una serie de mejoras en el ensayo de ETS y su aplicación en el fitoplancton (Kenner y Ahmed, 1975), zooplancton (Owens y King, 1975), bacterias (Christensen et al., 1980) y sedimentos (Christensen y Packard, 1977), y su relación con la respiración

(Arístegui y Montero, 1995; Finlay et al., 1983; King y Packard, 1975b), la temperatura (Martínez y Estrada, 1992; Packard et al., 1975) y la presión (King y Packard, 1975a).

El ensayo, como comúnmente se utiliza hoy en día (Packard et al., 2004; Gómez et al., 1996), fue diseñado como un índice para la respiración de todos los organismos que habitan en un volumen determinado de agua de mar (Packard et al., 1971) y para reducir la dependencia de otros métodos, que requieren mucho tiempo para la estimación de la respiración. (Barnes y Collias, 1961; Seiwell, 1937; Robinson y Williams, 2004). Esté método, utiliza preceptos básicos de enzimología, lo que significa y exige que una enzima se mida en su nivel máximo de actividad (V_{max}) (Fruton y Simmonds, 1958; Passonneau y Lowry 1993; Maldonado et al., 2012). Para esto se requiere la adición de sustratos (NADH y en algunas ocasiones NADPH y succinato) en concentraciones de saturación que proporcionan la sensibilidad necesaria para el Complejo I y Complejo II del ETS, así como para asegurar la reproducibilidad y la replicación de la medición. Históricamente, el ensayo del ETS se basa en la reducción de un aceptor artificial de electrones, 2- (p-yodofenil) -3- (p-nitrofenil) - cloruro de tetrazolio 5-fenil (INT) (Packard, 1971; Owens y King, 1975; Kenner y Ahmed, 1975; Gómez et al., 1996) (Figura 1.2).

A partir de la actividad ETS podemos calcular la respiración potencial (ϕ) pero estos valores tienen que ser convertidos a valores de respiración *in vivo* por algoritmos determinados

empíricamente (Arístegui y Montero, 1995), para ello se hace necesario realizar múltiples mediciones de respiración *in vivo* (R), en diferentes escenarios y relacionarlas con sus correspondientes valores de respiración potencial (ϕ), para así construir modelos e interpretaciones más sólidos y poder conocer los estados metabólicos de los diferentes organismos que componen el plancton.

El radio R/ϕ, es la relación entre la tasa de respiración *in vivo* (R) y el potencial de respiración celular (ϕ) calculada a través de la actividad enzimáticas *in vitro* que determinan la tasa máxima (V_{max}) de la respiración, también podría considerarse como una medida de la eficiencia del grado de control de las enzimas y transportadores de electrones en el ETS (Kenner y Ahmed, 1975b). Algunos autores consideran que los organismos respiran aproximadamente un 50% de su capacidad (Packard, 1985). A pesar que la actividad ETS estaba bien correlacionada (Packard, 1985a, b; Arístegui y Montero, 1996), la relación entre la respiración y las medidas enzimáticas puede ser variable, lo que ha impedido el uso de esta como un método de estandarización de la respiración *in situ*. La relación R/Φ también se puede usar como un índice de estado fisiológico (Christensen et al., 1980). Se cree que cuando esta relación es baja (< 0.5) los organismos tienen una alimentación limitada y probablemente un estado fisiológico desfavorable, mientras que cuando está entre 0.5 y 1, los organismos están bien alimentados y pueden responder a cualquier variación ambiental que genere un estrés biológico y si esta relación es igual a 1, probablemente están utilizando toda su

capacidad respiratoria. (Christensen et al, 1980; Packard et al, 1996). R/ϕ muy superiores a 1, podrían indicar errores en las mediciones, como subestimar ϕ o sobreestimar R. La relación R/ϕ muestra variaciones (Båmstedt, 1979; Christensen et al., 1980; Packard, 1985; Hernández-León y Gómez, 1996) debidas a la temperatura, la dieta, el estado fisiológico, el tamaño corporal, la edad, entre otras muchas, que podrían determinar esta variabilidad. No obstante, a pesar de la variabilidad encontrada en este cociente, del Giorgio (1992) mostró que el error asociado con la utilización del ETS es similar al error en otras técnicas standard ecológicas utilizadas tales como el método del ^{14}C, la incorporación de timidina, etc.

Otra de las críticas al método ETS, es que es un índice de la respiración, ya que como se definió anteriormente, mide la máxima capacidad respiratoria de los organismos, un estado que no es el habitual en ellos y menos durante tiempos prolongados. Este hecho, llevó a Båmstedt (2000) en un afán de tratar de desarrollar un método usando el sistema enzimático del ETS a que midiera la respiración *in vivo* de los organismos, omitiendo los sustratos NADH y NADPH, esperando que el INT sea reducido por las enzimas propias de la muestra. Este método no es un ensayo enzimático válido ya que viola algunos preceptos de la cinética enzimática. Martínez-García et al. (2009) va todavía más allá, recogiendo el testigo dejado por Båmstedt, añade directamente el INT al agua de mar para la determinación de la respiración *in situ* del microplancton allí presente. La ventaja hipotética es que el INT reacciona con el ETS en los niveles de sustratos naturales en las

células dando la respiración verdadera *in situ*. Smith y McFeters (1997) sugieren que al INT le cuesta trabajo penetrar en las zonas donde se produce la respiración, no obstante, Martínez-García et al. (2009) suponen que INT puede penetrar libremente las paredes celulares, el plasmalema bacteriano y las membranas mitocondriales, en una muestra de plancton. Sin embargo no se hizo verificación de este hecho al igual que tampoco se verificó la toxicidad del INT-formazan (Villegas-Mendoza et al., 2015). Además, existen otros problemas si utilizamos está metodología. El primero, es que el ensayo carece de especificidad y las sustancias reductoras con electropotenciales superiores a 170 mV pueden reducir el INT (Seidler, 1991). Otro inconveniente es que la cinética de las reacciones enzimáticas detectadas se modifica en una medida desconocida, debido a que los depósitos de formazan que se precipitaron al lado de la enzima y que reducirán la frecuencia de colisión de la enzima con su sustrato, cambiaran la cinética de la reacción y por lo tanto reducirán la velocidad de esta. Otra irregularidad, es que los valores de R/ETS que presenta, oscilan alrededor de 11, lo que significa que la medida de R excede su medición en un factor de 11. Esto indica que la medición de ETS es menor que la propia respiración lo que haría inútil la medición “enzimática”. Si el índice es verdaderamente la potencialidad máxima de la respiración, la relación R/ϕ debe ser igual o menor que uno.

La diversidad de los organismos puede estar también presente en las diferentes rutas metabólicas y actividades enzimáticas, que pueden depender de las condiciones fisiológicas o ambientales

adaptativas a su entorno particular. Los organismos tienen que controlar su propio sistema de transporte de electrones, a pesar de la universalidad del ETS. Esto es especialmente cierto en las bacterias, concretamente por ejemplo en las bacterias nitrificantes. En condiciones anóxicas estas bacterias, cambian de aceptor final de electrones, pasando del O_2 al nitrato, esto implica un cambio en los citocromos (Payne, 1976; Packard et al., 1983a; Packard, 1985). Asímismo Marrs y Gest (1973) llegan a la conclusión de que los electrones tienden a ir a través de rutas alternativas, en función de sus orígenes metabólicos.

Otra cuestión a tener en cuenta y que puede ser un inconveniente para el análisis de la respiración potencial, son las condiciones metabólicas inherentes a la especie y las condiciones ambientales a las que se encuentran sometidos los organismos. En el momento de la captura, se asume que todos los organismos allí presentes se comportan de la misma manera desde el punto de vista metabólico y fisiológico.

Los sustratos que históricamente se han empleado en esta metodología son el NADH, NADPH y el succinato (Packard et al., 1971; Garfield et al., 1979; Hobbie et al., 1972; Christensen and Packard, 1977; Gomez et al., 1996), bajo la premisa que el aporte de cada uno de estos es aditivo, dando como resultado el potencial maximo de la respiración. En esto sentido, diferentes autores bajo diferentes argumentos, realizan las pruebas con distintas combinaciones de sustratos. Owens y King (1975) no emplearon el succinato en muestras de zooplancton; Schalk (1988) en el

micronecton; Cammen et al. (1990) en la macrofauna bentonica y Toth et al. (1995) en larvas de peces entre otros. Madon et al. (1998), sólo emplean NADH, sustentando que la actividad del NAPDH no alcaza el 10% del aporte a la actividad total.

En esta tesis se pone de manifiesto la importancia de tener en cuenta según el tipo de muestra para conocer que sustratos se deben de añadir. Nuestros resultados demuestran que en algunos tipos de muestras, la adición de todos los sustratos genera algún tipo de inhibición en la reacción, impidiendo determinar el V_{max}, ya que como se ha puesto de manifiesto en la diversidad metabólica de los organismos, diferentes células pueden mantener grupos separados de NADPH y NADH, con diferentes potenciales redox. En general, el NADH transfiere electrones en las reacciones aeróbico-catabólicos al ETS y el NADPH generalmente suministra electrones para las reacciones anabólicas (Nelson y Cox, 2008). Con esto se podría explicar el por qué al realizar pruebas de ETS en diferentes organismos, bajo diferentes condiciones de sustratos, encontramos diferencias en las respiraciones potenciales.

Entendemos que realizar mediciones enzimáticas a cada grupo o especie de los organismos que conforman la comunidad planctónica a bordo de un buque oceanográfico e inclusive en el laboratorio, es una tarea titánica o prácticamente impensable. Sin embargo, es crucial hacer mediciones en el laboratorio para poder relacionarlas con estudios de muestras naturales.

El ensayo del ETS se ha mejorado continuamente a lo largo de los años, hasta el punto donde se requiere un tercio del tamaño de la muestra y cinco veces menos tiempo de cómo se diseñó originalmente (Packard y Christensen, 2004). A pesar de estas mejoras, las mediciones de la actividad ETS en aguas oligotróficas o profundas aún requieren la filtración de 10 o más litros de agua de mar (Packard et al., 1971; Hobbie et al., 1972; Packard and Williams, 1981; Packard et al., 1983b; Arístegui et al., 2005). Cualquier mejora en la sensibilidad analítica reduciría este inconveniente del volumen o la cantidad de biomasa necesaria para una buena detección de la señal, que se traducirá en mejorar la generación de secciones de respiración del océano y en los cálculos para los modelos de retención de nutrientes y la producción de energía (sensu Bindoff y Wunsch, 1992 y Packard et al., 2014).

En este sentido, se investigó las posibles mejoras potenciales asociadas con el uso de un receptor de electrones diferente al INT para el ETS. El escogido fue el reactivo no tóxico denominado, resazurin ($C_{12}H_6NNaO_4$).

El resazurin tiene la ventaja de ser fluorescente en su estado reducido (resorufin), lo que puede mejorar la detección analítica de la actividad de transporte de electrones en comparación con la detección que puede lograrse mediante espectrofotometría (Undenfriend, 1962; Cook y Ser, 1993; Passonneau y Lowry 1993;

Bisswanger, 2011). Esto se debe a que la fluorometría detecta una señal luminiscente de cero, mientras que con espectrofotometría se compara la reducción en la transmisión de luz desde una referencia de transmisión del 100% dando la diferencia entre dos grandes números (Oxtoby et al., 1999). Otra de las ventajas de la fluorescencia es que puede llegar a ser 100 veces más sensible que métodos espectrofotométricos, a lo que se le suma su especificidad, ya que los picos de longitudes de onda tanto de excitación como de emisión son muy precisos, lo que garantiza mayor especificidad en la lectura del compuesto, caso contrario de la espectrofotometría que lee solo la absorbancia o transmitancia de la solución en una sola longitud de onda.

El resazurin es de color azul en solución y cuando se reduce, como una respuesta a la actividad metabólica, como por ejemplo en las mitocondrias, se convierte en resorufin, un colorante fluorescente de color rosa intenso (Rasmussen, 1999; González y Tarloff, 2001; De Fries y Mistuhashi, 1995) (Figura 1.3).

El resazurin no es un reactivo nuevo, fue descubierto y desarrollado hace 145 años por Weselsky (1871) y el primero en usarlo fue Pesch y Simmert (1929) para la detección de bacterias en la leche. El tinte se ha utilizado en numerosos campos relacionados con el metabolismo celular, la mayoría de ellos en el campo médico de la citotoxicidad (eg: Cook y Ser, 1993; Gundemir y Johnson, 2009; Kumar et al., 2012; Abu-Amero et al., 2006), en la calidad espermática (eg: Erb y Ehlers, 1950; Strzeżek et al., 2013) y la industria de productos lácteos (eg: Ramsdell et al.,

1935; Moyer y Campbell, 1963; Ricciardi et al., 2014). Sin embargo su uso en la ecología, fisiología y estudios oceanográficos son menos comunes (eg: Morita y ZoBell, 1955; Bitton y Dutka, 1983; Thies y Grimme, 1995; Proctor, 1997; Barnes et al., 1998; Wellsbury et al., 2002). No obstante, en los últimos años algunos limnólogos han comenzado a utilizar resazurin para evaluar la respiración en sistemas lóticos (eg: Haggerty et al., 2008, 2009; Argerich et al., 2011; Stanaway et al., 2012; González-Pinzón et al., 2012).

De las diferentes maneras en que el resazurin se ha utilizado para evaluar la actividad metabólica, los que están más cerca de nuestro enfoque son los estudios realizados por Abu-Amero y Bosley (2005) y Zhang et al. (2004). Estos autores trabajan en la bioquímica relacionada con la respiración mitocondrial en ratas, pero no en su regulación. Por otra parte, Peroni y Rossi (1986), calcularon los equivalentes de oxígeno en muestras de sedimento marinos por medios espectrofotométricos, calculando el potencial de respiración a partir de la absorbancia del resorufin incubando sus muestras durante 1h sin la adición de sustratos. Además, calcularon el consumo de oxígeno suponiendo que la reacción es equivalente a la reducción de INT donde una molécula de formazan es reducida por dos electrones. Sin embargo este cálculo es incorrecto, ya que la reducción de resazurin sólo involucra un electrón.

El método ETS desde su creación y gracias a la universalidad de este complejo enzimático, ha logrado impactar en gran medida,

los estudios por los que se obtienen las mediciones de la respiración en los océanos y otros ecosistemas. También ha proporcionado una gran herramienta para desarrollar modelos oceánicos sobre la productividad o sobre los flujos de carbono. Es importante y necesario, seguir avanzando en el desarrollo de la técnica, así como en la profundización en el entendimiento de los mecanismos que regulan la respiración. Es indispensable coordinar el trabajo en el laboratorio con el de campo, entender que no todos los organismos se enfrentan de forma similar a las mismas condiciones ambientales y que poder descifrar estos mecanismos nos dará una visión más realista de los procesos biológicos oceánicos, que en gran medida afectan a los geofísicos.

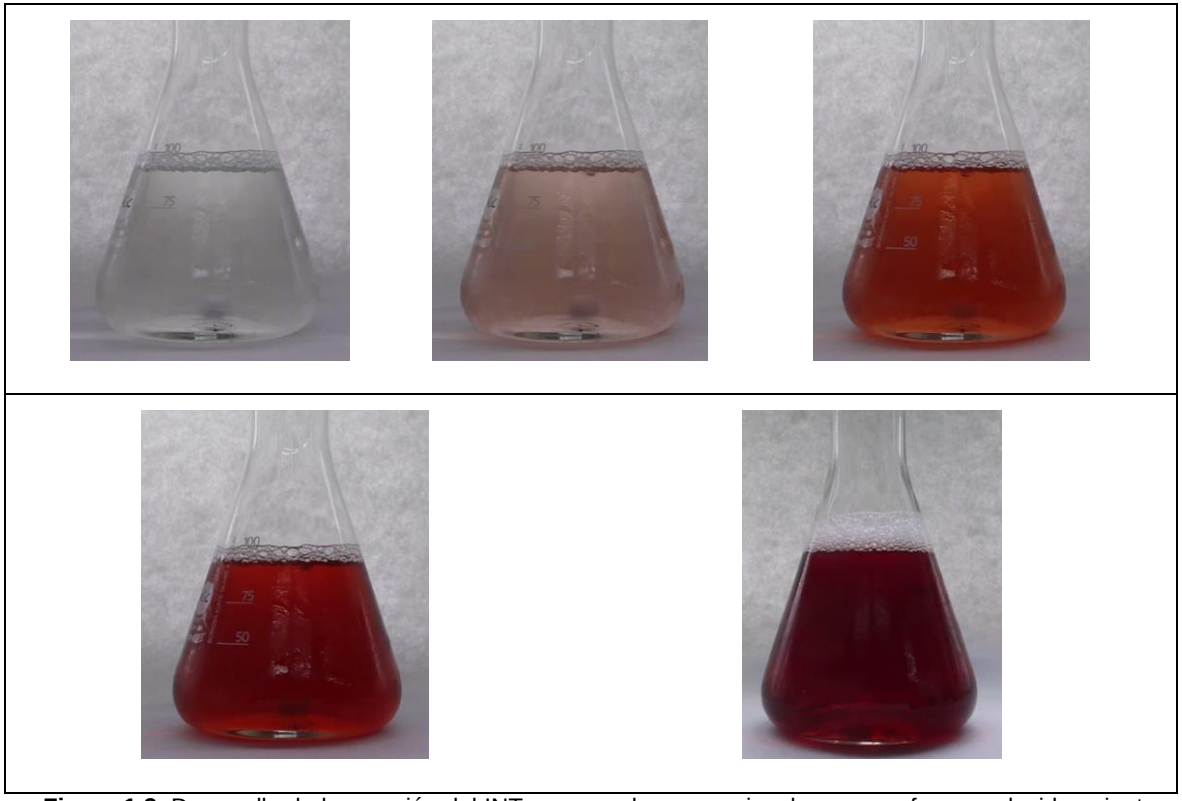


Figura 1.2. Desarrollo de la reacción del INT, que puede parecer incoloro, en su forma reducida, mientras que el formazan, es de color rojo o violeta, dependiendo de la concentración.

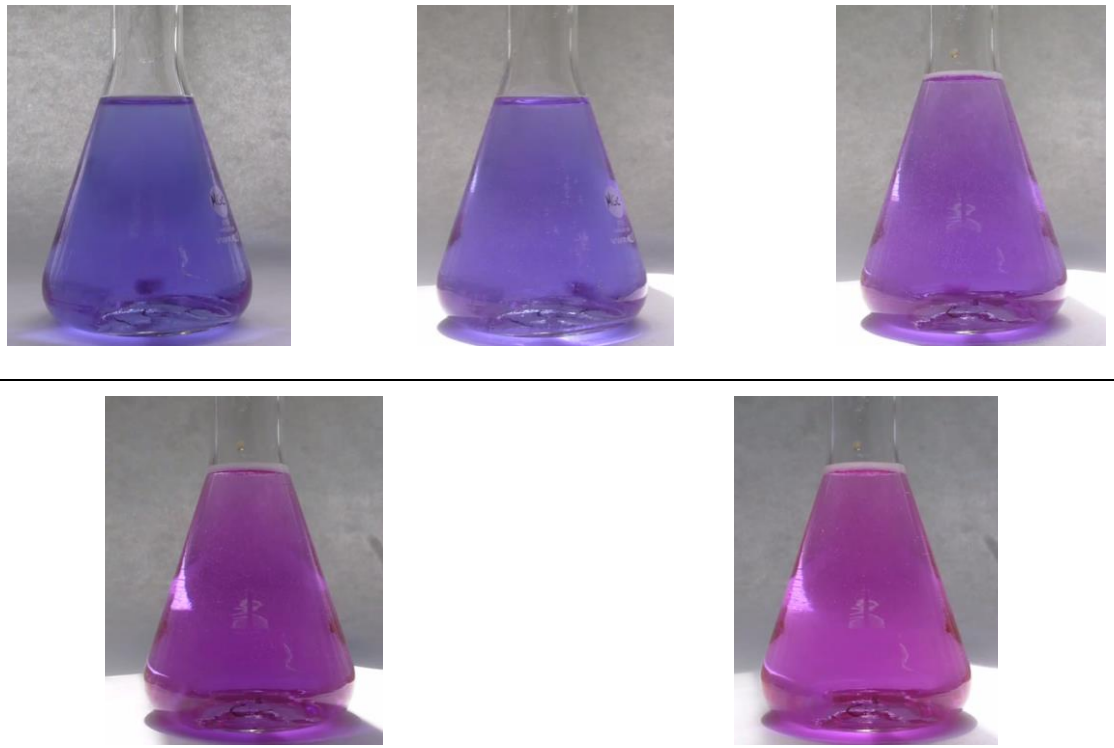


Figure 1.3. Desarrollo de la reacción del resazurin, de color azul-violáceo, en su forma reducida y fluorescente, el resazurin, de tonalidades rosáceas.

1.2. Objetivos

1.2.1. Capítulo 2

1. Determinar en el zooplancton, la relación entre la respiración *in vivo* (R), la potencial (ϕ) y la biomasa, en tres transectos en diferentes zonas geográficas, Cartagena de Indias–Cartagena (Océano Atlántico), Ciudad del Cabo–Perth (Océano Indico Sur) y Perth–Sídney (Océano Indico, cruzando la Gran Bahía Australiana).

2. Establecer el estado fisiológico del zooplancton a través de la relación R/ϕ en las zonas anteriormente citadas, y la posible variación de esta en relación con el tamaño (100-500, 500-100 y >1000 µm).

3. Explorar los efectos de la inanición sobre la respiración *in vivo* y determinar su variación con respecto a la respiración potencial en el zooplancton.

1.2.2. Capítulo 3

4. Comprobar que la realización del ETS *in vivo*, sin la adicción de sustratos, es un error conceptual, en el cual no se está detectado la actividad enzimática asociada al ETS.

5. Demostrar que algunas sustancias presentes en las células tienen la capacidad de reducir el aceptor artificial de electrones (INT) sin la necesidad de un proceso enzimático, como el del ETS.

1.2.3. Capítulo 4

6. Con el fin de aumentar la sensibilidad del método del ETS, se pretende desarrollar un método espectrofluorométrico.

7. Establecer para esta nueva metodología las condiciones óptimas de fuerza iónica, influencia del O_2, concentraciones óptimas de los sustratos (NADH, NADPH y succinato) y del resazurin.

8. Determinar, en muestras de zooplancton y diaforasa, si el método fluorescente es comparable con el método empleado históricamente y de referencia (Owens y King, 1975) para la determinación de la respiración potencial.

9. Determinar si el succinato tiene un efecto aditivo en la velocidad de la reacción.

10. Determinar la precisión, repetividad, el límite de detección (LOD) y cuantificación (LOQ) de los métodos en cuestión.

11. Generar una metodología para el calibrado de la actividad ETS.

12. Verificar si la enzima NADPH deshidrogenasa genera algún tipo de inhibición durante la reacción del ETS.

1.2.4. Capítulo 5

13. Estudiar la aplicación del método espectrofluorométrico y espectrofotométrico, sobre diferentes tipos de organismos y muestras ambientales, con distintas concentraciones de biomasa.

14. Determinar la actividad ETS en dos condiciones de sustratos, sólo con NADH o con la adición de los tres sustratos (NADH + NADPH + succinato) y observar si la actividad ETS es aditiva o existe algun tipo de inhibición.

1.3. Principales resultados obtenidos

1.3.1. Capítulo 2: Actividad Metabólica del Zooplancton (respiración y respiración potencial) en los Océanos Índico Sur y Atlántico.

El número de muestras tomadas en cada transecto estuvo en relación a la longitud del transecto, las prioridades a bordo del buque y las condiciones meteorológicas. En el Océano Índico meridional, desde Ciudad del Cabo (Sudáfrica)-Perth (Australia costa oeste) hubo un total de siete estaciones en las que se tomaron muestras. Cruzando la Gran Bahía Australiana (Perth-Sídney) se tomaron muestras de cuatro estaciones y en la travesía del Atlántico desde Cartagena (Colombia)-Cartagena, hubo un total de doce estaciones muestreadas. Sólo se tuvo en cuenta el zooplancton crustáceo, las salpas, medusas, quetognatos y larvas de peces fueron retirados. Se realizaron un total de 68 mediciones, 18 de estas durante el primer transecto, 14 en el segundo y 36 en la sección del Atlántico (Tabla 2.1).

A pesar de las diferencias entre el número de estaciones muestreadas (Tabla 2.2), el promedio de la biomasa (B) en la sección entre Ciudad del Cabo y Perth fue de 0.4 ±0.19 mg prot., entre Perth y Sídney fue 0.79 ±0.45 mg prot., y entre Cartagena-Cartagena fue de 0.78 ±0.60 mg prot. En general, la fracción de tamaño que más contribuyó fue la de 100-500 µm, seguida por la

de 500 a 1000 µm y por último la de >1000 µm (Tabla 2.1). La fracción más pequeña representó el 44.7% con una biomasa promedio de 0.85 ±0.65 mg prot., seguida del tamaño medio, con un 34.2%, con una media de 0.65 ±0.41 mg prot., y la menos abundante fue la talla mayor, con un 21.1% y un promedio de 0.40 ±0.20 mg prot. (Tabla 2.2).

Los valores específicos de la respiración (R) y la respiración potencial (Φ), variaron según las distintas áreas geográficas. Los valores más bajos, en ambas mediciones se presentaron en el Océano Atlántico (0.94 ±0.69 y 1.54 ±1.02. µmol $O_2 \cdot h^{-1} \cdot mg\ prot^{-1}$, respectivamente). La ϕ específica fue mayor en el transecto Perth-Sídney (1.97 ±0.56 µmol $O_2 \cdot h^{-1} \cdot mg\ prot^{-1}$), seguida de la sección Ciudad del Cabo-Perth (1.77 ±0.64 µmol $O_2 \cdot h^{-1} \cdot mg\ prot^{-1}$). Los valores más altos de la R específica fueron en el transecto Ciudad del Cabo-Perth (1.77 ±0,64 µmol $O_2 \cdot h^{-1} \cdot mg\ prot^{-1}$), seguido de Perth-Sídney (1.41 ±0.65 µmol $O_2 \cdot h^{-1} \cdot mg\ prot^{-1}$) (Figura 2.4, Tabla 2.1).

Desde el punto de vista de las fracciones de tamaño, los valores especificos más bajos para R y ϕ se presentaron en la fracción >1000 µm (0.93 ±0.61 y 1.13 ±0.62 µmol $O_2 \cdot h^{-1} \cdot mg\ prot^{-1}$, respectivamente), seguido por la de 500-1000 µm (0.98 ±0.53 y 1.69 ±0.41 µmol $O_2 \cdot h^{-1} \cdot mg\ prot^{-1}$, respectivamente) y la más alta se encontró en la talla más pequeña, 100-500µm (1.35 ±0.64 y 2.06 ±0.81 µmol $O_2 \cdot h^{-1} \cdot mg\ prot^{-1}$, respectivamente).

La relación de R/Φ es un índice de la capacidad respiratoria utilizada. Hay dos maneras de acceder a determinar R/ϕ, uno es a través de la división directa de los valores absolutos de R y ϕ (Tabla 2.1 y 2.2) o del valor de la regresión de las gráficas (Figura 2.2). En ocasiones, estos dos valores pueden diferir; debido a la variabilidad o dispersión de los datos, en esta ocasión consideramos usar el R/Φ de las gráficas, ya que de esta manera se tiene en cuenta la dispersión de los datos. Ambos valores de R/Φ se presentan en las Tablas 2.1 y 2.2. Desde el punto de vista geográfico, el cociente R/Φ varió entre 0.37 y 0.84 (Tabla 2.1) y en relación con los diferentes tamaños, este cociente varió entre 0.41 y 0.86 (Tabla 2.2). Los valores más altos de R/Φ fueron encontrados en el Océano Índico (0.84), seguido de la sección Perth-Sídney y la de Cartagena-Cartagena, que presentaron el mismo valor (0.37) (Tabla 2.2; Figura 2.4). Aun así, teniendo en cuenta todos los datos no hay ninguna diferencia significativa ($p > 0.05$) entre las diferentes zonas oceánicas (Figura 2.2). Desde el punto de vista de las clases de tallas, la relación R/Φ más alta se presentó en la fracción > 1000 µm (0.86), seguido por 100-500 µm (0.41) y luego por la de 500-1000 µm (0.30) (Tabla 2.2; Figura 2.4). No obstante, tampoco en este caso, existen diferencias significativas ($p > 0.05$) entre los valores obtenidos en las distintas clases de tallas.

Los resultados obtenidos en las las relaciones entre log R/Log B y log ϕ/log B, (Ley de Kleiber), fueron más altas en el Atlántico (1.09 y 1.32, respectivamente), seguida de Perth-Sídney (0.98 y 1.06, respectivamente), mientras que el valor más bajo se encontró

en el primer transecto del Océano Índico (0.71 y 0.85 respectivamente) (Figura 2.3). A pesar de las diferentes pendientes, no existen diferencias significativas entre ellas ($p > 0.05$). Teniendo en cuenta las diferentes clases de talla, estas relaciones logarítmicas son más altas en la talla >1000 µm donde tanto como para log R y log ϕ fue de 1.12. En la relación log R/log B (Figura 2.3) es ligeramente más alta en la talla 500-1000 µm (0.92) con respecto a la talla más pequeña (0.79); mientras que para Log ϕ/log B se registró que en 100-500 µm es mayor respecto a la de 500 a 1000 µm (1.09 y 0.80, respectivamente). No hay diferencia estadística significativa ($p > 0,05$) entre todas las relaciones, ya sean agrupadas por zonas o clases de tallas (Figura 2.3, 2.4).

En cuanto a los efectos debidos a la inanición, se esperaría que la R disminuyera y la ϕ se mantuviera más o menos estable, sin embargo no se encontraron diferencias estadísticamente significativas ($p > 0.05$) entre estos parámetros. La baja cantidad de experimentos a las 24h de inanición se debió a la escasez de biomasa en las muestras de océano abierto. Además, debido a la baja biomasa, hubo muchas ocasiones en las que sólo se podía medir la respiración en el tiempo inicial (0h). Es por eso que hay cerca de ocho veces más mediciones en 0h respecto a 24h (Tabla 2.2). Por estas razones los resultados se analizaron conjuntamente utilizando todas las mediciones, sin discriminar por regiones o clases de talla.

Los valores específicos de R y ϕ (Tabla 2.2), fueron similares ($p > 0.05$) tanto para 24 horas (1.28 ±0.45 and 1.71 ±0.58 μmol O_2 h^{-1} mg prot.$^{-1}$, respectivamente) como para 0h 1.13 ±0.61 and 1.70 ±0.65 μmol O_2 h^{-1} mg prot.$^{-1}$, respectivamente.

1.3.2. Capítulo 3: Reducción del INT y la importancia de los sustratos en la medición del sistema de transporte de electrones

Cuando comparamos la actividad de la respiración potencial (Φ) con y sin sustratos, se observa que cuando estos están presentes, la Φ aumenta en relación al incremento de la biomasa. Mientras que en el ensayo donde no se añaden sustratos, siguiendo lo propuesto por Båmstedt, 2000, la señal se mantuvo constante, sin mostrar un aumento y con niveles cercanos a cero; estos resultados son muy similares al blanco de la prueba inicial (reactivos sin muestra). Los valores de Φ entre métodos presentan unas diferencias hasta de un orden de magnitud (Figura 3.4).

Tres de los compuestos ensayados (cisteina, glutatión y ácido ascórbico) reaccionaron con el INT para producir formazan. La producción de formazan fue dependiente del tiempo hasta que la reacción se completó, pero esta linealidad no debe ser considerada como consecuencia de una actividad enzimática, ya que no hay enzimas presentes en las reacción (Figura 3.3 y 3.5).

La cisteína y el glutatión mostraron tasas de producción de formazan similares, pero la producción debida al ácido ascórbico fue de tres órdenes de magnitud más altas (Figura 3.5). El fenol y la glucosa no mostraron ninguna reacción con el INT. La reacción del INT con la vitamina B_{12} fue difícil de rastrear

espectrofotométricamente porque tiene un coeficiente de extinción a 490 nm de 5.350 cm^{-1} M^{-1} y por lo tanto compite con la absorción de INT a esta misma longitud de onda (Du et al., 1998; Hill et al., 1964), pero no se descarta su posible capacidad reductora sobre el INT.

1.3.3. Capítulo 4: Optimización de un método fluorescente para la determinación del ETS

Se ratifica que el resorufin disponible comercialmente y el resorufin como consecuencia de la reducción del resazurin son idénticos haciéndolos comparables al momento de realizar la curva de calibrado. El resorufin bajo las condiciones de nuestro experimento, presenta picos de excitación (λ_{ex}) a 520 nm y de emisión (λ_{em}) a 594 nm (Figura 4.2). Además se comprueba que los demás reactivos presentes en la reacción no comprometen la detección del resorufin (Figura 4.2).

Para determinar todas las condiciones del nuevo método se empleó la diaforasa como catalizador de la reacción. Este compuesto pertenece al grupo de las deshidrogenasas que son capaces de reducir principalmente a los piridin nucleótidos.

Existen indicios de que el oxígeno genera una competencia por los electrones que deberían de reducir al resazurin durante la reacción. También en ciertas circunstancias el incremento de la fuerza iónica puede generar algún tipo de influencia sobre la señal. Aun así no se encuentran evidencias de que estas variables tengan influencia sobre la señal del ETS (Figura 4.3), por ende para las pruebas subsiguientes no se añadirá NaCN (que evitaría la competencia del O_2 por los electrones) y NaCl (empleado para incrementar la fuerza iónica.

También se determinó cuál es la concentración adecuada de cada sustrato (NADH, NADPH y succinato), sin perder los niveles de saturación de estos en la reacción, requisito imprescindible para la realización de una prueba enzimática. Al momento de determinar dicha concentración óptima para el succinato, no se encontró ninguna evidencia de transferencia de electrones desde el sustrato al resazurin, en ninguna de las concentraciones ensayadas (Figura 4.4). Caso contrario que con el NADH y el NADPH, donde se evidenció actividad enzimática. La actividad de la NADPH-dh fue aproximadamente 1,5 veces menor que la de la NADH-dh. La extensa meseta para cada piridin nucleótido indica que las actividades de las deshidrogenasas no están limitadas por estos sustratos. En consecuencia, las concentraciones elegidas para el ensayo espectrofluorométrico del ETS fueron de 1 mM para el NADH y 0,15 mM para el NADPH (Figura 4.6). Estos valores son menores a los empleados en el método espectrofotométrico, lo que repercute en un ahorro de costos.

Se realizaron asimismo pruebas para saber si al estar todos los sustratos juntos en la reacción, el succinato genera un efecto sinérgico en la señal total del ETS, ya que por sí solo no mostraba ninguna actividad. En este caso se observó que el succinato no aumenta la reducción del resazurin a través de la catálisis de la diaforasa (Figura 4.5). Por consiguiente, no se incluyó en las sucesivas pruebas.

También fue necesario determinar la concentración óptima de resazurin, ya que este es un compuesto que colorea fuertemente la solución inclusive a bajas concentraciones. Por ende fue necesario determinar un punto de equilibrio en el cual se evitara que fuera un compuesto limitante o por el contrario generara un "inner cell effect", que puede ser definido como la disminución en la intensidad del haz de excitación a causa de la absorción óptica del cromóforo (resorufin) en la región de excitación. Entonces la señal de emisión experimenta una aparente disminución en el rendimiento cuántico y/o distorsión de la banda como resultado de la re-absorción de la radiación emitida (Pawlak et al., 2011).

Se encontró que existe linealidad (r^2 0.996) hasta los 5 µM (concentración final de resorufin en la cubeta) luego de este punto existe una disminución en la señal (Figura 4.8 y 4.9). Para los ensayos se estimó conveniente utilizar la concentración inicial de 10µM de resazurin. Esta concentración está en el rango de la señal de fluorescencia máxima según lo observado por Catomeris y Thibert (1988) y Barnes y Spenney (1980). Además, durante el tiempo de medición de la reacción (10 minutos), la velocidad de la reacción se mantiene lineal, lo que garantiza que esta concentración empleada del aceptor artificial de electrones, es la apropiada para evitar una posible limitación.

Se determinó el límite de detección (LOD) y cuantificación (LOQ) bajo los criterios de la IUPAC (Inczedy et al., 1998; Irving et al, 1978). El LOD y LOQ, para el Tris buffer (del método espectrofluorométrico) fueron de 1.91 y 3.33 nM, respectivamente.

No se pudo determinar estos parámetros para el tampón fosfato con el espectrofotómetro, ya que en él la mayoría de los resultados dieron mediciones negativas. La desviación estándar relativa (RSD) del F_m muestra que los datos varían respecto a la media, tan sólo un 4% aproximadamente (Tabla 4.7).

Con el fin de comparar el método clásico espectrofotométrico (S_m) de ETS (Owens y King, 1975) con el espectrofluorométrico (F_m), se realizaron dos series de pruebas. Primero con diaforasa, una deshidrogenasa capaz de catalizar la reacción, ya que esta enzima oxida los dos piridin nucleótidos, NADH y NADPH. (Shapiro y Silanikove, 2010; Silanikove y Shapiro, 2012). Tanto para el INT como para el resazurin, la velocidad de reacción fue lineal con las concentraciones crecientes de la diaforasa. Teniendo en cuenta las pendientes, el F_m fue ~1.68 veces mayor que el S_m, presentándose una diferencia estadística significativa (p-valor: $8.46e^{-15}$) (Figura 4.12). Una segunda serie de pruebas, se hicieron con zooplancton, realizando diluciones en serie. Las pendientes presentaron una relación de ~1,14. Entre las pendientes no hubo diferencias significativas (p-valor: 0.3151). Sim embargo hay que señalar que con el S_m, a bajas biomasa la actividad empieza a ser cercana a cero, mientras que con el resazurin la señal permanece alta (Figura 4.12).

Para conocer el papel que desempeñan los sustratos en la reacción del ETS, se repitió la prueba anterior pero esta vez con diferentes concentraciones y combinaciones de NADH y NADPH. Inicialmente se esperaba que el comportamiento del ETS fuera

similar, cualquiera que fuese la combinación de sustratos con diaforasa o zooplancton, pero no fue el caso. El NADH, en presencia de la diaforasa, presentó mayores tasas de reducción que el NADPH para los dos métodos (Figura 4.13). En los misidáceos con cualquiera de los dos métodos, el NADPH prácticamente no presenta ninguna actividad (Figura 4.13). Sumado a esto, cuando los dos sustratos están presentes la señal no es aditiva. Por el contrario, a medida que la concentración de NADPH aumenta la inhibición es mayor (Figura 4.13), confirmando que este piridin nucleótido generó algún tipo de inhibición en ambos métodos, especialmente en los homogeneizados de misidáceos. El F_m siempre mostró actividades ETS más altas.

No se pudo calcular las K_m bi-sustrato para la diaforasa, ya que las diferentes concentraciones de NADH y NADPH no fueron lo suficientemente bajas como para determinar la linealidad de la reacción. No obstante, se puede observar la clara influencia del NADPH en la reacción ya que al estar este presente, la inhibición era proporcional a su concentración (Figura 4.10 y 4.11).

1.3.4. Capítulo 5: Actividad ETS en diferentes organismos, así como en muestras de plancton: comparación de los métodos e influencia de los sustratos

Se comparó las posibles diferencias entre el método espectrofotométrico (S_m) y el espectrofluorométrico (F_m) y al mismo tiempo el posible efecto de la presencia de los sustrato en la reacción en dos escenario (1, NADH; 2, $NADH^+$ NADPH + succinato [SB]).

Para ello se emplearon seis organismos diferentes; misidáceos adultos machos, *Artemia salina*, abalón (*Haliotis tuberculata*), juvenil de mejillón (*Mytilus* sp.), alevines de caballitos de mar (*Hippocampus guttulatus)*, y alevines de dorada (*Sparus aurata*). Ambos métodos muestran el mismo patrón entre los organismos. El método F_m da siempre los valores más altos, y aunque existen diferencias significativas (0.05 >p) entre ambas señales (excepto en el mejillón, Figura 5.6), el S_m presenta el mismo comportamiento (Figuras 5.1 a 5.5; Tabla 5.2). En ambos métodos, existe una ligera tendencia del ϕ a ser mayor con el NADH, la excepción es en *H. guttulatus*, donde las máximas actividades están presentes con la SB.

Para probar esta nueva metodología en muestras oceanográficas, utilizamos muestras obtenidas en la Bahía de

Taliarte (este de Gran Canaria) desde el buque oceanográfico Poseidón. Se realizó un perfil vertical entre 5 y 600m. Con el fin de detectar la sensibilidad de los métodos y las actividades obtenidas con las diferentes condiciones de adición de sustratos a bajas concentraciones de biomasas, se filtraron 5 l, lo que supone un volumen menor del usual para mediciones de la actividad ETS en estas condiciones oceánicas (Packard et al., 1971; Hobbie et al., 1972; Arístegui et al., 2005).

Se tomaron ocho muestras en los primeros 100 metros de la columna de agua. Con el S_m todas las señales fueron ligeramente superiores con NADH, aunque ambos seguían el mismo comportamiento (Figura 5.7). La actividad ETS específica con SB va de $2.17x10^{-4}$ a $4.08\ x10^{-4}$ M O_2 h^{-1} mg prot.$^{-1}$ l^{-1} y con NADH entre $2.57x10^{-4}$ a $5.36\ x10^{-4}$ M O_2 h^{-1} mg prot.$^{-1}$ l^{-1}. Se presentan dos picos de actividad a 100 y 300 m. En la F_m los valores con NADH oscilan entre $4.24x10^{-4}$ y $4.48x10^{-3}$ M O_2 h^{-1} mg prot.$^{-1}$ l^{-1} y con SB entre $8.67\ x10^{-4}$ y $4.37\ x10^{-3}$ M O_2 h^{-1} mg prot.$^{-1}$ l^{-1}; ambos con un pico a 100 m (Figura 5.8; Tabla 5.2). Cuando se compararon los dos métodos, los resultados obtenidos por el método F_m fueron siempre mayores que los obtenidos con el método S_m, siendo en su mayoría de un orden de magnitud mayor (Tabla 5.2), debido a la mayor sensibilidad del F_m ($p < 0.05$).

La relación NADH:SB, podría suponer una aproximación al grado en el que los sustratos contribuyen a la donación de electrones en la actividad ETS. Cuando esta relación es cercana a uno se puede inferir que el NADPH y el succinato no contribuyen a

la reacción, esto es especialmente evidente, para ambos métodos, cuando usamos muestras de animales (*A. salina, S. aurata*, *Leptomysis* sp. y *Mytilus* sp.*)* y en general para el perfil de profundidad con el S_m. Cuando los valores son menores que uno, indicaría que el NADPH y/o el succinato contribuyen en cierto grado a la reacción, esto se evidencia en F_m, con *H. guttulatus* y las muestras más superficiales y más profundas del perfil (500 y 600 m). En el caso contrario, valores superiores a uno indican que el NADH por sí mismo es más eficaz para obtener la respiración potencial (Figura 5.9).

Chapter 2

Zooplankton metabolic activity (respiration and ETS) from the Southern Indian and Atlantic Ocean

F. Maldonado , T.T. Packard, M. Gómez

Plankton Ecophysiology Group, Instituto de Oceanografía y Cambio Global, Universidad de Las Palmas de Gran Canaria, Canary Islands, Spain

Abstract

Three ocean transects were sampled for ETS activity and respiration, two from the Indian Ocean (Cape Town-Perth and Perth-Sidney) and one from the Atlantic Ocean (Cartagena de Indias-Cartagena). Zooplankton samples were captured from the upper 150 m with a WP2 100µm mesh net. Biomass, respiration (R), and potential respiration (Φ) were measured on three size fractions. In addition, starvation experiments with R and Φ were conducted. In the southern Indian Ocean they were 1.41 ±0.65 and 1.77 ±0.64 µmol O_2 h^{-1}·mg $prot.^{-1}$, in the Great Australian Bight the

protein-specific R and Φ were 1.23 ±0.60 and 1.97 ±0.56 μmol O_2 h^{-1}·mg prot.$^{-1}$, and during the Atlantic crossing they were 0.94 ±0.69 and 1.54 ±1.02 μmol O_2 ·h^{-1} ·mg prot.$^{-1}$. Challenging Kleiber's law, we found that the Kleiber exponent, *b*, for the southern Indian Ocean zooplankton was 1.06, for the Great Australian Bight, was 1.23, and for the Atlantic crossing, 0.94. These values are supported by the isometric theory of Glazier (2005), suggesting that the organisms were in good health. The starvation results showed no significant difference ($p > 0.05$) in the R/Φ or log ϕ/log biomass ratios between recently caught organisms (0h) and the starved ones (24h). In contrast with the difference ($p < 0.05$) between the high log R/log biomass ratio in the 24h organisms (1.40), possibly generated by external stimuli and the 0h measurements (0.89). This results proves how variable is the respiration and how difficult it is to obtain acceptable measures opposite case with the stability and relative easy measurement of the ETS.

2.1 Introduction

Key to understanding ocean ecosystems is learning how environment affects zooplankton respiration. One approach towards this end is through the metabolic theory of ecology (Allan and Gillooly, 2007; Brown *et al.*, 2004) and Kleiber's law (Kleiber, 1932, 1961; Glazier, 2005, 2006) that argue the significance of biomass in respiration and growth. Another is through the

enzymology that controls the chemistry of respiration. Here we explore the latter approach.

Respiration is what separates life from all else. It is the prevalent metabolic process in all marine organisms, which, along with primary productivity, is paramount in balancing anabolism and catabolism in the ocean. Furthermore, determination of zooplankton respiration provides secondary production indicator. Accordingly, it is essential to understand and predict respiration in all the different regions and depths of the ocean. Here, we focus of the respiration in the zooplankton, but unfortunately, existing techniques for the direct determination of zooplankton respiration is difficult to measure. For one reason, zooplankton have to be captured by nets and in doing so they are injured and crowded in extreme conditions, respiration measurements also involves the incubation in a controlled environment, and the determination of a time-dependent change in oxygen concentration (Owens and King, 1975). As a result, physiological measurements reflect these extreme conditions and not real, *in situ* conditions. Even after appropriate dilution and acclimatization the measurements are likely to be distorted. That is why the ETS method was developed (Packard, 1969; Packard *et al*., 1971) and applied to zooplankton (Packard *et al*., 1974). ETS activity measures potential respiration. It is a reaction rate, but a biological reaction rate and as such depends on the physiological state and nutritional state of the zooplankton before they were rendered into a cell-free emulsion. If the zooplankton were nutrient-limited or starved the *in vivo* enzyme activity in their living cells may be low. If they were nutrient-

sufficient or well-fed the *in vivo* enzyme activity may be high. However, measuring *in vivo* enzyme activities is extremely difficult. Normally, enzymes are extracted from cells and their activities measured *in vitro* by supplying unlimited amounts of all reactants (substrates). This *in vitro* activity is the V_{max} (sensu Michaelis-Menten). Relating this *in vitro* respiration capacity to the true *in vivo* rate (respiration) is a challenge that is usually met by seeking a statistical relationship between the two processes The *in vivo* flux cannot be measured with current technology, but it can be calculated linearly from *in vivo* respirations (Kenner and Ahmed, 1976; Gómez et al, 1996; Packard and Christensen, 2004) or from theoretical considerations of Michaelis-Menten kinetics (Aguiar-González et al., 2012). We measured *in vivo* respiration and potential respiration in an effort to calibrate the ETS method for zooplankton and to further our understanding of the metabolic state of zooplankton.

Here we examine some of these relations between *in vivo* respiration, potential respiration, biomass and the effects of 24h starvation in zooplankton from different ocean regions.

2.2. Methods

From the oceanographic cruise Malapina 2010, we took zooplankton samples and conducted physiological experiments

during three stages. Two transects were from the Indian Ocean, Cape Town South Africa) to Perth (west coast of Australia) and from Perth to Sydney (east coast of Australia) and the last one in the Atlantic Ocean from Cartagena de Indias (Colombia) to Cartagena (Spain) (Figure 2.1).

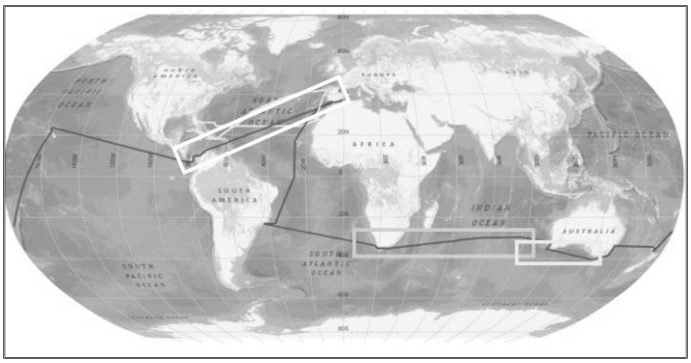


Figure 2.1. Transects from which the zooplankton samples were acquired. Vertical hauls using a WP2 net down to 200m were made. Indian Ocean samples were taken between February 12 to March 13, and 17 to 30 March 2011. Atlantic samples were taken from June 20 to July 14, 2011.

Samples were collected with a WP2 100 µm mesh size, with a physiological purpose collector as described by UNESCO (1968). Vertical hauls were made between 200 or 150 m to surface. Samples were immediately fractionated into three size classes (100-500 µm, 500-1000 µm and >1000 µm). Each one of these was placed individually in half-litter plastic vessels with filtered seawater (2 µm) and maintained at the average temperature of the water column sampled by the net haul. Then, with extreme care gelatinous organisms were removed. For the respiration measurements, the remaining zooplankton, in good health, were removed by a spoon, one-by-one for the bigger sizes, and by

siphoning, for the smaller ones. These healthy zooplankters were put in bottles with filtered sea water. Respiration was measured from the oxygen consumption in these bottles using O_2 electrodes (Strathkelvin Instrument Oxygen Interface 928) over a time period of an hour in the dark. Each bottle had an individual stirring mechanism, isolated from the organisms to assure no damage and to ensure homogenous distribution of oxygen. The temperature of the experiments was controlled all the time by a thermostatic waterbath, maintaining the temperature at which the animals had been adapted, assuring less stress and more realistic data. At the end of the measurement the zooplankton were immediately frozen in liquid nitrogen and stored at -80 °C to preserve enzymatic activity (Gómez *et al*., 1996). While the measurement was being performed, the other size samples are kept aerated, light protected and at seawater temperature.

When time and weather conditions permitted and there was sufficient quality biomass, the samples were split in two parts in order to measure the effects of starvation. One part was used immediately for the respiration assays (0h), the other was kept in 2 l bottles with filtered sea water with continuous oxygenation and controlled temperature, for 24 hours. Storage and respiration were carried out as described above.

In the laboratory, the determination of the potential respiration (Φ) was done according to Owens and King (1975). Protein was determined as a measure of biomass (B) by the method of Lowry *et al*. (1951) modified by Rutter (1967).

2.2.1. Statistical analysis

Data was analyzed using *R*-Development Core Team 2010 (R Foundation for Statistical Computing, Vienna, Austria). To confirm normality, ETS activity (Φ), specific Φ, respiration (R) and specific R, was treated statistically by the Shapiro-Wilk test (Shapiro and Wilk, 1965) and the homoscedasticity of the residuals was assessed graphically. The results showed that the data was not normally distributed. Consequently, the non-parametric Kruskal-Wallis test was used to test differences in the R, Φ, B, specific R and specific Φ data between the different oceanic zones and different size classes. The correlation between B, Φ and R was estimated with the Pearson test, at a confidence level of 95%. For the starvation data a non-parametric statistical hypothesis test (Mann–Whitney *U test)* for assessing whether one of two samples of independent observations tended to have larger values than the other, was used.

2.3. Results

In the southern Indian Ocean, from Cape Town-Perth there were a total of seven stations at which we were able to take samples. Crossing the Great Australian Bight (Perth to Sidney) there were four stations and on the Atlantic crossing from Cartagena-Cartagena, there were total of twelve stations sampled. The

differences in the number of samples taken on each transect was due to the transect length, the priorities on board the ship, and the weather conditions. For example during the crossing of the southern Indian Ocean, the weather was often too terrible for samples to be taken. Also, in the >1000 µm size class, only the crustacean zooplankton were taken into account, the salps, jellyfishes, chaetognaths and fish larvae were removed from the samples. This impacted the number of measurements (Table 2.1). A total of 18 measurement were made on the first transect, 14 on the second one and 36 on the Atlantic section.

2.3.1. Specific activities

Despite the differences between the number of stations sampled (Table 2.2) the biomass (B) average on the Cape Town Perth transect was 0.4 ±0.19 mg prot., on the Perth-Sydney transect it was 0.79 ±0.45 mg prot., and on the Cartagena-Cartagena,, it was 0.78 ±0.60 mg prot.. In general, the main contributing size-class was the 100-500µm group, followed by the 500-1000 µm group (Table 2.1). The small size class accounted for 44.7% of B with an average of 0.85 ±0.65 mg prot., followed by medium size with 34.2% with an average of 0.65 ±0.41 mg prot., and the least abundant was the largest size with 21.1% and an average of 0.40 ±0.20 mg prot. (Table 2.2).

R and Φ specific activities vary according to the geographical location. The lowest specific activities, with both physiological measurements were in the Atlantic Ocean (0.94 ±0.69 and 1.54

±1.02 µmol $O_2 \cdot h^{-1} \cdot$mg prot.$^{-1}$ respectively). The specific ϕ was higher on the Perth-Sidney transect (1.97 ±0.56 µmol $O_2 \cdot h^{-1} \cdot$mg prot.$^{-1}$) followed by Cape Town-Perth section (1.77 ±0.64 µmol $O_2 \cdot h^{-1} \cdot$mg prot.$^{-1}$). The specific R was the higher on the Cape Town–Perth transect (1.77 ±0.64 µmol $O_2 \cdot h^{-1} \cdot$mg prot.$^{-1}$) followed by Perth-Sidney transect (1.41 ±0.65 µmol $O_2 \cdot h^{-1} \cdot$mg prot.$^{-1}$) (Figure 2.4; Table 2.1). We also observed that for both specific R and specific ϕ, there was an inverse relation with size (Table 2.1; Figure 2.2).

From the point of view of the size classes, the lowest activities for R and ϕ were present in the >1000µm (0.93 ±0.61 and 1.13 ±0.62 µmol $O_2 \cdot h^{-1} \cdot$mg prot.$^{-1}$ respectively), followed by the 500-1000 µm size (0.98 ±0.53 and 1.69 ±0.41 µmol $O_2 \cdot h^{-1} \cdot$mg prot.$^{-1}$ respectively) and the higher was present in the smallest size, 100-500µm (1.35 ±0.64 and 2.06 ±0.81 µmol $O_2 \cdot h^{-1} \cdot$mg prot.$^{-1}$ respectively).

2.3.2. R/Φ

The ratio of R/Φ is an index of the amount of respiratory capacity used. If R/Φ = 1 the zooplankton are using all their respiratory capacity to live and have no reserve. If R/Φ < 1 the zooplankton has a reserve with which they can respond to some biological stress. Ratios higher than 1 indicate either a flaw in the theory, the presence of unknown biochemistry operating, or errors in the measurements (underestimating ϕ or over estimating R). There are two ways to access R/Φ ratios, one from the direct division of the

absolute values (Table 2.1 and 2.2) and from the regression line from the plots (Figure 2.2). Sometimes these two calculations can differ; this is due the variability of the data that is why we considered using the R/Φ from the plots, since this takes into consideration the dispersion of the data. Both estimates of R/Φ are presented in Tables 2.1 and 2.2.

From the geographical point of view, the R/Φ ranged from 0.37 to 0.84 (Table 2.1) and with regard to the different size classes, the ratio ranged from 0.41 to 0.86 (Table 2.2). The higher ratios of R/Φ were found in the Indian Ocean between Cape Town–Perth (0.84), the Perth–Sidney and the ones from the Atlantic crossing, share the same ratio (0.37) (Table 2.2; Figure 2.4). There is a conundrum on the 100-500 and 500-1000 µm size-fraction of the Perth Sidney section, since the R/Φ calculated from the regression plot were negative. This is due to a disparity in the data. The physiological measurements are similar but the biomass are not (Table 2.1). However, considering all the data and their dispersion, there is no significant difference between the ratios from the different oceanic zones ($p > 0.05$).

Also, from the size point of view, the R/Φ ratio in the >1000 µm fraction was the highest (0.86) followed by the 100-500 µm fraction (0.41) and then by the 500-1000 µm size-fraction (0.30) (Table 2.2; Figure 2.4). Here too, there was no significant differences ($p > 0.05$) between the ratios from the different size fractions.

Table 2.1. Mean biomass (B, in mg protein), R, and Φ on each transect. standard deviations are given in parenthesis. R/Φ ratio may signal the metabolic state of the zooplankters. It can be calculated two ways: (1) by direct division or from the slope of a regression (plot). Note that the difference is due to the correlation of the data given in the parenthesis (r^2).

	n	B (mg prot. sample^{-1})	R (µmol $O_2 \cdot h^{-1}$ mg prot.$^{-1}$)	Φ (µmol $O_2 \cdot h^{-1}$ mg prot.^{1})	R/Φ division	R/Φ slope (r^2)
Cape Town-Perth						
100-500 µm	9	0.40 (0.22)	1.61 (0.68)	1.89 (0.65)	0.85	0.89 (0.73)
500-1000 µm	4	0.36 (0.15)	1.32 (0.84)	1.98 (0.64)	0.66	1.03 (0.63)
>1000 µm	5	0.45 (0.28)	1.10 (0.38)	1.37 (0.56)	0.80	0.62 (0.85)
TOTAL	**18**	0.40 (0.19)	**1.41 (0.65)**	**1.77 (0.64)**	**0.80**	**0.84 (0.67)**
Perth-Sidney						
100-500 µm	5	0.68 (0.46)	1.23 (0.63)	2.15 (0.57)	0.57	-0.16 (0.02)
500-1000 µm	4	1.06 (0.70)	0.99 (0.35)	2.18 (0.36)	0.45	-0.71 (0.52)
>1000 µm	5	0.44 (0.28)	1.42 (0.92)	1.63 (0.78)	0.87	1.04 (0.78)
TOTAL	**14**	**0.79 (0.45)**	**1.23 (0.60)**	**1.97 (0.56)**	**0.62**	**0.37 (0.12)**
Cartagena Col-Cartagena						
100-500 µm	15	1.18 (0.73)	1.24 (0.68)	2.12 (1.02)	0.58	0.37 (0.31)
500-1000 µm	12	0.62 (0.24)	0.86 (0.45)	1.43 (0.92)	0.60	0.50 (0.43)
>1000 µm	9	0.34 (0.15)	0.56 (0.17)	0.72 (0.35)	0.78	0.59 (0.17)
TOTAL	**36**	**0.78 (0.60)**	**0.94 (0.58)**	**1.54 (1.02)**	**0.61**	**0.37 (0.43)**

Table 2.2. Mean B (in mg protein), R and Φ, grouped by size class. Standard deviations are given in parenthesis. Since the starvation data for 24h was sparse, they are pooled, regardless of size or area. The R/Φ ratio signifies the metabolic state of the organisms; there are two ways to calculate it. One is by direct division of the values (division); the other is by the slope of a regression (plot). Note that the difference is due to the correlation of the data in the parenthesis (r^2).

	n	B (mg prot. sample^{-1})	R (µmol O_2 h^{-1} mg $prot^{-1}$)	Φ (µmol O_2 h^{-1} mg $prot.^{-1}$)	R/Φ	
					division	slope (r^2)
Size Class						
100-500 µm	29	0.85 (0.65)	1.35 (0.64)	2.06 (0.81)	0.66	0.41 (0.27)
500-1000 µm	20	0.65 (0.41)	0.98 (0.53)	1.69 (0. 41)	0.58	0.30 (0.21)
>1000 µm	19	0.40 (0.20)	0.93 (0.61)	1.13 (0.65)	0.82	0.86 (0.83)
Treatment						
0 h	68	0.67 (0.52)	1.13 (0.61)	1.70 (0.65)	0.66	0.48 (0.40)
24 h	8	1.10 (0.53)	1.28 (0.45)	1.71 (0.58)	0.65	0.64 (0.67)

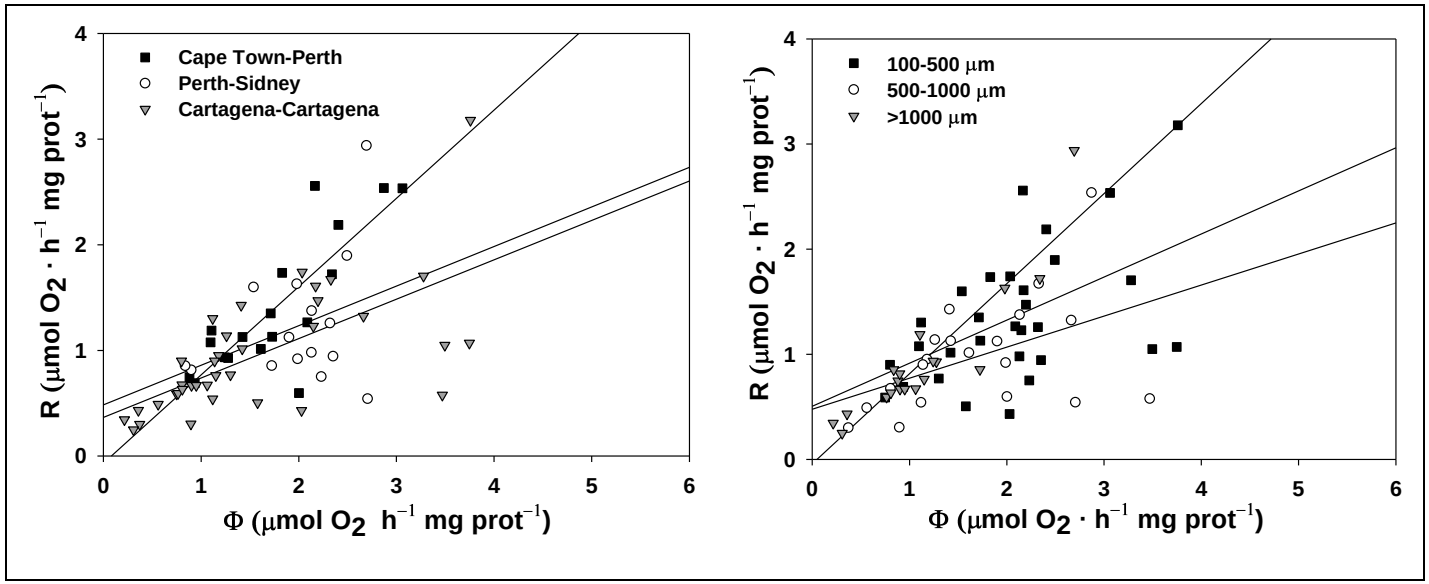


Figure 2.2. Relation between R and ϕ, by ocean region, where the highest rates were found on the Cape Town-Perth transect (R=0.84ϕ - 0.07, r^2 =0.67). The next highest rates were found on the Perth-Sidney transect (R=0.37ϕ + 0.5, r^2 =0.12), similar values are present in the Atlantic region (R = 0.37ϕ + 0.4, r^2 =0.43). Ranked according to size class, the higher rates were found in >1000 μm size-class (R=0.86ϕ - 0.04, r^2 =0.83), followed by those in the 100-500 μm size-class (R=0.41ϕ + 0.5, r^2 =0.27), and finally by those in the 500-1000 μm size-class (R=0.30ϕ + 0.5, r^2 =0.21). It is important to note that low R/ϕ in the Atlantic region was influenced by the large number of measurements in the small size-class. No significant difference (p >0.05) was found between the three ocean regions nor between the three size classes,

2.3.3. Kleiber's law

The relationships among log B and log R or log ϕ, was obtained by regression plots (Figure 2.3 and 2.4). These ratios were highest in the Atlantic (1.09 and 1.32 respectively), followed by ratios in the Perth-Sidney section (0.98 and 1.06, respectively). The lowest ratio occurred in the first transect of the Indian Ocean (0.71 and 0.85 respectively) (Figure 2.3). Despite the different slopes, especially the Atlantic transect, there was no statistical differences ($p > 0.05$) between them, this was probably due to the dispersion of the data.

The relationships by size class between log R and logΦ versus log B was higher in the >1000 µm size class (0.97 and 1.12 respectively) followed by the relationships in the 100-500 µm size class (0.79 and 1.09 respectively) and finally in the 500-1000 µm size class (0.92 and 0.80 respectively) (Figure 2.4).

If Kleiber's law is tested within size classes, the behavior is very similar. The logarithmic relations are higher in the >1000 µm size class as well with non-transformed R and ϕ (1.12 both equally). In the Log R/log B (Figure 2.3) the ratio is slightly higher in the mid size class (0.92) with respect to the small one (0.79),on the other hand, for the Log ϕ/log biomass ratio, the 100-500 µm size class is higher respect to the ratio from the 500-1000 µm size-class (1.09

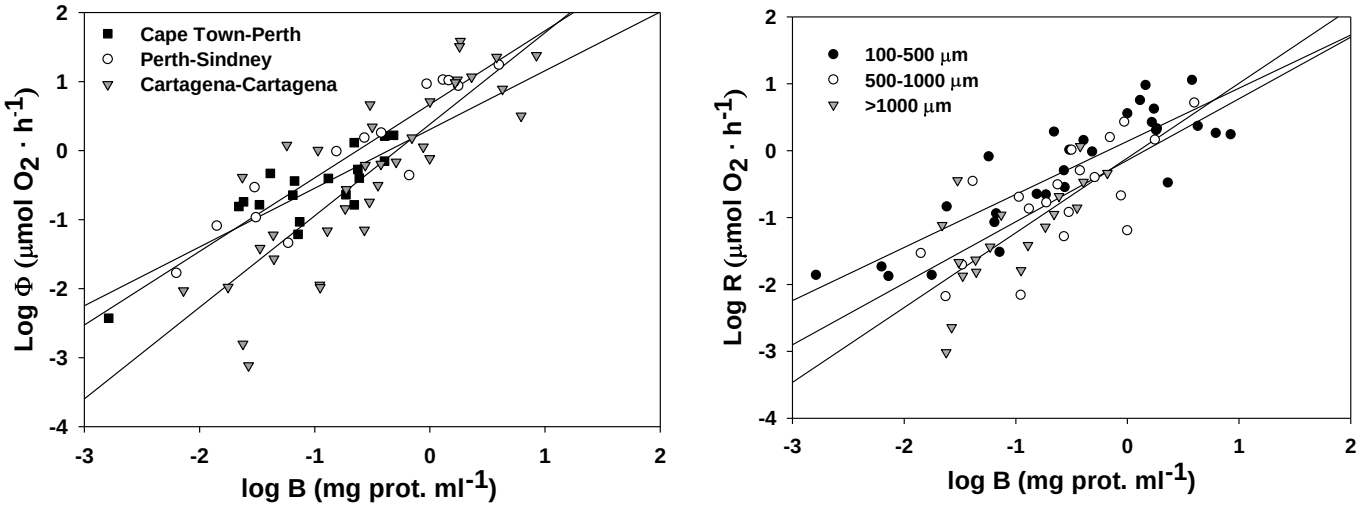


Figure 2.3. Relationships, between either log Φ or log R against log B, of the different sections were determined to establish the scaling ratios (slope) or *b* (the correlation inside the parenthesis). The higher ratio for the LogR/Log B was 1.33 (r^2 =0.71) in the Atlantic section, followed by 0.98 (r^2 =0.83) during Perth-Sidney and 0.71 (r^2 =0.55) in the Cape Town–Perth transect. For the Logϕ/Log B is, 1.33 (r^2 =0.71) for Cartagena-Cartagena, followed by 1.07 (r^2 =0.87) inside the Great Australian Bight and finally 0.85 (r^2 =0.71) in the Indian Ocean. In general, R is more dispersed due to the rapid changes that can occur in these measurements due to manipulation or other environmental factors. However, for the different slopes, no statistical differences (p >0.05), neither with Log ϕ and Log R, could be found.

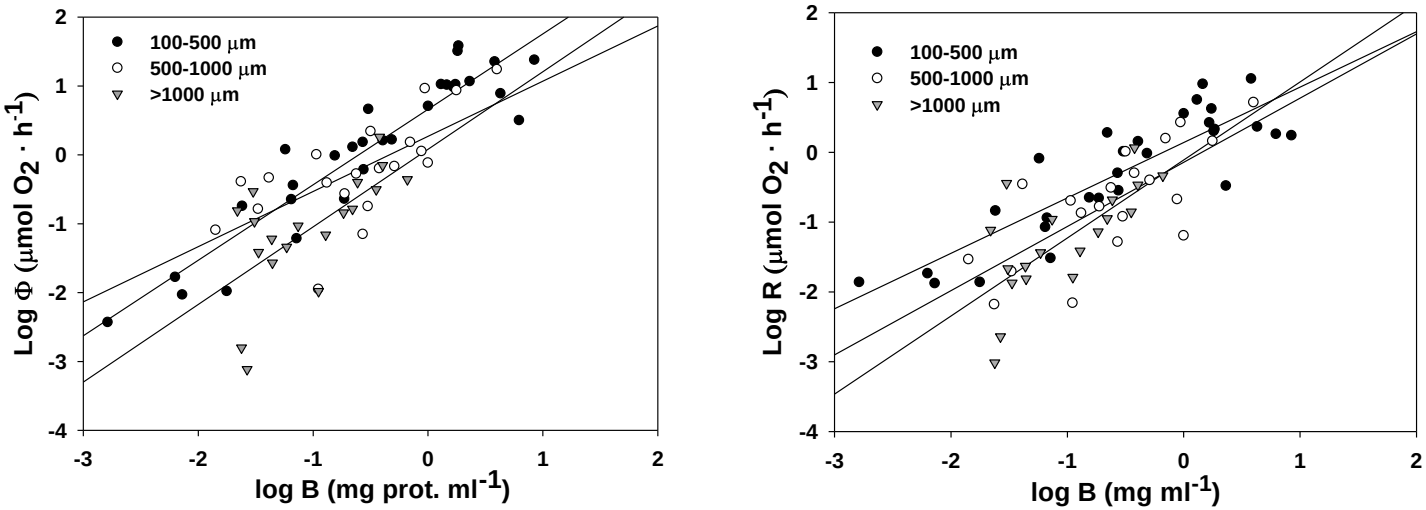


Figure 2.4. Relationships between either log Φ or log R versus log B, in the different size classes were made to establish the scaling ratios (slopes) or *b* (the correlation inside the parenthesis). The higher ratio for the LogR/Log B was 1.12 (r^2 =0.48) for >1000 μm, followed by 0.92 (r^2 =0.54) for 500-1000 μm and 0.78 (r^2 =0.75) in 100-500 μm size. For the Logϕ/Log B it was 1.12 (r^2 =0.42) for the >1000 μm size, followed by 1.09 (r^2 =0.87) in the 100-500 μm size and finally 0.80 (r^2 =0.46) in 500-1000 μm size class. The dispersion in both measurements was similar due to the variability of the zones. However, for the different plots no statistical differences (p >0.05) were found.

and 0.80 respectively) (Figure 2.3). The difficulty of making good respiration measurements with the oxygen electrode in the field, yields to less precise measurements, these are reflected in their low correlation (r^2) with respect to ϕ. Even so there is no statistical difference ($p > 0.05$) between all the ratios either in Log R or Log ϕ (Figure 2.3, 2.4).

2.3.4. Food limitation (starvation experiments)

It was expected that R would fall and ϕ would maintain at its original level during these experiments. The results were otherwise, R increased after 24h. The experiments were hampered by the paucity of biomass in the open ocean samples. This problem might have been obviated with a larger net. More seawater could have then been sampled. Unfortunately, that was not the case. In addition, because of the low biomass, there were many occasions when we could only manage to measure respiration in the initial sample. That is why there are nearly eight times more initial measurements (0h) than measurements after 24 hours (24h) (Table 2.2). Furthermore, the problem was compounded by the high mortality rate of the zooplankters during the first 24h after capture. For these reasons the starvation experiments were conducted on pooled samples rather than on individual size classes.

In Table 2.2, the average values of both R and ϕ were similar ($p > 0.05$), at 24h was 1.28 ±0.45 and 1.71 ±0.58 μmol O_2 h^{-1} mg prot.$^{-}$

^{1}, respectively, while at 0h they were 1.13 ±0.61 and 1.70 ±0.65 µmol O_2 h^{-1} mg prot.$^{-1}$, respectively.

When transforming data to establish the Kleiber factor, its variability is again evident, especially at 24h in in the log R/log B, were the ratio was higher ($p < 0.05$) than those in the recently caught (0h) organisms. The logϕ/log B data, were not significantly different ($p > 0.05$) between 0h and 24h, demonstrating once again the stability of the ETS in time (Figure 2.5).

The R/Φ ratios were compared determine if the starvation had some influence on the physiological state. We found that the R/Φ rates were higher at 24h (0.64), as calculated from Figure 2.6, than they were at 0h (0.48). Even so, the slopes were significantly different ($p > 0.05$).

2.4. Discussion

2.4.1. Zooplankton physiology state

The relationship between R, and M, as expressed by the exponent b in the allometric equation $R = aM^b$, has been accepted by many authors to be 0.75 (Hemmingsen, 1960; Kleiber, 1932, 1961; Savage *et al*., 2004). However, Glazier (2005, 2006), cautioned that this value is only a statistical mean and should not

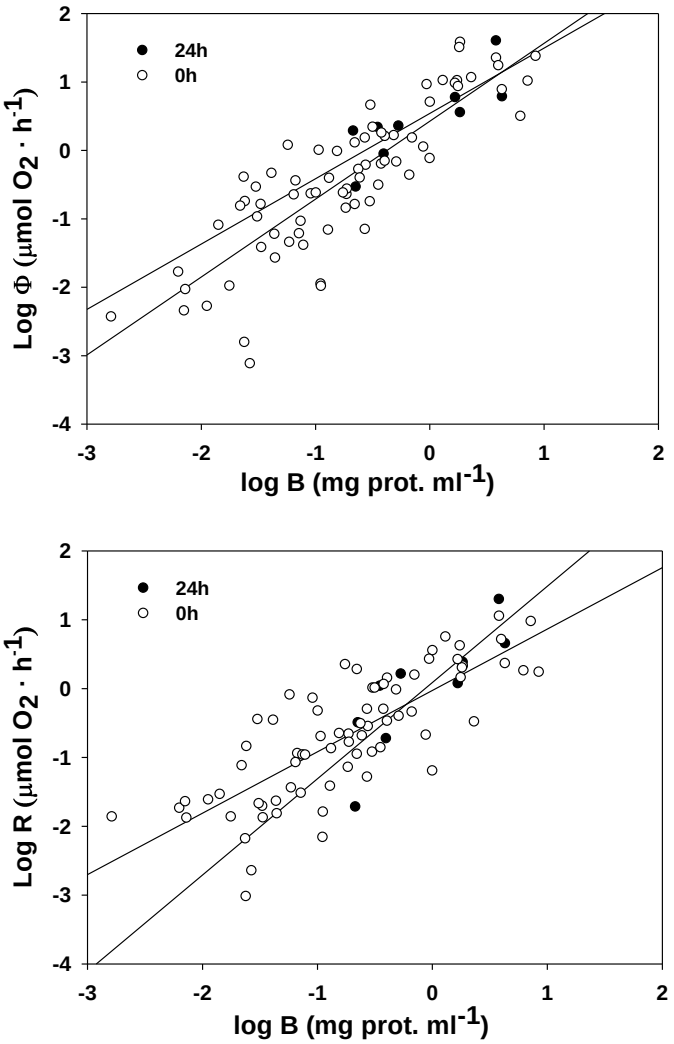


Figure 2.5. Relationships between either log Φ or log R versus log B in recently caught organisms (0h) and in starved organisms (24h) were calculated in order to establish the scaling ratios (slope) or *b* (the correlation inside the parenthesis). The ratio for the Log R/Log B at 0h was 0.89 (r^2 =0.62) whereas the ratio for 24h was 1.40 (r^2 =0.69). These ratios were statistical different ($p < 0.05$). The ratio, Log ϕ/Log B was 0.95 (r^2 =0.68) and 1.13, (r^2 =0.72) respectively, for the two different nutrient conditions, no statistical differences ($p > 0.05$) between these ratios was found.

be considered invariant. He found that for many pelagic organisms, b was around 1 and that for pelagic crustacean it was approximately 0.88. Our values fit in this range, for the relation with R, b ranged from 0.71 to 1.09 in the different regions and for ϕ, between 0.85 and 1.33. From the point of view of the size classes, it ranged from 0.79 to 1.12 and 0.80 to 1.12, respectively (Figure 2.3, 2.4). This isometric metabolic scaling is characterized by high sustained production costs, resulting in a mass-specific metabolic rate that does not decrease with increasing body mass (Glazier 2006). This scaling, according to Glazier (2006), could be attributed to energy expensive activities like regulating buoyancy (in general planktonic animals must continuously swim or pump ions across membranes) and surviving (high mortality and predation). This pressure to avoid mortality could select for rapid growth rates and large reproductive outputs. Also, these planktonic organisms have short lives forcing them to grow rapidly and reproduce rapidly. This leads to high energy expenditures. Another explanation is that high production requires a high food intake which, in turn, requires locomotor activity.

Our results are not precise enough to establish which power law of biomass-metabolism applies to the zooplankton captured on our different transects. Nevertheless, they do establish the range of the exponent b with R (0.71-1.09) and with ϕ (0.85-1.33) for zooplankton metabolism in these waters and they demonstrate the difficulty in making physiological measurements on oceanographic expeditions. As Glazier (2005, 2006) points out, scaling in pelagic animals shows that it is not simply the result of physical or

geometric constraints, but can be profoundly influenced by ecological circumstances.

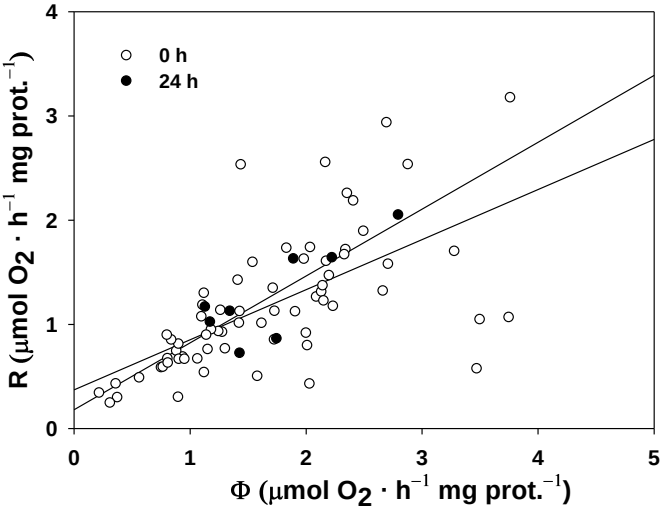


Figure 2.6. Relationship between R and Φ from the pooled data for the two treatments. The 0h give the lower ratio (R= 0.48 Φ +0.4; r^2 =0.40) compared to the 24h ratio (R= 0.64 Φ +0.2; r^2 =0.67). Even so, there was no significant differences (p >0,05) between the two treatments.

Variations of b are well documented, but not understood. It is likely that evolutionary, ecological, physiological, and biochemical factors need to be considered before this variability can be explained (Ikeda, 1970); Martínez *et al*. (2010); Herrera *et al*. (2011). These authors have pointed out that elevated departures from b = 0.75 may indicate healthy and well-fed organisms while values of b <0.75 may indicate starved or otherwise unhealthy organisms.

The foregoing is relative concordant with the R/Φ ratio as an index of physiological state as stated by Christensen *et al*. (1980). It is believed that when this ratio is low (< 0.5) organisms are nutrient-limited and when it is between 0.5 and 1, organisms are well fed (Christensen *et al*., 1980; Packard *et al*., 1996). Hernández-León and Gómez (1996) found that for zooplankton, temperature, diet, physiological state, and age could determine the variability of the ratio. Our results show no differences ($p > 0.05$) between the geographical regions. The dispersion present in the data. This could lead to a misinterpretation since in the Perth-Sidney and Cartagena-Cartagena transects the R/Φ ratios are low (0.37). In the first zone the values are vitiated by the variation of biomass in the two smaller sizes, while in the second region the predominant size (100-500 μm) the ϕ has high levels in relation R (Table 2.1). Still, if the R/ϕ is taken from the calculated division of these measurements it seems in general that the sampled zooplankton community in these regions is in good physiological condition (Table 2.1).

Again for the sake of argument, from the point of view of the size classes, either detailed by geographic area (Table 2.1) or grouped by size (Table 2.2) our metabolic activity measurements fit the pattern in which the smallest zooplankton has higher metabolic rates in proportion to their body mass (Kleiber 1932; Gillooly *et al*., 2001; Glazier, 2006; Kolokotrones *et al*., 2010). This type of relationship is also found in other poikilothermal animals (Weymouth *et al*., 1944) as well as in bacteria and big mammals (Zeuthen, 1953).

But in general, independently if the data is grouped by zone or size the R/Φ ratio was equal or greater than 0.5, concluding that this zooplankton in our samples were in good physiological state, apparently in oligothophic environments the zooplankton manage to maintain their good health.

2.4.2. Food limitation (starvation)

As shown, there is no difference ($p > 0.05$) for the measurements Φ. This is expected since the system that controls the ETS is more conservative (Packard et al., 1996). On the other hand with the R, there are differences between slopes ($p < 0.05$) this can be expected since the respiration measurement is difficult, besides it can be affected (Hernández-León and Ikeda, 2004) and can immediately respond to external stimuli, in our case bottle effect, increased densities and handling of the organisms.

Theory argues that the R/Φ ratio should decrease with time, as starvation reduces the levels of Krebs cycle intermediates and other essential donors to the ETS (NADH and NADPH). This in turn will reduce respiration. However, in our experiments this does not occur (Figure 2.6). Perhaps R decays after 24h, but the new conditions as mentioned, previously trigger respiration, masking what the true basal respiration should be under these conditions because the organisms need more time to feel stress. Or, perhaps they are adapted in some way to maintain normal metabolic activity in spite of a scarce food supply so no differences between R/ϕ (p

>0.05) can be detected in this elapsed time. Herrera *et al*. (2011) found the same phenomena in cultured mysids where the ratios are equal or higher than 0.75, indicating well-fed or good physiological condition of the organism independently of the time of measurement.

2.5. Summary

1. In the Great Australian Bight the protein-specific R and Φ were 1.23 ±0.60 and 1.97 ±0.56 μmol O_2 h^{-1} mg $prot.^{-1}$, in the southern Indian Ocean they were 1.41 ±0.65 and 1.77 ±0.64 μmol O_2 h^{-1} ·mg $prot.^{-1}$, and during the Atlantic crossing they were 0.94 ±0.69 and 1.54 ±1.02 μmol O_2 h^{-1} mg $prot.^{-1}$. No differences were found between any of the regions ($p > 0.05$)

2. Values of metabolic scaling and R/Φ points to a zooplankton community in good conditions, despite the variability in the data.

3. The scaling of the samples varies between 0.71 to 1.09 for the logR /log biomass and 0.85 to 1.32for logϕ /log biomass indicating more an isometric scaling.

3. There is no effect of the starvation on the metabolic behavior of the zooplankton collected from the Indian Ocean (Cape Town-Perth and Perth-Sidney) and Atlantic Ocean (Cartagena (Colombia)-Cartagena), pointing to a well adapted condition to the environment or insufficient time to see any changes in the decrease of respiration.

Acknowledgments

We are thankful to have been part of the MALASPINA 2010 (CSD2008-00077) program. Many thanks to Alicia Herrera Ulibarri for her help with the statistical software and data analysis, to Natalia Osma Prado and Igor Fernández Urruzola for taking samples of the Malaspina cruise. We acknowledge the Agencia Canaria de Investigación, Innovación y Sociedad de la Investigación as well as EXMAR SE-539 10/17 (Proyecto Estructurante en Ciencias Marinas) and the ULPGC, Biology Department and the Basic Science Library “Carlos Bas” for their economic support.

Chapter 3

Understanding tetrazolium reduction and the importance of substrates in measuring respiratory electron transport activity

F. Maldonado , T.T. Packard, M. Gómez

Plankton Ecophysiology Group, Instituto de Oceanografía y Cambio Global, Universidad de Las Palmas de Gran Canaria, Canary Islands, Spain

Abstract

Most of the oxygen consumption in plankton is controlled by an enzymatic complex called the electron transport system (ETS) or the electron transport chain. To detect and measure this ETS in a biologically diverse plankton community it is common practice to add, in addition to an artificial electron acceptor, the various

substrates that donate reducing equivalents to the ETS. Specifically, pyridine nucleotides (nicotine adenine dinucleotide (NADH) and nicotine adenine dinucleotide phosphate (NADPH)) and succinate are routinely used. The addition of these substrates to the ETS stimulates its activity to capacity and hence serves as a measure of potential respiration (Φ). This proxy is then used in ecological and oceanographic studies as an index for respiration. Unfortunately in an attempt to align this proxy more closely with *in vivo* physiological respiration and to simplify the ETS assay the required substrates were omitted from the analysis. The consequences of this shortcut are demonstrated and explained here. In effect, because some basic rules of biochemistry were broken, the simplified assay yields misleading results that are, in fact, the equivalent of the control (the blank) in a normal ETS assay. Here we explain some of the chemistry of this background reaction, most of which can be found in textbooks. We demonstrate that the use of the artificial electron acceptor, the tetrazolium salt, INT, is not specific for cell respiratory ETS reactions, but is sensitive to many, substances present in all cells. We show that, among these common cellular substances, ascorbic acid, cysteine, and glutathione readily reduce INT non enzymatically to its formazan. Such INT reduction is part of the normal blank reaction in the ETS assay and is far removed from the desired respiratory capacity of the cell.

3.1 Introduction

Respiration consists of the consumption of oxygen, the production of CO_2, and the biological consumption of organic carbon (Lane, 2010). Priestley and Lavoisier discovered this with clever experiments during the age of enlightenment (Bell, 2005; Lane, 2002, 2005; Uglow, 2002). It is a key physiological process in all marine organisms and as such it is fundamental, along with primary productivity, in assessing the metabolic balance in the ocean (del Giorgio and Duarte, 2002). It is essential in understanding and predicting the development and maintenance of oxygen minimum zones (Falkowski et al., 2011) as well as the so-called "dead zones" at the mouths of estuaries (Rabalais et al., 2010). It determines the oxygen concentration and the apparent oxygen utilization (AOU). It strongly influences the level of dissolved organic carbon (DOC) and particulate organic carbon (POC) the pH, and the rest of the carbonate system (CO_2, HCO_3, CO_3). Furthermore, it can be used to calculate new production and carbon flux in the ocean. But, for all its utility it is rarely measured in an oceanographic sense (in ocean sections and in ocean time series, sensu Sverdrup et al., 1942) on time and space-scales compatible with nutrient and hydrographic measurements. This is because the origin of most oceanic respiration is the microscopic-sized plankton and obtaining physiologically acceptable samples is so difficult and costly. Furthermore, because the respiration measurement itself, is almost impossible to take except in eutrophic surface waters, where the respiration signal is very

strong. On a volume basis, the respiration of larger organisms (i.e., nekton) has a negligible impact on the oceanic rates of O_2 consumption and CO_2 production. It is the plankton that impacts ocean chemistry and it is their small size, fragility, and low concentration that make ocean respiration so difficult to measure. In the deep-dark ocean, which is 98% of the ocean by volume, zooplankton respiration cannot be measured directly by existing technology because of two problems. The first is that zooplankton is so dispersed in ocean space that its oxygen consumption rate cannot be detected by existing technology. To solve this, nets are used to capture and concentrate the zooplankton. However, this introduces the second problem. During capture, the zooplankters are packed in extremely crowded conditions and are injured so that even after suitable dilution, measures of their respiration would not be representative of their *in situ* respiration. It is true that some individuals pass the ordeal of capture relatively unscathed and their respiration can be directly measured by changes in oxygen. However, it is very difficult to scale these measurements up to the integrated zooplankton community respiration per liter of sea water which is needed for oceanographic and ecological purposes.

To study respiratory physiology, marine copepods, euphausids, mysids, chaetognaths can be carefully collected in the field and selected if they appear healthy. Then, their respiration can be measured in a respirometer. These measurements are considered accurate assessments of the respiration of individual specimen of zooplankton living in a controlled environment (Ikeda and

Takahashi, 2012). However, these physiological measurements will only with great labor yield a measure of the temporal and spatial variability of zooplankton respiration in ocean. The data acquisition rate is simply too low for the task. For these reasons the ETS technique was developed (Packard, 1971; Packard et al., 1971 and Packard et al., 1974). It measures potential respiration by detecting the respiratory electron transfer activity in the dehydrogenases and cytochromes that biochemically control respiration. Collectively these respiratory control enzymes are known as the respiratory chain, the cytochrome chain, or the respiratory electron transport system (Fruton and Simmonds, 1958). We use the term ETS because the components of electron transport from the dehydrogenase enzymes of intermediary metabolism to the oxygen consuming cytochrome oxidase are not arrayed linearly (Gnaiger, 2009a; Packard, 1985a,b). These electrons flow non-linearly between the iron–sulfur lipo-protein complexes via quinone transporters in the membrane and via cytochrome-c outside the membrane. Furthermore, the number of complexes and entry points depends on the organism and its terminal electron acceptor. It is a system and not a chain (Gnaiger, 2009b).

Since its inception, the ETS assay has been used widely in aquatic ecosystems (e.g. Cammen et al., 1990; del Giorgio, 1992; Hernández-León et al., 2001; Ikeda and Takahashi, 2012; Packard and Codispoti, 2007; Packard et al., 1971) and in other research areas (e.g. Christensen et al., 1989; Fanslow et al., 2001; Ikeda, 1989; Peñuelas et al., 1988). It was designed as a proxy for the

respiration of all the organisms in a parcel of seawater (Packard et al., 1971) to replace more laborious methods for estimating seawater respiration (Barnes and Collias, 1958; Seiwell, 1937). It incorporated the basic precepts of enzymology in which an enzyme is measured at its maximal level of activity (V_{max}) and this required adding saturating levels of substrates (Fruton and Simmonds, 1958). This was done to provide the necessary sensitivity to Complex I and Complex II of the ETS as well as to ensure reproducibility and replication of the measurement. Without specifying the chemical conditions of an enzyme reaction the measurement cannot be reproduced. Not including the substrates the assay loses its specificity and may detect the activity of a host of other enzymes (Fruton and Simmonds, 1958). If it had been possible to know the *in situ* substrate concentration, as we know the *in situ* temperature, we would have designed (in 1970) an ETS enzyme assay for that *in situ in vivo* concentration. However measuring *in vivo* concentrations of substrates in 1970 was and still is notoriously difficult to achieve (Segel, 1993), so the assay has remained close to its original form (Gómez et al., 1996; Packard and Christensen, 2004). However the substrates for the ETS are expensive (Sigma-Aldrich Catalogue, 2011). Furthermore, the physiological and ecological interpretation of an enzymological V_{max} from the classic ETS assay hinders its use in oceanographic and limnological surveys. In an attempt to reduce the cost, to simplify the measurement, and to find a measurement more closely linked to physiological respiration, Båmstedt (2000) introduced a version of the measurement that did not require the expensive ETS

substrates (NADH, NADPH) and simplified the chemical steps in the assay. In addition, the author claimed that the activity measured by this so-called “zero-substrate assay” was theoretically closer to the *in vivo* ETS activity, which, according to Packard et al. (1996a) is thought to be the true *in vivo* respiration rate.

The true respiratory oxygen consumption rate, associated with energy production in eukaryotes, can be calculated from the electron flux through the ETS to O_2. This is associated with, but not stoichiometrically tied to the respiratory CO_2 production rate (CPR) (La Ferla and Azzaro, 2004). The CPR is the sum of the enzymatic reactions that catalyze the production of CO_2 (Packard et al., 1996b). These reactions produce reduced pyridine nucleotides that support the electron flux through the ETS, but other enzymes also contribute to this electron flux. However, the common electron collecting point and the control point of the electron flux is the entry to the ETS at NADH dehydrogenase. Cytochrome oxidase, at the terminal end of the ETS, controls the reaction with oxygen, but its activity is thought to be controlled by the electron flux through the NADH dehydrogenase at the other end of the ETS (Chance and Williams, 1955, 1956; Gnaiger, 2009a) That is why the NADH dehydrogenase was targeted for measurement by the original ETS activity measurement (Packard et al., 1971). Note that there are other cellular enzymes that consume O_2, oxygenases, hydroxylases, and oxidases (other than cytochrome oxidase), but these enzymes are not linked to energy production nor do they contribute much to the total oxygen consumption rate.

A characteristic of NADH dehydrogenase, the control point of the ETS, is the type of reaction it catalyses. It facilitates the transfer of 2 electrons from the nearly universal electron transfer molecule, NADH ($NADH + H^+ \rightarrow NAD^+ + 2e^- + 2H^+$), to another universal membrane bound electron transferring molecule, ubiquinone ($2e^- + 2H^{++} Q \rightarrow QH_2$). In this reaction the quinone form (Q) is reduced by the two electrons and protons to its quinol form, ubiquinol (QH_2). The important factor here, from our point of view, is that whether studied inside the cell (*in vivo*) or outside the cell (*in vitro*) this redox reaction can be detected and its activity measured by the reduction of 2-(ρ-iodophenyl)-3-(ρ-nitrophenyl)-5phenyl tetrazolium chloride salt, INT (Figure 3.1).

Confusion surrounding the terms, “enzyme activity”, “*in vitro*”, and “*in vivo*” has caused problems in the application of enzymology development of the zero-substrate enzyme assays. These assays are designed to measure *in vivo* enzyme activity, but actually measure activity levels far below the *in vivo* ones. Enzyme activity is a chemical reaction rate that requires substrates (reactants), and is defined by the disappearance rate of these substrates or by the appearance rate of products per unit time. As with all reaction rates, it begs additional qualification as to whether it is a molecular, molar, mass, or volume specific rate. In biochemistry, enzyme activity is reported simply as “units” or “katals” which are defined as the amount of the enzyme that catalyzes the conversion of 1 μmol of substrate per minute (U or unit) or the amount of enzyme that converts 1 mol of substrate per second (katal). In oceanography, it

is more helpful to specify the true chemical units for each case. For example since ETS activity concerns electrons, we can report our results in micro mol e^- per liter of seawater per minute (μmol e^- l^{-1} min^{-1}) or for zooplankton micro mol e^- per milligram protein per minute (μmol e^- mg $prot.^{-1}$ min^{-1}).

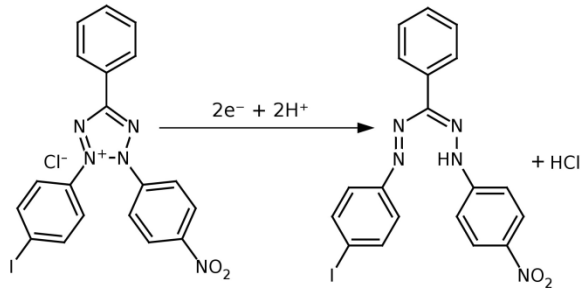


Figure 3.1. Reaction of tetrazolium reduction and formazan production in INT. Modified from Rogers (1953) and Packard (1971).

Confusion surrounding the terms, "enzyme activity", "*in vitro*", and "*in vivo*" has caused problems in the application of enzymology development of the zero-substrate enzyme assays. These assays are designed to measure *in vivo* enzyme activity, but actually measure activity levels far below the *in vivo* ones. Enzyme activity is a chemical reaction rate that requires substrates (reactants), and is defined by the disappearance rate of these substrates or by the appearance rate of products per unit time. As with all reaction rates, it begs additional qualification as to whether it is a molecular, molar, mass, or volume specific rate. In biochemistry, enzyme

activity is reported simply as “units” or “katals” which are defined as the amount of the enzyme that catalyzes the conversion of 1 μmol of substrate per minute (U or unit) or the amount of enzyme that converts 1 mol of substrate per second (katal). In oceanography, it is more helpful to specify the true chemical units for each case. For example since ETS activity concerns electrons, we can report our results in micro mol e^- per liter of seawater per minute (μmol e^- l^{-1} min^{-1}) or for zooplankton micro mol e^- per milligram protein per minute (μmol e^- mg $prot.^{-1}$ min^{-1}).

Furthermore, although enzyme activity is a chemical reaction rate, it is also a biological reaction rate and as such, depends on the physiological state of the organism. If the organism is nutrient limited or starved, the *in vivo* enzyme activity in its living cells may be low. If these cells are nutrient-sufficient or well-fed the *in vivo* enzyme activity may be high. However, measuring *in vivo* enzyme activities is extremely difficult (Cornish-Bowden, 2004; Fruton and Simmonds, 1958; Segel, 1993). Normally, enzymes are extracted from the cells and their activities measured *in vitro*, in a cell-free media designed to simulate the chemical environment of the cytoplasm during nutrient-saturation (Kornberg, 1989). Since it is nearly impossible to know or measure the concentrations of the enzyme's *in vivo* substrates at the same time that the enzyme's activity is being measured, these substrates are added to the enzyme extract (*in vitro*) at saturating levels at a concentration well above their K_m (sensu Michaelis–Menten). Then when the enzyme's activity is measured, the measurement will be the

maximal velocity (rate) of the reaction, its V_{max} (sensu Michaelis–Menten). This, in turn, thought to be the potential physiological rate which is controlled or partially controlled by the enzyme in question (Packard et al., 1996b). With this V_{max} in hand it then becomes a challenge to interpret the results and relate it to the true *in vivo* physiological rate. In the case of respiration the simplest approach is to find the statistical relationship between the *in vivo* respiration and the *in vitro* potential respiration. This was the approach taken by Christensen et al., 1980 and Packard and Williams (1981) for the Gulf of Maine phytoplankton communities and much more extensively, for the euphotic zone-microplankton (Figure 3.2) in many parts of the ocean by Arístegui and Montero (1995). A more accurate and satisfying way of relating the potential to the *in vivo* rate may be in the approach taken by Aguiar-González et al. (2012) in which the *in vivo* rate is calculated from the *in vitro* rate via enzyme kinetics. However, although the feasibility of this kinetic approach has been demonstrated in culture, it has yet to be applied to an ocean ecosystem.

It was to avoid this challenge in interpreting an *in vitro* based enzyme assay that the first zero-substrate assay was developed (Båmstedt, 2000). The author thought that by not adding substrates to the cell-free homogenates (extracted enzyme preparations) they would preserve the *in vivo* concentrations of the natural ETS substrates and thus the measured ETS activity would be the true *in vivo* rate. Unfortunately during the sample preparation, when zooplankton is homogenized, the amount of biological material may

vary by a factor of 10 and the final reaction volume may vary by a factor of 3. Under these conditions it is impossible to know the *in vivo* concentration of the substrates, consequently, the assay could not represent the *in vivo* ETS activity.

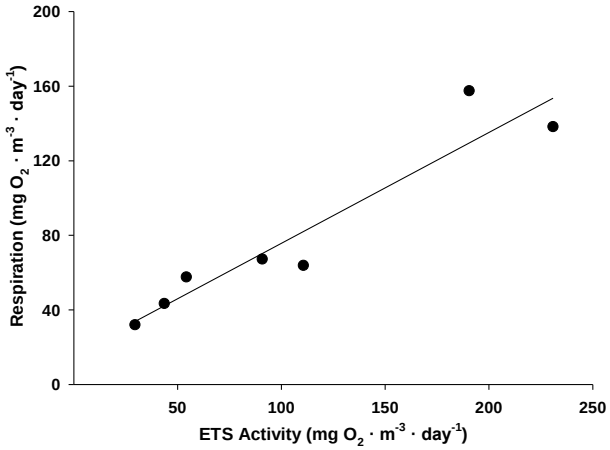


Figure 3.2. Respiration–ETS activity relationship from the data of Arístegui and Montero (1995). Each data point represents the average data for each of the 7 different ocean expeditions. The regression equation is: R=0.5949(ETS) + 16.223 (r^2 =0.8967). The R/ETS ratio is 0.6, close to a prediction from enzyme kinetics of 0.5 if ETS is potential respiration (V_{max}).

In addition to the substrate problem, confusion over the specificity of the tetrazolium salt used in the analysis, has caused problems. INT is an artificial electron acceptor (as contrasted to a natural physiological one such as O_2) with a half-wave reduction potential of −0.09 V that reacts with complexes 1 and 3 in the ETS

independent of the presence of O_2 (Smith and McFeters, 1997). However, it can also detect the activity of many other enzymes and it can react with many other substances, inorganic and organic. Among the inorganic substances are the ferrous form of iron (Fe^{+2}), the hydrogen sulfide ion (HS^-), the sulfide form of sulfur (S^{-2}), the dithionite form of sulfur (SO_3^{-2}) (SO_3^{-2}), stannite ($Sn(OH)_3^-$), and lead dioxide (Cheronis and Stein, 1956). The reactions with Fe^{++}, HS^-, and S^{-2} can take place in anoxic waters and sediments and is a partial reason for the high background observed when analyzing ETS samples from these sources. Among the organic substances that can react with INT are ascorbic acid and possibly mercaptans, glutathione, cysteine and other thiol containing organic constituents of the cell. Since none of these reactions would contribute to respiration they were considered background noise and their measurement was always incorporated (as blanks) in the early ETS assays in order to quantify and subtract off this signal (Devol, 1975; Packard, 1969).

Here we examine some of these organic reactions to document their presence in zooplankton samples. Then we: (i) investigate the Båmstedt measurement; (ii) show that it measures only a small and variable fraction of the ETS activity; (iii) demonstrate that it measures the reduce g power of many background biochemical substances in the cell; (iv) and explain why it doesn't measure *in vivo* ETS activity.

3.2. Materials and methods

All experiments were conducted with adult male *Leptomysis lingvura*, a planktonic Canary Island mysid collected in autumn at Risco Verde, according to Herrera et al. (2011) and Fernández-Urruzola et al. (2011). The organisms were kept for no more than 3 days under controlled conditions at 20 ±1 °C and fed with *Artemia salina* nauplii enriched with Easy-DHA Selco® as described by Herrera et al. (2011). Each mysid was frozen in liquid nitrogen and stored at −80 °C for later analysis for ETS activity and biomass. Protein was determined as a measure of biomass by the method of Lowry et al. (1951) modified by Rutter (1967).

3.2.1. The assays

For both assays, mysids were homogenized in a glass-teflon tissue grinder for 2 min at 2000 rpm and maintain in an ice-bath at 0–4 °C to preserve the enzymatic activity (Gómez et al., 1996). ETS activity was determined by the zooplankton ETS assay of Owens and King (1975) designed to saturate the electron flux by adding high levels of the natural substrates, NADH and NADPH. The tetrazolium-reduction assay of Båmstedt (2000) was performed as described without the addition of NADH, NADPH, or succinate (another natural substrate). From here on, we will term

this assay, the Båmstedt assay. Table 3.1 describes the solutions and the conditions used for the two assays. Potential respiration activity (Φ) is expressed as μmol O_2 h^{-1} and for Owens and King (1975) method, Φ is calculated from the expression:

$$\Phi = \frac{(A \text{ x } H \text{ x } V)}{(v \text{ x } 15.9)} \qquad (2.1)$$

where A is the rate of formazan production measured spectrophotometrically in kinetic mode (absorbance increase per hour per centimeter cell). H is total volume (ml) of the sample homogenate; V is the total volume of the reaction mixture (for these experiments, 2.5 ml) in a 1 cm (light path) cuvette; v is the subsampled homogenate volume used in the reaction (0.5 ml here). The specific absorptivity of the INT-formazan is 15.9 absorbance units (μmol of formazan)$^{-1}$ ml^{-1} cm^{-1}. This specific absorptivity of the INT is the equivalent to 15.9x10^3 absorbance units (μmol of formazan)$^{-1}$ l^{-1}cm^{-1}, the value that was determined by Kenner and Ahmed (1975). To calculate INT reduction for the zero-substrate method, we followed the Båmstedt (2000) procedure. Accordingly, the formula for μmol of O_2 was:

$$\Phi = \left[\frac{((47.455_{475} - 0.109) \text{ x } V/1000 \text{ x } 1/l)}{2}\right] \qquad (2.2)$$

Note that the regression equation ($47.455A_{475}$ − 0.109), representing μM formazan, is our equivalent to the Båmstedt (2000) equation for the standard. In Eq. (2.2), A_{475} is the absorbance of the standard solution in a 1 cm cell. V is the final reagent volume in ml (for these experiments, 3 ml), L is the cell length of the cuvette in which the measurement took place (1 cm). Dividing by 1000 converts the milliliters to liters. (Note that M signifies mol l^{-1}). Dividing by 2 is the stoichiometric conversion factor from μmol formazan to μmol O_2. Two electrons are needed to reduce INT to formazan and four electrons are needed to reduce O_2 to H_2O (Packard et al., 1983). Note that the factor 16 in the Båmstedt equation is not needed because here, we are working in molar units rather than in grams.

ETS activity, as calculated from Eqs. (2.1) and (2.2), was corrected for any differences in the temperature between the *in situ* and the incubation temperatures using the Arrhenius equation (Aguiar-González et al., 2012; Seiwell, 1937; Yurista, 1999) with an activation energy of 15 kcal mol^{-1} (Packard et al., 1975). Note that the Båmstedt assay is not measured at *in situ* temperature and does not correct for the difference between the assay temperature (40 °C) and the *in situ* temperature.

3.2.2. INT reduction in cell-free homogenates

Because the INT is a non-specific oxidizing agent it can react with any reducing agent in the cell with an electropotential negative enough to reduce INT to its formazan (Smith and McFeters, 1997). So it can be reduced by many different cellular compounds that have nothing to do with the ETS. To demonstrate this reduction six compounds where tested, ascorbic acid (Panreac 131013.1208), cysteine (Panreac Sintesis 15B512.1206), phenol (Merck 8.22296.0100), glucose (Panreac 131341.1210), reduced glutathione (Merck 4090) and vitamin B_{12} (Sigma-Aldrich 117K1520). These substances are known for their wide distribution and universal importance in cell metabolism (e.g. De Roeck-Holtzhauer et al., 1991; Mæland et al., 2000; Nelson and Cox, 2008; Wang et al., 2008). For example, glutathione in marine phytoplankton ranges from 0.08 to 3.1 mM (Rijstenbil and Wijnholds, 1996) for phytoplankton, and 45.7 ±0.92 mg prot./g protein for marine Decapoda (Wang et al., 2008). Cysteine in phytoplankton ranges from 0.04 to 2.23 mM, (Rijstenbil and Wijnholds, 1996; Satoh et al., 2002), Miller, 1992; Maldonado, 2009). In our experiments we selected concentrations within these ranges.

Table 3.1. Comparison of reagents and conditions used in the ETS and zero-substrate assays. Abbreviations: PVP (polyvinyl pyrrolidone); Tris (Tris (hydroxymethyl) aminomethane); NADH (nicotinamide adenine dinucleotide); NADPH (nicotinamide adenine dinucleotide phosphate (reduced)); INT (2-(ρ-iodophenyl)-3-(ρ-nitrophenyl)-5phenyl tetrazolium chloride). Note that the zero-substrate assay does not incorporate a substrate buffer and the Owens and King kinetic assay does not incorporate a quench or an extraction buffer.

	Båmstedt (2000)	Owens and King (1975)
Homogenate buffer		
Phosphate (M)	0.01	0.1
pH	8.5	8.5
PVP (mg prot. ml^{-1})	1.5	1.5
Mg prot.SO_4 (μM)	75	75
Tris (mM)	50	–
Triton X-100 (ml l^{-1})	2	2
INT (mg prot. ml^{-1})	2	2[a]
Substrate buffer		
NADH (mM)	–	1.7
NADPH (mM)	–	0.25
Quench		
Formaldehyde (M)	1	–
Phosphoric acid (M)	1	–
Extraction medium Chloroform/methanol	2:1	–
Essay incubation conditions		
Temperature (°C)	40	*In situ*
Time (min)	60	20 (or less)
Measurement	End point	Kinetic
Temperature correction	No	Yes

[a] In this method the INT reacts while the reaction mixture is in the spectrophotometer.

Test solutions of different concentrations (1.75, 1.5, 1.25, 1.0, 0.75, 0.5, 0.25 and 0.005 mM), of each of the above substances, were prepared in 0.5 M phosphate buffer pH: 8.0 (6.72 g Na_2HPO_4,

0.36 g KH_2PO_4, 18.5 mg prot. Mg prot.$SO_4 \cdot 7H_2O$, 1.5 g/ml polyvinyl pyrrolidone and 2 ml/l Triton X-100). In the case of vitamin B_{12}, test solutions of different concentrations (0.1, 0.01 and 0.001 mM) were used because higher concentrations generated absorbances that challenged the upper limits of the spectrophotometer and masked the formazan absorbance. For each reaction, 1 ml of each test solution was added to, 0.5 ml of INT (2 mg prot./l) 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. An example of this type of kinetic analysis for the reaction-rate dependence on concentration is given in Figure 3.3 for the case of the peptide, glutathione.

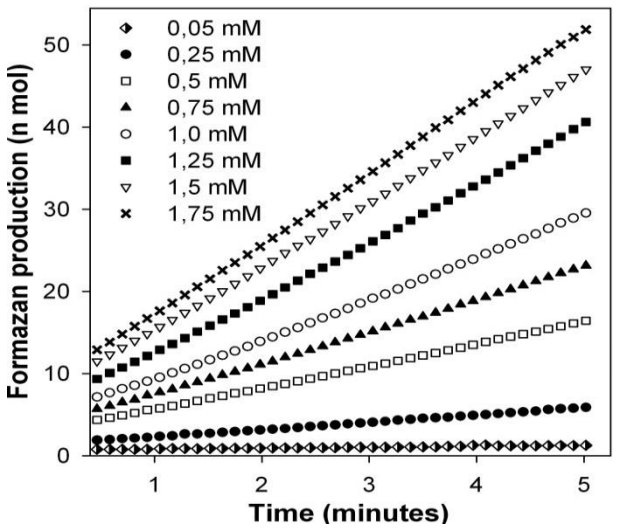


Figure 3.3. The glutathione reaction with INT, as a function of time, for eight different glutathione concentrations. Only the first 5 min of the formazan production is shown. The velocity of the reaction is proportional to concentration. Note that no enzyme is involved in this reaction.

3.3 Results

3.3.1 Comparison of methods

Figure 3.4 shows Φ as measured by the Owens and King (1975) methodology rising strongly with increasing biomass while its blank and the Båmstedt INT reduction assay remained constant. This behavior was consistent with the studies of Savenkoff et al. (1995), Packard et al. (1996a, 1996b), and Packard and Gómez (2008). Upon examination the signal from Φ was an order of magnitude larger than both its blank assay and the signal from the Båmstedt assay. Figure 3.4 (inset) is a blow-up showing the fine-scale variability in the later two measurements.

From many studies (Hernández-León and Gómez, 1996; King and Packard, 1975b; Martínez et al., 2010; Packard and Gómez, 2008) it is known that ETS activity is closely related to biomass. Figure 3.4 shows that this relationship is stronger when specific substrates are added to the homogenate otherwise, both the ETS blank and the Båmstedt assay would show stronger biomass dependency.

3.3.2. Non enzymatic formazan production

Three of the compounds tested reacted with INT to produce formazan non enzymatically. The formazan production was time dependent until the reaction was complete, but this linearity should not be considered an enzymatic reaction (Figure 3.3), but no enzymes were present in the reaction mixtures. Cysteine and glutathione showed similar formazan production rates, but ascorbic acid production rates were three orders of magnitude faster (Figure 3.5). Phenol and glucose did not show any reaction with INT. The reaction of INT with vitamin B_{12} was difficult to trace spectrophotometrically because it has an extinction coefficient at 490 nm of 5.350 cm^{-1} M^{-1} and thus competes with INT absorption at this same wavelength (Du et al., 1998; Hill et al., 1964).

3.4. Discussion

Because enzyme activity is a characteristic property of living cells, Aleem (1955) proposed to use triphenyltetrazolium chloride to quantify the living biomass of plankton populations in the Baltic Sea. Later, considering that enzyme activity is the basis of cellular physiology, Davey (1964) used malic dehydrogenase to assess phytoplankton respiration and Curl and Sandberg (1961) used succinate dehydrogenase (SDH) to measure zooplankton

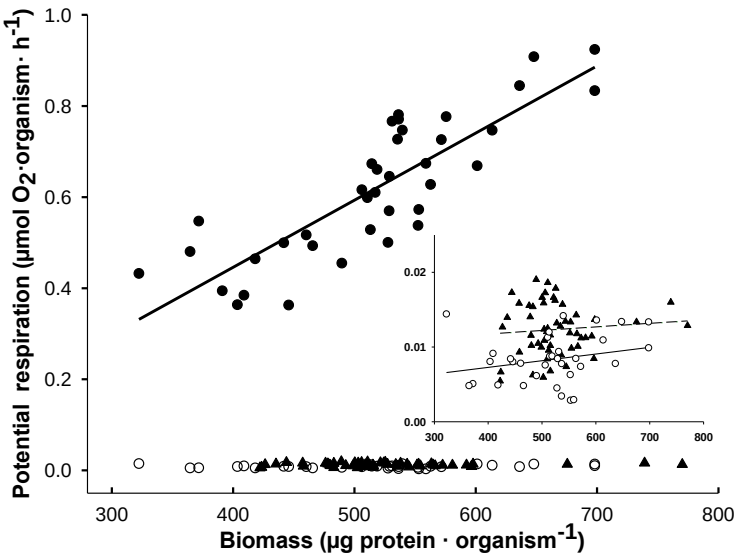


Figure 3.4. Potential respiration comparison between the Owens and King (●) assay, the Owens and King blank (○) (n =39), and the Båmstedt assay (▲) (n =53). The Båmstedt assay was done at 40 °C and the Owens and King assay and blank were corrected to *in situ* temperature using the Arrhenius equation. There is a clear difference in the biomass relationship between the two assays. The regression equation for the biomass dependence assay is: $y=9\times10^{-4}x - 0.06$ (n=49, $r^2=0.62$ (p>0.001)). The equivalent equation for the Båmstedt essay is $y=5\times10^{-6}$ x −0.003 (n=55, $r^2=0.022$ (p >0.001)). (Inset) Note the coherence between the Owens and King blank ($y =9\times10^{-6}x$ -0.006) (n =49, r^2 =0.03 (p >0.001)) and the Båmstedt assay (equation above). Note also, that the Båmstedt essay and the Owens and King blank are only weakly sensitive to biomass variations.

respiration. In both cases, the enzymes were part of the Krebs citric acid cycle and from the early investigations of A. Szent-Györgyi and H. Krebs, these enzymes were known to be associated with respiration (Keilin, 1966). On this basis, they were a logical choice. Pearre (1964) tested the use of SDH in field samples and found that it could roughly predict zooplankton respiration, but not

phytoplankton respiration. Packard and Taylor (1968) found a correlation between SDH and respiration in *A. salina*, but in the process of this investigation realized that a better index of respiratory oxygen consumption would be the electron transport system (ETS) activity. The research of Chance and Williams (1955, 1956) showed that this cytochrome–enzyme system, and especially the NADH dehydrogenase complex in it, controlled the oxygen consumption rate. Building on this idea,

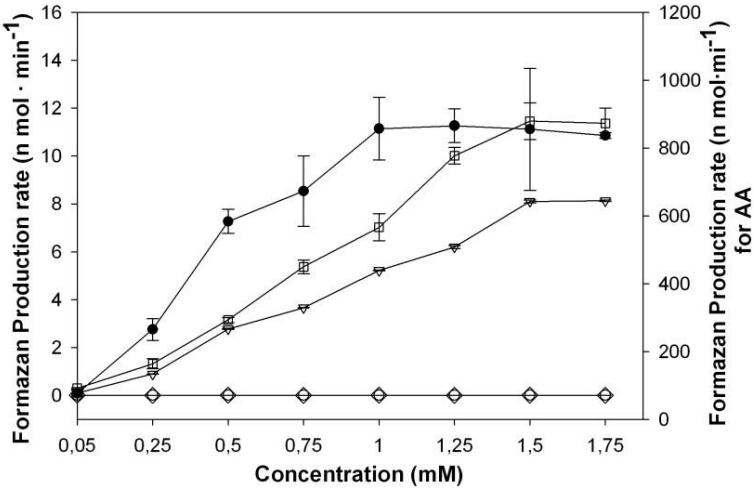


Figure 3.5. Formazan production as a function of concentration for ascorbic acid (AA), cysteine, glutathione, glucose and phenol. Ascorbic acid (●) was the most powerful reducing agent, two orders of magnitude higher (Y axis right) than cysteine (□) and the glutathione (▽) (Y axis left). Both of which had a similar capability to reduce INT to its formazan form. Phenol (○) and the glucose (◊) did not show any reaction with INT. Note that for ascorbic acid the reaction becomes saturated above 1 mM.

Packard et al. (1971) showed that ETS activity measurements in seawater samples could be used to calculate oxygen consumption rates in the ocean water column. This led to a series of improvements in the ETS assay and its application to phytoplankton (Kenner and Ahmed, 1975), zooplankton (Owens and King, 1975), bacteria (Christensen et al., 1980) and sediments (Christensen and Packard, 1977), and its relationship to respiration (Arístegui and Montero, 1995; Finlay et al., 1983; King and Packard, 1975b), temperature (Martinez and Estrada, 1992; Packard et al., 1975), and pressure (King and Packard, 1975a).

With the intent of directly measuring the fraction of the ETS activity (*in vivo* activity) that is being used for marine zooplankton respiration *in situ*, the Båmstedt assay introduced an INT-reduction method that eliminates the use of the ETS substrates (NADH and NADPH). A priori, from considerations of enzyme kinetics, this method is not a valid enzyme assay. An enzyme or an enzyme system, whether of the ETS or another enzymatic pathway, is very specific in its catalytic action. An assay designed to measure its activity with some detector, must include the enzyme's substrate or substrates, otherwise the assay is not specific (Cornish-Bowden, 2004; Fruton and Simmonds, 1958; Segel, 1993). Without substrates it would measure any number of reactions that can react with the detector, in this case INT. Table 3.2 lists some reducing agents that can react with INT, but have nothing to do with respiration. In the case where only INT is added to a plankton homogenate, both the enzymatic and non enzymatic reactions will

stop when the natural substrates or reactants in the homogenate are exhausted. The INT would react non-specifically with many reducing agents and many different enzymes in the homogenate as well as with ETS enzymes. The second factor that can be deduced from a priori analysis is that the assay does not measure the ETS or any of the homogenate's enzymes at their *in vivo* substrate concentration. The process of homogenization in a buffer dilutes the substrates by an unknown amount. In effect, the enzymes are working far below their K_m.

Table 3.2. Additional applications of the tetrazolium salt INT in enzymology and cytology.

Determination of lactate dehydrogenase activity	Nachlas et al., 1960; Babson and Babson, 1973
Effects of the succinic dehydrogenase activity by Coenzyme Q_{10}	Wattenberg and Leong, 1960
Differentiation of non-viable cells helping to detect primary tumor cells	Alley et al., 1982
Helps enumerate viable and metabolically active bacteria	King and Parker, 1988
Detection of NADPH-diaphorase activity in neurons	Hope and Vincent, 1989
Measurement relative growth of microorganisms in microplates	Gabrielson et al., 2002
Revealing LDH released by lysis of the spermatogonial steam cells	Braydich-Stolle et al., 2005
Detection of lactate production	Shen et al., 2008
Cytotoxicity of MβCD on mice neuroblastoma N18 by the release of cytoplasmic enzyme lactate dehydrogenase (LDH)	Lee et al., 2008

Both factors can be deduced a priori from a knowledge of enzymology (Cornish-Bowden, 2004; Mahler and Cordes, 1971; Segel, 1976, 1993), but in addition, a steady stream of reports starting at least with Rogers (1953), as well as the results here (Figure 3.3; 3.4) confirm their validity. This is why a classic enzyme assay incorporates excess substrate.

In addition to the Båmstedt assay for zooplankton, Martínez-García et al. (2009) introduced a microplankton tetrazolium reduction method for determining respiration *in situ* by directly adding INT to seawater. As with the Båmstedt assay, this technique does not require substrates, which are expensive, and it does not destroy the biological integrity of the sample. The hypothetical advantage is that the INT reacts with the ETS at natural substrate levels in the cells giving the true *in situ* respiration rate. However, it assumes that INT can freely penetrate cell walls, bacterial plasmalemmas, and mitochondrial membranes in a plankton sample. This, however, was not verified. Furthermore, there are several other problems with this approach. The first is that, like the Båmstedt assay, it lacks specificity and reducing substances with electropotentials higher than 170 mV can reduce INT (Seidler, 1991). So, the ETS-based reduction cannot be separated from other INT reducing reactions. Second, seawater incubation ofmany hour-duration is required rendering the data acquisition rate slow and comparable, but less sensitive than Winkler-based respiration measurements (Bryan et al., 1976; Carrit and Carpenter, 1966). Third, the kinetics of the enzyme reactions

detected is modified to an unknown extent by the deposits of formazan that are precipitated beside the enzyme. These deposits will reduce the collision frequency of the enzyme with its substrate, change the reaction kinetics and hence reduce the reaction rate. Fourth, the ratio of respiration to their assay is around 11 signifying that R exceeds their measurement by a factor of 11. This indicates that the measurement lead is less sensitive than direct respiration measurements. Also when the ratio of respiration to its proxy is as high as 11 theory argues that the INT reduction, as measured, is clearly not potential respiration. If the proxy is truly potential respiration, the R-toproxy ratio must be equal or less than 1.

The results here show that when no specific substrate is added, the formazan production signal is small (Figure 3.4) and equivalent to the blank in a classic ETS assay. The measurement is the product of many signals from unidentified enzymatic and non-enzymatic reactions and so it cannot be called an ETS assay. In fact is not an enzymatic essay. Furthermore, because the Båmstedt assay is measured at 40 °C and is calibrated with respiration rates measured over a range of temperature from6 to 17 °C it cannot predict respiration at any single *in situ* temperature. To do that, the Båmstedt assay should be calibrated against respiration measured at a single *in situ* temperature or use the Arrhenius equation to make the temperature correction.

Consequently, we do not recommend using this Båmstedt (2000) methodology to estimate respiration because it does not measure what it purports to measure, it is not specific for any biochemical reaction, it is insensitive, and can never be reproduced. The Båmstedt method clearly is measuring glutathione, cysteine, and ascorbic acid, substances that are universally found in all eukaryotes and prokaryotes (Merchie et al., 1995; Nelson and Cox, 2008; Newton et al., 1996). In addition, it is probably measuring vitamin B_{12}, mercaptans, and other reduced organic compounds with similar reducing capacity measuring the background activity of a plethora of dehydrogenases, oxidoreductases, and hydroxylases present in all cells. Alpha-keto glutarate dehydrogenase and any enzyme that reduces NAD^+ or $NADP^+$ to NADH and NADPH will also reduce INT to its formazan in the Båmstedt assay. Thus the assay has no specificity.

3.5. Summary

1. Three of the six intracellular biochemical compounds tested (glutathione, cysteine and ascorbic acid) reacted non enzymatically with INT resulting in both concentration and time dependent formazan production.

2. The formazan production signal from the Båmstedt assay is lower by a factor of ten than the formazan production signal of the

Owens and King (1975) zooplankton, substrate-based ETS method.

3. The Båmstedt assay measures the natural capacity of a zooplankton sample to reduce the INT. It is not measuring potential respiration or ETS activity.

Acknowledgments

This investigation was supported by Projects EXZOME (CTM2008-01616/MAR) and CAMVALEX (CTM2010-09515-E) granted to M. Gómez and by the Canary Islands Government grant to F. Maldonado. T. Packard was supported by contract EXMAR SE-539 10/17. We thank I. Martínez Sánchez, C. Voß, N. Osma Prado, A. Herrera Ulibarri, and I. Fernandez Urruzola for their encouragement, support, and assistance. [SS].

Journal of Experimental Marine Biology and Ecology 434–435 (2012) 110–118

Contents lists available at SciVerse ScienceDirect

Journal of Experimental Marine Biology and Ecology

journal homepage: www.elsevier.com/locate/jembe

Understanding tetrazolium reduction and the importance of substrates in measuring respiratory electron transport activity

F. Maldonado *, T.T. Packard, M. Gómez

Plankton Ecophysiology Group, Instituto de Oceanografía y Cambio Global, Universidad de Las Palmas de Gran Canaria, Canary Islands, Spain

ARTICLE INFO

Article history:
Received 2 February 2012
Received in revised form 13 August 2012
Accepted 13 August 2012
Available online 28 August 2012

Keywords:
Electron transport system
Enzyme reaction
ETS
Potential respiration
Respiration
Zooplankton

ABSTRACT

Most of the oxygen consumption in plankton is controlled by an enzymatic complex called the electron transport system (ETS) or the electron transport chain. To detect and measure this ETS in a biologically diverse plankton community it is common practice to add, in addition to an artificial electron acceptor, the various substrates that donate reducing equivalents to the ETS. Specifically, pyridine nucleotides (nicotine adenine dinucleotide (NADH) and nicotine adenine dinucleotide phosphate (NADPH)) and succinate are routinely used. The addition of these substrates to the ETS stimulates its activity to capacity and hence serves as a measure of potential respiration (Φ). This proxy is then used in ecological and oceanographic studies as an index for respiration. Unfortunately in an attempt to align this proxy more closely with in vivo physiological respiration and to simplify the ETS assay the required substrates were omitted from the analysis. The consequences of this shortcut are demonstrated and explained here. In effect, because some basic rules of biochemistry were broken, the simplified assay yields misleading results that are, in fact, the equivalent of the control (the blank) in a normal ETS assay. Here we explain some of the chemistry of this background reaction, most of which can be found in textbooks. We demonstrate that the use of the artificial electron acceptor, the tetrazolium salt, INT, is not specific for cell respiratory ETS reactions, but is sensitive to many substances present in all cells. We show that, among these common cellular substances, ascorbic acid, cysteine, and glutathione readily reduce INT non enzymatically to its formazan. Such INT reduction is part of the normal blank reaction in the ETS assay and is far removed from the desired respiratory capacity of the cell.

Contents

1. Introduction . . . 110
2. Material and methods . . . 113
2.1. The assays . . . 113
2.2. INT reduction in cell-free homogenates . . . 113
3. Results . . . 114
3.1. Comparison of methods . . . 114
3.2. Non enzymatic formazan production . . . 115
4. Discussion . . . 115
5. Summary . . . 116
Acknowledgments . . . 116
References . . . 116

1. Introduction

Respiration consists of the consumption of oxygen, the production of CO_2, and the biological consumption of organic carbon (Lane, 2010). Priestley and Lavoisier discovered this with clever experiments during the age of enlightenment (Bell, 2005; Lane, 2002, 2005; Uglow, 2002). It is a key physiological process in all marine organisms

* Corresponding author at: Plankton Ecophysiology Group, Instituto de Oceanografía y Cambio Global, Campus Universitario de Tafira, Universidad de las Palmas de Gran Canaria, 35017 Las Palmas de Gran Canaria, Canary Islands, Spain. Tel.: +34 928 454 471; fax: +34 928 45 2922.
E-mail address: federico.maldonado@ulpgc.es (F. Maldonado).

Chapter 4

A fluorescent method for determination of the respiratory electron transport system activity

Maldonado, F[*]., Packard, T. T[*]., Osma, N[*]., Gómez, M[*]., and Santana-Rodríguez, J. J.[+]

*Plankton Ecophysiology Group, Instituto de Oceanografía y Cambio Global, Universidad de Las Palmas de Gran Canaria, Canary Islands, Spain

[+]Departmento de Química, Universidad de Las Palmas de Gran Canaria, 35017 Las Palmas de Gran Canaria, Spain.

Abstract

Measuring respiratory electron transport system (ETS) activity has changed little since its introduction in the early 70'. It is based on measuring the reduction of 2-(p-iodophenyl) -3- (p-nitrophenyl) -5-phenyl tetrazolium chloride (INT) spectrophotometrically. Here, we employ fluorometry switching the artificial electron acceptor from INT to a fluorescent dye, resorufin. This methodological shift in some cases improve sensitivity up to twelve times compared to spectrophotometry, enabling trace levels of respiratory metabolism in low-biomass areas of the ocean to be detected.

Fluorescence and ETS activity are affected by many parameters, some of which we investigated. For the conditions of this ETS assay, the concentrations of the pyridine nucleotides, NADH and NADPH, were optimal at levels of 1.0 and 0.15 mM respectively. Succinate, another ETS substrate, did not contribute to the ETS activity signal, so it was not included as a substrate. Oxygen quenching of the electron flux could not be detected. The limit of detection (LOD) and limit of quantification (LOQ), for reduced fluorescent electron-acceptor, resorufin, were 1.91 and 3.33 nM, respectively. Spectrophotometrically, it was impossible to detect resorufin at these concentrations. The spectrophotometric ETS assay and this new espectrofluorometric assay were compared on zooplankton samples and found to return equivalent ETS activity levels ($p > 0.05$). The espectrofluorometric assay is reproducible, cost-effective, and more sensitive than the spectrophotometric, especially at low plankton biomass.

4.1. Introduction

Two of the key physiological processes in the ocean, primary productivity and respiration, are fundamental to assessing the metabolic balance of the ocean (del Giorgio and Duarte, 2002); this research focuses on the latter process. Respiration consists of oxygen consumption, CO_2 production, the biological consumption of organic carbon, and the production of ATP (Lane, 2010; 2015). It

can be used to calculate new production, carbon flux in the ocean (Packard et al., 1988), nutrient retention efficiency (Osma et al., 2014), and the heterotrophic energy production (Packard et al., 2014). However, in spite of its utility, respiration is rarely measured oceanographically (sensu Sverdrup et al., 1942) on time and space-scales compatible with nutrient and hydrographic measurements, to generate ocean sections and ocean time-series. This is because most oceanic respiration originates with microscopic-sized plankton and obtaining physiologically acceptable samples of these organisms is difficult. The respiration signal is generally subtle and weak, especially when taking samples from the open or deep ocean, which is almost ≥80% (Garrison, 2012) of this ecosystem. The measurement itself is difficult to accomplish except in eutrophic surface waters. That is one the few places where the respiration signal is strong. However, even in these waters collecting enough sample material or water for the measurements can be stressing, difficult and costly. To obtain sufficient biological material to perform physiological measurements, the dispersed organisms have to be concentrated either by net, filtering, or other procedures that normally injure delicate metazoans. This highlights an additional criteria, that planktonic organisms must be captured unharmed. Otherwise, any measurement will not be representative of the true *in situ* respiration. Complying with this requirement is, again, difficult, time consuming, and expensive. For these reasons the ETS technique, in which the biochemistry of the respiratory electron transport system (ETS) was used to supplement the physiological approach, was developed (Packard, 1971; Packard et al., 1971; Packard et

al., 1974). The ETS measurement determines the potential respiration by measuring the biochemical redox reactions (Grey and Ellis, 1994) that control respiration. For metazoans, this is via detection of the electron transfer and transport activity in the dehydrogenases, quinones, and cytochromes present in the mitochondrial inner membrane. Collectively these respiratory control enzymes are known as the respiratory electron transport system (Fruton and Simmonds, 1958; Scheffler, 2008; Gnaiger, 2009a and b). Historically, because of the pioneering work of David Keilin, the ETS was called the electron transport chain (ETC, Keilin, 1966), but because research over the subsequent 70 years revealed it to be more complex than a chain, we, and some others (Gnaiger, 2009a and b), use the term, electron transport system, in preference. The assay, as commonly used today (Packard et al., 2004), was designed as a proxy for the respiration of all the organisms in a parcel of seawater (Packard et al., 1971; Packard, 1985a, b). It was intended to reduce dependence on more time-consuming methods for estimating seawater respiration (Barnes and Collias, 1961; Seiwell, 1937; Robinson and Williams, 2004), but it still requires incubation-type of methodology for calibration (Packard and Williams, 1981). The ETS method incorporated the geosciences' practice of using proxies for intractable unknown quantities as well as the basic precepts of enzymology, one of which requires that an enzyme be measured at its maximal level of activity (V_{max}; Fruton and Simmonds, 1958; Passonneau and Lowry 1993; Maldonado et al., 2012). Following this latter practice required adding saturating levels of substrates to sustain the reaction requirements of Complex I and Complex II of the ETS as

well as to ensure reproducibility and replication of the measurement. Currently, the spectrophotometric ETS assay is based on the reduction of an artificial electron acceptor, 2-(p-iodophenyl) -3- (p-nitrophenyl) - 5-phenyl tetrazolium chloride (INT) (Packard, 1971; Owens and King, 1975; Kenner and Ahmed, 1975; Gómez *et al*., 1996). As with all technology, the ETS assay has been continually improved with time to the point where it requires 1/3 the sample size, 1/4 the time, and 1/5 the expense as it did originally (Packard and Christensen, 2004). These improvements have been achieved by advances in solution chemistry and engineering, however the tetrazolium redox detector (INT) has remained the same. In spite of these improvements, deep-sea measurements of microplankton ETS activity still require the filtration of 10 liters of seawater. Any improvement in analytical sensitivity would reduce this volume requirement and improve the ETS-activity data-acquisition rate. Such an improvement, in turn, would facilitate the generation of synoptic sections of ocean respiration and the calculation of modeled sections of nutrient retention efficiency and heterotrophic energy production (sensu Bindoff and Wunsch, 1992 and Packard et al, 2015). Here, we investigate potential improvements associated with using a different electron acceptor for the ETS, the non-toxic dye, resazurin ($C_{12}H_6NNaO_4$).

Resazurin has the advantage of fluorescing only in its reduced state, resorufin. In addition resorufin has the advantage over the reduced tetrazolium salt, 5-cyano-2,3-ditolyl tetrazolium chloride (CTC), in that resorufin fluoresces while in solution. The formazan

of CTC does not, meaning that it can not be used as a stoichiometric indicator of electron transfer. This fluorescent characteristic of resorufin can improve analytical detection of electron transport activity over what can be achieved by INT reduction and spectrophotometry (Undenfriend, 1962; Cook and Self, 1993; Passonneau and Lowry 1993; Bisswanger, 2011). This is because fluorometry compares a luminescent signal with zero while spectrophotometry compares the reduction in light transmission from a reference of 100% transmission yielding the difference between two large numbers (Oxtoby et al., 1999).

Resazurin is not a new redox dye; it was discovered and developed 145 years ago by Weselsky (1871) and first used by Pesch and Simmert (1929) for detecting bacteria in milk. It is blue in solution and when reduced, as a response to metabolic activity, as, for example, in mitochondria, it changes to resorufin, a pink fluorescent dye, (Rasmussen, 1999; González and Tarloff, 2001; De Fries and Mistuhashi, 1995). In green light, resazurin does not fluoresce, but in this same green light, resorufin fluoresces the color orange (585nm). Resazurin has been used in numerous fields related to cell metabolism (Table 4.1), most of them in medicine-cytotoxicity (eg. Cook and Self, 1993; Gundemir and Johnson, 2009; Kumar et al., 2012; Abu-Amero et al., 2006), in detecting spermatic quality (eg. Erb and Ehlers, 1950; Strzeżek et al., 2013), and in the dairy industry (eg. Ramsdell et al., 1935; Moyer and Campbell, 1963; Ricciardi et al., 2014). However, in proportion to the previous topics mentioned, its use in ecological, physiological, and oceanographic studies is less common (eg. Morita and ZoBell,

Table 4.1. Some uses of resazurin-resorufin are not related to measurements of mitochondrial respiration. Regardless of the field in which they are used, most of them are employed to determine the viability of the cells. Here are some examples.

Dairy industry	Pesch and Simmert, 1929; Ramsdell et al., 1935; Johns and Howson, 1940; Nixon and Lamb, 1945; Shahani and Badami, 1958; Moyer and Campbell, 1963; Ali-Vehmas et al., 1991; Larsen et al., 2011; Chandrasekaran et al., 2014; Ricciardi et al., 2014.
Sperm quality-fecundity	Erb and Ehlers, 1950; Erb et al., 1952; Glass et al., 1991; Fuse et al., 1993; Dart et al., 1994; Glass et al., 1991; Strzeżek et al., 2013
Wastewaters	Liu, 1983, 1986; Eker and Kargi, 2006, 2009; Wei et al., 2006; McNicholl et al., 2007; Kalathil et al., 2012; Zare et al., 2015;
Biofilms	Kennedy and Droste, 1987;Peeters et al., 2008; Mariscal et al., 2009; Sandberg et al., 2009; Jiang et al., 2011; Van den Driessche et al., 2014; Salcedo et al., 2015
Cytotoxicity	Schuurmans et al., 1964; Rein et al., 1980; Namabar et al., 1983; Jenneman et al., 1986; Koontongkaew etal., 1986; Babich and Borenfreund, 1997; O'Brien et al., 2000; Poulsen et al., 2015, Connolli et al., 2015
Medicine	Stark and Lee, 1981; Cook and Self, 1993; Palomino et al., 2002; Gundemir and Johnson, 2009; Dienstknecht, et al., 2010; Kumar et al., 2012;
Oncology	Chen and Koren, 1985; Wong et al., 2008; Anoopkumar-Dukie et al., 2009; Abu-Amero et al., 2006; Treeck et al., 2006
Ecology	Liu and Strachan, 1979; Bitton and Dutka, 1983; Thomsom et al., 1986; Bakermans et al., 2003; Steven et al., 2006; Haggerty et al., 2008, 2009a, 2009b; Lemke et al., 2013; Liao et al., 2013
Oceanography/ Marine Biology	Morita and ZoBell, 1955; Oremland, 1979; Schropp et al., 1987; Jannasch et al., 1988; Proctor, 1997; Barnes et al., 1998; Wellsbury et al., 2002; Bertoldo and Antranikian, 2006.
Physiology	Thies and Grimme, 1995
Respiration	Peroni and Rossi, 1986; Gumprecht et al., 1995; Zhang et al., 2004; Abu-Amero and Bosley, 2005; Argerich et al., 2011; González-Pinzón et al., 2012; Stanaway et al., 2012.

1955; Bitton and Dutka, 1983; Thies and Grimme, 1995; Proctor, 1997; Barnes et al., 1998; Wellsbury et al., 2002). However, in recent years limnologists have started to use resazurin to assess freshwater respiration (eg. Haggerty et al., 2008, 2009; Argerich et al., 2011; Stanaway et al., 2012; González-Pinzón et al., 2012), but none of them used a biochemical approach and none accurately measure respiration.

Of the different ways that resazurin has been used to assess metabolic activity, the ones that are closer to our approach are the studies of Abu-Amero and Bosley (2005) and Zhang et al. (2004). They worked on the biochemistry related to mitochondrial respiration in rats, but not on its regulation. Peroni and Rossi (1986), calculated oxygen equivalents in ocean sediment samples.

The difference is that they used spectrophotometry. They calculated potential respiration from the resazurin absorbance incubating their samples for 1h without adding substrates. Furthermore, they calculated the oxygen consumption assuming that the reaction is equivalent to the INT reduction (one molecule of formazan is reduced by 2 electrons), which is incorrect. The resazurin reduction only involves one electron (Figure. 4.1). Here, we try using resazurin as an alternative electron acceptor in different forms of respiratory electron transport system in plankton, with the intent to measure their potential respiration.

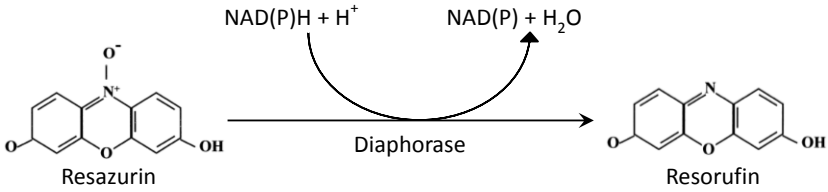


Figure 4.1. One electron enzymatic reduction of the non-fluorescent blue-colored resazurin as catalysed by diaphorase. The pyridine nucleotides, NADH and NADPH were tested as substrates in generating the fluorescent pink-colored resorufin. (Modified from O'Brien et al., 2000; Catomeris and Thibert, 1988).

4.2. Materials, Methods, and procedures

Developing or modifying enzyme assays requires the establishment of baselines, initial conditions and reaction characteristics. Here we describe the steps taken to achieve a robust and sensitive ETS assay proper for ocean exploration purposes.

We set out to develop a new more sensitive ETS assay to detect the low levels of respiratory metabolism in nearly pristine ocean waters. Towards this end, the traditionally used tetrazolium, INT, that turns vermillion upon reduction, was replaced by resazurin, that upon reduction fluoresces bright green. Both compounds are artificial electron acceptor that are reduced by the electrons flowing through the ETS. Since fluorescence is affected by various parameters, such pH, temperature, reagent concentration, ion

strength (Guilbault, 1973), experiments were performed to establish optimum conditions for the resorufin detection. This was accomplished by a progressive series of tests, where the determination of each optimum condition was established. Then the method was tested on field and laboratory samples, and finally it was compared with the spectrophotometric assay that has been used for zooplankton over the last 40 years (Owens and King, 1975).

4.2.1. Instruments

Fluorescence was measured, over time (kinetically), with a Horiba FluoroMax-4 spectrofluorometer equipped with a xenon lamp and a temperature controlled water bath. The values for the exit and entrance slits that, in turn, determined the excitation and emission wavelengths, were controlled by the software Datamax. In this study, only the signal of the sample was taken into account (S). Lamp checks were done periodically to assure the comparability of the measurements. Three-ml Helma® GS quartz cuvettes were used for the reagent optimum determinations; 1.5-ml Helma® GS quartz cuvettes were used for comparing the methods.

Spectrophotometric measurements for the classical method (Owens and King, 1975) were carried out, in kinetic mode, with a Beckman DU® 650 spectrophotometer. The reaction temperature was controlled with a Frigiterm 600382 Spectra water bath. For

each reaction, a 1 cm (light path) disposable plastic spectrophotometer cuvette Semi-Micro LP was used.

Homogenization of the zooplankton sample was performed with a Teflon-glass tissue grinder (Glas-Col 099CK54). Centrifugation of the samples was done in a Sorvall centrifuge (Legend 21R, Thomas Scientific), with internal temperature control. The pH of both buffers and samples was measured with a Testr30 pH meter (Eutech Instruments).

4.2.2. Reagents.

The Reagents used as well as their manufacturer as listed in Table 4 .2

Tris Buffer. All solutions for the resazurin reaction were prepared in 0.1 M Tris (Hydroxymethyl) Aminomethane PA-ACS, 75 µM Mg prot.$S0_4 \cdot 7H_2O$, 1.5 mg prot.$\cdot ml^{-1}$ polyvinyl pyrrolidone (PVP). pH was adjusted to 8.0 adding small aliquots of 0.1 M HCl.

Phosphate Buffer. This buffer was basically the Sorensen's buffer used by Packard et al., 1971, but incorporating a detergent (Triton X-100) to solubilize the formazan and polyvinyl pyrrolidone to protect enzymatic SH groups (Packard, 1969). The final spectrophotometric method incorporating these improvements

Table 4.2. List of reagents employed in these experiments. The manufacturer, the corresponding Chemical Abstracts Service (CAS) Registry Number, and the concentration used are included.

Reagent	Name	Origin	CAS	Method	
				INT	Resazurin
Diaphorase	Lipoamide Dehydrogenase from *Clostridium kluyvery*	SIGMA	EC:1.8.1.4		0.002 U/ml
INT	2-(p-iodophenyl) -3- (p-nitrophenyl) - 5-phenyl tetrazolium chloride		146-68-9	2 mg prot.·ml^{-1}	-
NADH	β-Nicotinamide adenine dinucleotide, reduced disodium salt hydrate		606-68-8	1.7 mM	1.0 mM
NADPH	β-Nicotinamide adenine dinucleotide 2′-phosphate reduced tetrasodium salt hydrate		2646-71-1	0.25 mM	0.15 mM
PVP	Polyvinylpyrrolidone		9003-39-8	1.5 mg prot.·ml^{-1}	
Resazurin	7-Hydroxy-3H-phenoxazin-3-one-10-oxide sodium salt		62758-13-8	-	10 uM

Continuance of table 4.2

Reagent	Name	Origin	CAS	Method	
				INT	Resazurin
Resorufin	7-Hydroxy-3*H*-phenoxazin-3-one		635-78-9	-	
KH_2PO_4	Potassium di-hydrogen phosphate	Panreac	7778-77-0	0.1 M	-
Mg prot.$S0_4 \cdot 7H_2O$	Magnesium sulphate 7-hydrate		10034-99-8	75 µM	
Na_2HPO_4	di-Sodium hydrogen phosphate anhydrous		7558-79-4	0.1 M	-
NaCl	Sodium chloride		7647-14-5		
Succinate	Sodium succinate 6-hydrate		6106-21-4	0.133 M	-
Tris	Tris-(Hydroxymethyl)-Aminomethane		77-86-1		0.1 M
Triton X-100	4-(1,1,3,3-Tetramethylbutyl)phenyl-polyethylene glycol		9002-93-1	2 $ml \cdot l^{-1}$	-

consisted of 0.1 M Na_2HPO_4, 75 µM Mg prot.$S0_4 \cdot 7H_2O$, 1.5 mg prot.$\cdot ml^{-1}$ PVP, and 2 $ml \cdot l^{-1}$ Triton X-100, with the pH adjusted to 8.5 with H_3PO_4 (Owens and King, 1975).

Both buffers are light protected and kept at ~4°C also during experiments.

Substrate Solution. Depending on the assay, substrates (NADH and NADPH) were dissolved just prior to the measurement in the corresponding cold buffer (0-4ºC). To determine the peaks of emission and absorbance of resorufin, and the influence of oxygen and ionic strength on the reaction, 0.133 M disodium succinate, 1.7 mM reduced nicotinamide adenine dinucleotide (NADH), and 0.25 mM reduced nicotinamide adenine dinucleotide phosphate (NADPH), were included, according to Packard et al. (1971), Owens and King (1975), and Kenner and Ahmed (1975). After establishing the optima (Figure 4.6), 1 mM NADH and 0.15 mM NADPH were used for the following experiments. Because the ETS activity was neither stimulated nor repressed by disodium succinate it was excluded from the reaction (see results).

Resorufin Solution. A 500 µM stock solution was prepared with Tris buffer and used in serial dilutions to generate the calibration curve. This curve was done for each group of experiments.

Resazurin Solution. A 100 µM solution was prepared in Tris buffer and usedafter reduction, to determine the resorufin excitation, emission and absorbance peaks, as well as to determine the influences of oxygen and ionic strength on the reaction. In all cases the concentrations suggested by Barnes and Spenney (1980) were used. For studying the diaphorase catalyzed reaction and the natural ETS activity in fresh biological material, a 10 µM solution was used.

Even though resorufin is stable for weeks, in natural waters (Haggerty et al., 2008) and resazurin is stable for more than 12 months at ~4 °C (Guilbault, 1975; Castillo and Thibert, 1988; Rasmussen, 1999), a fresh solution of each were prepared every 10 days and saved at ~4 °C. During experiments solutions were maintained cold (0-4 °C) and light protected.

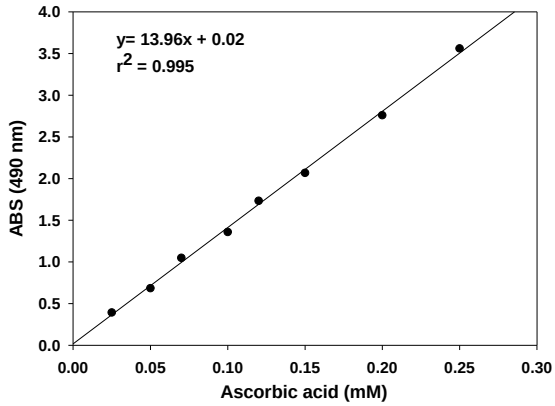
INT Solution. For the spectrophotometric ETS assay, a 2 mg prot.$\cdot ml^{-1}$ solution of 2-(p-iodophenyl) -3- (p-nitrophenyl) - 5-phenyl tetrazolium chloride (INT) was made up in Milli-Q water. Upon opening a new bottle of INT (1 gr) the extinction coefficient was determined and applied to the results from that the batch of tetrazolium (Table 4.3). From each INT batch 20 ml aliquots of were stored in plastic bottles at -20 °C. The solution during tests was maintained cold (0-4 °C) and light protected.

The initial extinction coefficient for INT (15.9 absorbance units [µmol of formazan]$^{-1}\cdot ml^{-1}cm^{-1}$) reported initially by Gomez et al.

(1996), we found it varies, so it had to be recalculated each time a new bottle of INT was purchased. To do this we added 0.5 ml of INT (2 g l^{-1}), to 1.5 ml phosphate buffer, plus 10 µl of ascorbic acid in different concentrations (0 – 50 mM). The redox reaction in this mixture produced formazan with an absorbance of 490 nm. The time-course of the reaction was linear until 0.25 mM ascorbic acid. However, for the region where the absorbances are higher than 2.0, it is better to dilute the solution. The slope yields the formazan extinction coefficient that was employed in the calculations.

Table 4.3. An example of the extinction coefficient determination for INT. Ascorbic acid (AA) was used to reduce INT from its tetrazolium-form to its formazan-form. $[AA]_i$ is the initial concentration of ascorbic acid and, $[AA]_f$ is the final concentration of (AA) in the reaction and, ABS is the Absorbance.

$[AA]_i$ mM	$[AA]_f$ mM	ABS (490 nm)
5	0.025	0.39145
10	0.05	0.68275
15	0.07	1.0474
20	0.10	1.3569
25	0.12	1.73115
30	0.15	2.06785
40	0.20	2.7588
50	0.25	3.55985



In the plot associated with Table 4.3 the extinction coefficient (*e*) (see ETS calculations) for the INT-formazan solution was 13.96 µmol of formazan·ml^{-1} cm^{-1}. This means that if we put 1 ml of a formazan solution in a 1 cm cuvette in the spectrophotometer, and

find a reading of 13.96 at 490 nm, we would have one µmol of formazan in the cuvette.

Enzyme Solution. To prepare the diaphorase solution, 0.002 units (U)·ml^{-1} of diaphorase from *Clostridium kluyvery* (EC Number 1.8.1.4) was diluted in the corresponding cold buffer (Tris for flurometric and PO_4 for the spectrophotometric) immediately before the analysis. Then a 0.004 U ml^{-1} was used for the K_m determinations. Diaphorase solution was prepared daily to maintain its enzymatic activity and maintained cold (0-4 °C) and light protected at all times.

Reaction mixture. The ETS assay was carried out in the corresponding cuvettes. The substrate solution, the artificial electron acceptor solution (resazurin or INT), and the diaphorase solution or the natural ETS sample (Table 4.4) was added directly to the cuvettes in a 3:1:1 ratio, respectively and mixed (Owens and King, 1975). The cuvettes were read immediately in kinetic mode in either the spectrophotometer or the spectrofluorometer. A control was prepared, in each case, by replacing substrate solution with the appropriate buffer (Tris or phosphate) (Table 4.4). The final volume for the experimental determination of emission peaks, resorufin absorbance, influences of oxygen and ionic strength on the reaction was 2.5 ml. For all other experiments the final volume of the reaction was 1.25 ml.

Table 4.4 Reagents used in the Owens and King (1975) spectrophotometric ETS method (S_m) and the new resazurin-based spectrofluorometric ETS assay (F_m). The values are the initial concentrations, not the final concentrations in the cuvette.

	F_m	S_m
Homogenate buffer		
Phosphate (M)	-	0.10
Tris (M)	0.10	-
pH	8.00	8.50
PVP (mg prot.·ml^{-1})	1.50	1.50
Mg prot.SO_4 (µM)	75.00	75.00
Triton X-100 (ml·l^{-1})	-	2.00
Dye (electron acceptor)		
INT (mg prot.·ml^{-1})	-	2.00
Resazurin (µM)	10.00	-
Substrate (electron donor)		
NADH (mM)	1.00	1.70
NADPH (mM)	0.15	0.25

4.2.3. Fluorescence characteristics of resorufin

To determine the excitation peak, the fluorescence intensity was measured at wavelengths ranging from 300 to 565 nm; and for the emission peak, ranging between 575 and 650 nm. Entrance and exit slits were held at 2 nm; an integration time of 0.5 s was used.

To confirm that the commercially available resorufin (R_p) was identical with the resorufin generated in our laboratory by either diaphorase catalysis or biological resazurin reduction (R_r), we examined their fluorescent characteristics and found them to be identical.

4.2.4. Influence of oxygen on the reaction

Molecular oxygen can be a strong quencher of electron flux (Lakowicz, 1983) competing with resazurin for electrons from the ETS. Barnes and Spenney (1980) showed that at pH 9.0, O_2 is preferred 2:1 over resazurin as an electron acceptor converting oxygen in an additional as well as an alternative electron acceptor. To establish if oxygen is competing with resazurin for electrons under our conditions, 2 mM Cyanide (NaCN) (Kenner and Ahmed, 1975a) was added to the substrate solution and blank buffer, just prior the ETS activity measurement. The cyanide should inhibit cytochrome oxidase and block O_2 from being an electron acceptor in our reaction. The reaction was followed in kinetic mode for 10 minutes with the entrance and exit slits adjusted to 2 nm and the integration time set to 0.5 s.

4.2.5. Effect of ionic strength

The phenomenon of fluorescence quenching refers to processes that decrease fluorescence (Lakowicz, 1983). The reaction rate can be controlled by the ionic strength of the medium in which it proceeds, since oppositely-charged ions attract one another whilst like-charged ions repel (Bigger et al., 2003). The effect of ionic strength was tested on the Sigma resorufin by measuring the signal strength and the velocity of the kinetic reaction. Five different concentrations of NaCl where used (0, 0.2, 0.4, 0.6, 0.8 and 1M). Each of these was added to the resorufin, the resazurin, substrate and blank solutions, to maintain the concentration. The reaction

was followed in kinetic mode for 10 minutes with the entrance and exit slits adjusted to 2 nm and the integration time set to 0.5 s.

4.2.6. Optimum substrates, diaphorase, and resazurin concentrations

To determine how the resazurin-reduction reaction was limited by the substrate(s), a range of concentrations was chosen that fell between those reported by Owens and King (1975) and Christensen and Packard (1979) (Table 4.5) and each concentration was reacted with diaphorase and resazurin. Diaphorase can be taken as a standard for the calibration of the ETS measurements. Then five concentrations of diaphorase (Table 4.5), were tested in the reaction mixture, in order to detect if the concentration were enough to give a good signal and linear activity. In experimenting with diaphorase as a standard for ETS activity, ten concentrations of resazurin solution (Table 4.5), were tested in order to detect the concentration limit of the linear relationship. All measurements where measured kinetically for 10 minutes under the same conditions describe previously. Entrance and exit slits 2 nm; integration time of 0.5 s.

4.2.7. Resorufin calibration curve

A good linear calibration curve is needed to assure a proper interpretation of the resorufin production in biological samples; the fluorescence of resorufin should be linear with concentration before reaching the inner-cell effect (Guilbault, 1973). This inner-cell effect

occurs when, at high reagent concentrations, emitted photons from the fluorophore are absorbed by the solution before they reach the detector (a photomultiplier tube). Here, a broad range of resorufin concentrations were used to locate the zone of linearity (Table 4.5).

Table 4.5. Concentrations of the reagents used to determine their optimum conditions. All concentrations are initials.

Resazurin µM	**Resorufin** µM	**NADH** mM	**NADPH** mM	**Succinate** M	**Diaphorase** $U \cdot ml^{-1}$
0	0	0	0	0	0
0.025	0.003	0.1	0.05	0.050	0.0024
0.05	0.050	0.5	0.15	0.085	0.0034
0.10	0.100	1.0	0.25*	0.133*	0.0084
0.25	0.500	1.7*	0.35	0.200	0.0170
0.50	1.000	2.0	0.50	0.500	-
1.00	5.000	2.5	-	-	-
2.50	10.000	3.0	-	-	-
4.00	-	-	-	-	-
5.00	-	-	-	-	-
10.00	-	-	-	-	-

*Concentration reported by the previous cited authors.

4.2.8. Resorufin reproducibility

Eleven measurements were made to the mid-concentration value of the resorufin calibration plot (Figure 4.9). This concentration was 4 µM. However, since this value will be the highest concentration for the future calibration plots, another midpoint was chosen, 1 µM. To these measurements the following statistical parameters (Snedecor, 1934) were used to determine reproducibility:

Variance $$V = \frac{\sum(x_i - \bar{x})^2}{n-1} \quad (3.1)$$

Standard Deviation $$\sigma = \pm\sqrt{V} \quad (3.2)$$

Relative Standard Deviation (RSD) $RSD = \frac{\sigma}{\bar{x}} \cdot 100$ (3.3)

4.2.9. Limits of Detection and Quantification

Limit of detection (LOD) and the limit of quantification (LOQ) were calculated from 12 measurements of the absorbance (490 nm) of PO_4 buffer and the fluorescence (λ_{ex} 520 nm; λ_{em} 594 nm) of Tris buffer. The calculations were made according to the IUPAC criteria (Inczedy et al., 1998; Irving et al., 1978).

LOD $Signal = \bar{x}_B + 3\sigma_B$ (3.4)

LOQ $Signal = \bar{x}_B + 10\sigma_B$ (3.5)

Where, the *Signal* will be the absorbance for the phosphate buffer and fluorescence units for Tris buffer; $\bar{x}_B$ and σ_B were the mean and the standard deviation of the blank measures.

3.2.10. Diaphorase kinetics

In an attempt to calculate K_m values for diaphorase, different concentrations of NADH and NADPH were prepared (Table 4.6). The range of concentrations was developed from previous experiments (data not show). The minimum values tested were 0.1 mM for NADPH and 0.2 mM for the NADH, where the reactions still showed a plateau. From here, we decreased the concentrations to

one third of the original value. Each of these substrate concentration was cross tested with the 0.004 $U \cdot ml^{-1}$ enzyme solution and 10 µM resazurin. Each reaction was followed for seven minutes. Entrance and exit slits were held at 2 nm; integration time was 0.5 s. Measurements were done under the same temperature conditions (20 ±1ºC). With all these combinations, it was possible to determine how the activity (rate) changed by increasing one substrate while holding the other one constant and vice versa.

Table 4.6. Pyridine nucleotide concentrations used to determine Michaelis-Menten constants (K_m) for diaphorase.

NADH (µM)	NADPH (µM)
0	0
0.274	0.045
0.820	0.137
0.091	0.412
2.470	1.230
7.400	3.700
22.20	11.10
66.70	33.30
200.00	100.00

4.2.11. Biological samples (Methods comparison)

To determine if the classic spectrophotometric ETS method (Owens and King, 1975) and this spectrofluorometric ETS assay

are equivalent, identical zooplankton samples were taken from the CAMVALEX cruise in Canary Islands waters.

Each zooplankton sample was ground in Milli-Q water at 0-4 °C for two min in a teflon-glass tissue homogenizer. The homogenate was centrifuged at 2000 rpm for 8 min, at 0-4°C. The supernatant fluid was divided into 2 parts and then diluted 6 times in 1:5 serial dilutions in the respective buffer solution (Sorenson's PO_4^{-3} buffer for the spectrophotometric assay and Tris buffer for the spectrophotometric assay). One part was used to measure the ETS activity spectrofluorometrically; the other half was assayed by the Owens and King (1975) spectrophotometric method (Table 4.6). The reaction for each was followed for 8 minutes in kinetic mode. If assays were incubated at other than *in situ* temperatures, activities were corrected using the Arrhenius equation and energy of activation of 15 kcal per mol-degree (Packard et al., 1975). The remaining homogenate was used for protein determination by the Lowry et al. (1951) method as modified by Rutter (1967).

Potential respiration (Φ) was expressed as μmol O_2 min^{-1}. For the Owens and King (1975) method, Φ was calculated from the expression:

$$\Phi = \left[\left((A \cdot H \cdot V)\right)/(v \cdot \varepsilon)\right]/2 \qquad (3.6)$$

For the resazurin method:

$$\Phi = [A \cdot H \cdot V/v]/4 \quad (3.7)$$

Where, *A*, for the S_m is the rate of formazan (absorbance units $\cdot min^{-1}$) or for the F_m, is the resorufin (counts per sec (CPS) min^{-1}) production measured in kinetic mode; *H* is total homogenate volume (ml) of the sample; *V* is the total volume (ml) of the reaction mixture in a 1 cm (light pathlength) cuvette; *v* is the subsampled homogenate volume (ml) used in the reaction; ε is extinction coefficient of INT in absorbance units (µmol of formazan)$^{-1}$ ml^{-1} cm^{-1} (Table 4.4). Dividing by 2 is the stoichiometric conversion factor from µmol formazan to µmol O_2, since two electrons are needed to reduce INT to formazan and 4 electrons are needed to reduce O_2 to H_2O (INT + $2e^-$ + $2H^+$ → INT-formazan; O_2 + $4e^-$ + $4H^+$ → $2H_2O$; Packard et al., 1983). Since resazurin reduction involves only 1 electron, the molar resorufin production rate has to be divided by 4 to calculate the molar equivalence in units of potential oxygen consumption (Twigg, 1945; Rasmussen, 1999). A calibration plot (Fig 4.9) is used to determine the formazan equivalents, in µmol, of the resazurin reduction reaction (CPS units).

4.2.12. Statistical analysis

Differences between the spectrophotometric and espectrofluorometric methods were tested by a 2 factor ANCOVA.

Included were the factors: “protein” or “diaphorase” (numerical predictor variable) depending on the case and the “method” (categorical predictor variable). The analysis was made using the R statistic program.

4.3. Results

4.3.1. Fluorescence characteristics of resorufin

Under our experimental conditions the peaks of excitation and emission were 520 nm and 594 nm respectively (Figure 4.2). Since, at the moment of the fluorescence measurement there are other components (different from resorufin) in the solution, such as resazurin (that is not yet reduced) and the buffer, we made a scan with these, components using the excitation and emission peaks of resorufin. We found that none of these reagents caused distortion or overlapping with the resorufin signal (Figure 4.2). In all measurements, controls were run.

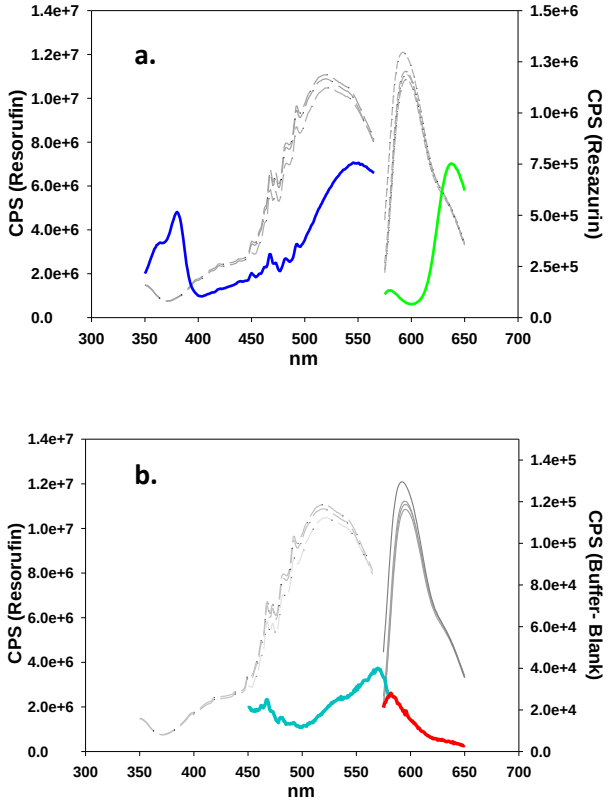


Figure 4.2. Comparison of the emission and excitation spectra of resorufin (gray lines) with, a. resazurin (blue and light green line) and, b. Tris buffer (light blue and red line). Note that in both cases the peaks of emission and excitation do not coincide with the resorufin peaks and that the resazurin and Tris Buffer fluorescence are scaled on the secondary ordinate axis (right) where the magnitude is smaller than it is on the primary ordinate (left).

4.3.2. Influence of oxygen and the effect of ionic strength

To check if oxygen generates a quenching effect during an ETS assay, NaCN was added (See section 4.2.4). No evidence was found that CN improved the resorufin production rate as would be

expect if oxygen were competing with resazurin for electrons. In fact the reaction was slower with the cyanide (Figure 4.3). As a result it was not included in the reaction mixture. The effect of ionic strength, based on NaCl solutions varying from 0 to 1M (See section 4.2.5), on the ETS activity was not conclusive (data not shown); so for further experiments it will be not considered. Accordingly, we adopted the buffer and other reagent concentrations as recommended by Owens and King (1975) for our assay.

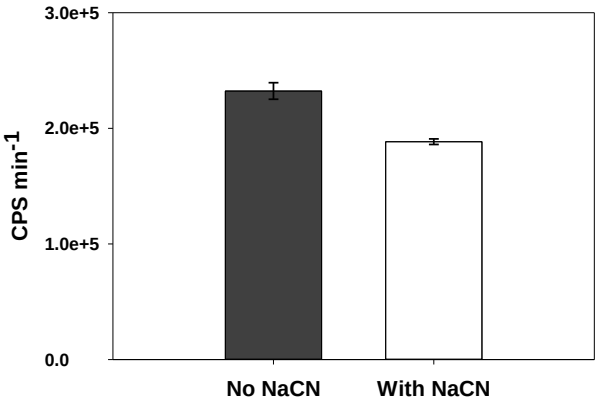


Figure 4.3. Effect of cyanide and indirectly, oxygen, on the enzymatic reduction of resazurin to resorufin. The addition of NaCN (blocking cytochrome oxidase) generated a decrease in the velocity of the reaction, arguing that O_2 does not compete with resazurin for electrons from the ETS. So cyanide was omitted from the reaction.

We continued to use pH 8.0 to be consistent with past ETS analyses and because Guibault and Kramer (1965) reported that for the resazurin-NADH oxidoreductase reaction the optimum pH was between 8 and 9. Also Bueno et al. (2002), suggested that at pH values higher than 7.5, resorufin is in the anionic form, which is

more fluorescent; at pH values lower than 7.5, the fluorescence decreases.

4.3.3. Substrates conditions

Although succinate is known to contribute reducing equivalents to the ETS (Packard and Taylor, 1968; Kenner and Ahmed, 1975; Savenkoff et al., 1995; Gómez et al., 1996). When succinate was included in a diaphorase-based reaction, there was no sign of electron transfer, from succinate to the electron acceptor. The signal was so low that sometimes the net slope was negative (Figure 4.4).

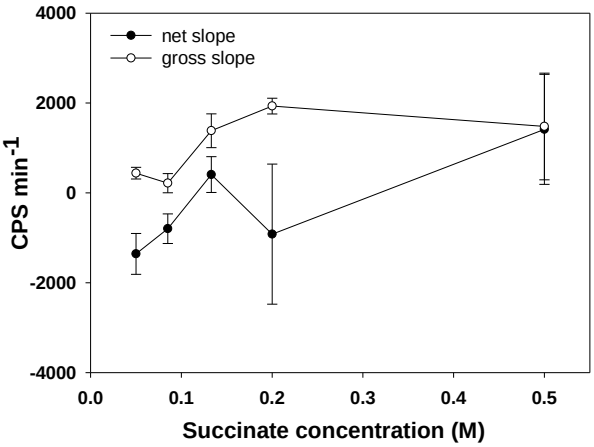


Figure 4.4. Fluorescence intensity in counts per second (CPS) over time (slope), for different succinate concentrations. Note the generally low fluorescent signal. The net slope (reaction-mixture slope minus the buffer-blank slope) showed some negative values, this means that there was no reaction. The spectrofluorometer was measuring background noise.

We concluded that succinate does not react with diaphorase. Slater et al. (1963) found similar results. In their study, INT did not react with soluble succinate dehydrogenase. However, to know if succinate impacts the activity when all substrates are together, as shown by Kenner and Ahmed (1975) for phytoplankton or by Gómez et al. (1996) for zooplankton, an experiment was required. For this evaluation, we compared an ETS assay using all three substrates (NADH, NADPH, and succinate) and one using only the two pyridine-nucleotides (Figure 4.5). The result showed that succinate does not augment the reduction of resazurin via diaphorase catalysis. Consequently, succinate was not included in the reaction mixture.

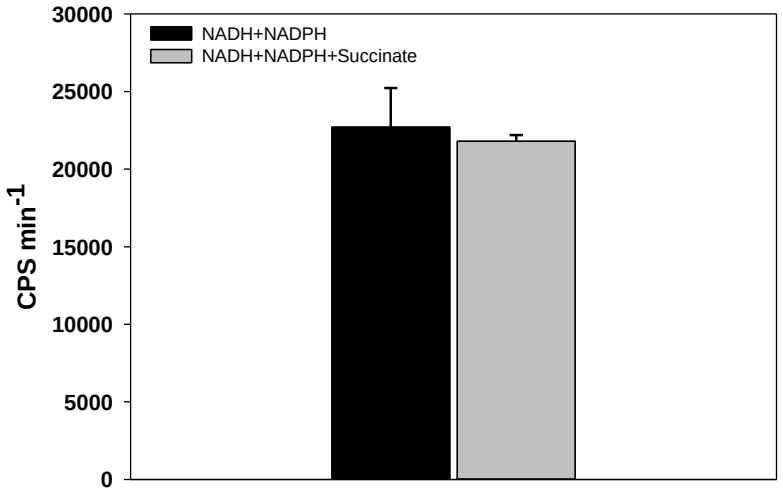


Figure 4.5. Influence of succinate on the velocity of the enzymatic reaction. There was no evidence of the contribution of succinate to the overall reaction.

Since NADH is the expected gatekeeper for the ETS (Gnaiger, 2009a) and NADPH is thought to be involved in some prokaryote

and algal respiratory electron transport systems, we tested the two reduced pyridine nucleotides for their contribution to the overall ETS activity. As expected they were both contributors. During 10 minutes of monitoring, ETS activity remained constant with NADPH dehydrogenase activity about 1.5 times lower than NADH dehydrogenase activity (Figure 4.6). Catomeris and Thibert (1988) and Rasmussen (1999) found that NADH was slightly more reactive than NADPH in the presence of resazurin. The extensive plateau for each pyridine nucleotide indicates that over the range tested (0.1- 2.5 mM NADH and 0.05 to 0.5 mM NADPH) the

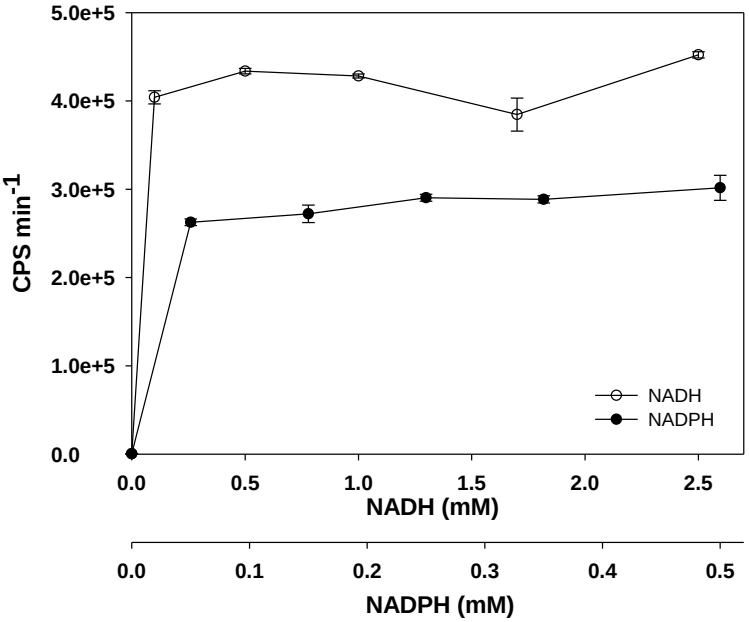


Figure 4.6. Fluorescence intensity production with time (slope), at different NADH and NADPH concentrations. With diaphorase catalyzing the oxidation of the substrates, NADH yielded higher activities than did NADPH. In the range tested there was no inhibition of the reaction by either pyridine nucleotide at high concentrations.

dehydrogenase activities will not be limited by these substrates. Accordingly, the concentrations chosen for the ETS assay were 1 mM NADH and 0.15 mM NADPH. The same pattern was found with the corresponding values from the diaphorase kinetics experiments (Figure 4.10; 4.11).

4.3.4. Diaphorase concentration

Linearity of the enzyme activity with enzyme concentration is an important property in developing an enzyme assay. Figure 4.7, is an example of one of the experiments done to determine if there was linearity. In subsequent experiments, 0.002 U·mg protein^{-1} of diaphorase from *C. kluyvery* was used. This was the minimum concentration we could accurately obtain and the minimum concentration that would also assure a strong reaction signal. With this concentration selection we complied with the suggestion of Bisswanger (2011) that in enzymatic assays, the enzyme concentration should be "as low as possible" ([enzyme]<<[substrate]), but "must be sufficient to detect initial velocity". Also, this value is above the limit of detection found by Catomeris and Thibert (1988) i.e., 10^{-4} units of enzyme activity. From a practical point of view, this is important because enzymes are expensive and need to be prepared each time an experiment is performed.

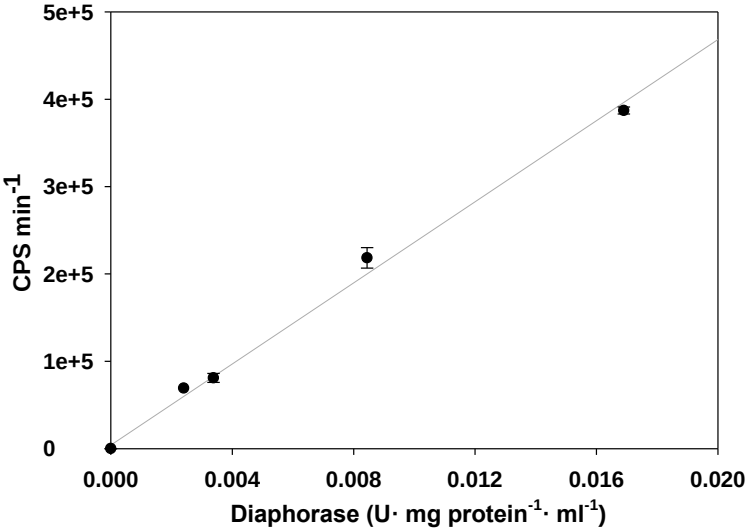


Figure 4.7. Fluorescence intensity production with time, at different diaphorase concentrations. Linearity without limitation or interference was observed ($y= 2.37e^7 + 4.08\ e^3$; $r^2 =0.993$) between zero and 0.016 U (mg protein)$^{-1}$ ml^{-1}.

4.3.5. Optimum resazurin –resorufin concentrations

The dependence of fluorescence on the resazurin concentration showed that at some point after 2 µM final concentration in the cuvette, decay of the fluorescence sets in. This is probably caused by self-quenching by the inner-cell effect as reported by Barnes and Spenney (1980), and McNicholl et al, (2007). Linearity was found up to 2 µM ($r^2 =0.996$), after that concentration, fluorescence starts to decay (Figure 4.8 in box). For the assay, 10 µM resazurin was chosen (initial concentration, Table 4.4). This concentration is in the range of the maximum fluorescence signal as reported by Catomeris and Thibert (1988) and Barnes and Spenney (1980).

This is important to have a good balance between having enough dye without being limited by it. During the reaction time (10 minutes) no plateau was reached at any diaphorase or natural-enzyme concentration. This means that resazurin concentrations in the cuvette, between 0 and 2 □M, were not limiting the resorufin formation.

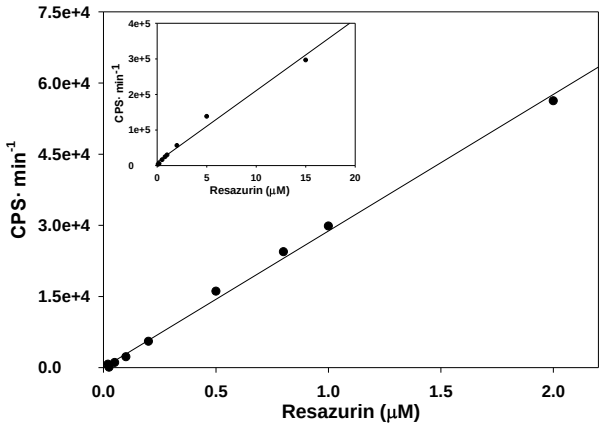


Figure 4.8. Rate of reduction of resazurin to resorufin. In the range tested, in the box is shown the self-quenching by the inner cell effect (y= 2.01e^{4}x + 1.02e4; r^2 = 0.978) starts to show at some point after 5µM. Until 2 µM there is a good linearity (2.88e^4 + 16.3; r^2 = 0.995).

The calibration plot of pure resorufin achieved linearity around 1µM (r^2 =0.999), and was close to 2 µM when self-quenching by the inner cell effect started to manifest itself (Figure 4.9 in box). This was concordant with the resazurin test (Figure 4.8), It is important to note that even though we worked at 2 µM with resazurin, with the enzyme concentrations or biological biomass tested and with the reaction time (~8 min) used, there was no opportunity to produce 2 µM of resorufin, so the inner cell effect

was avoided and sub-estimation of the reaction velocity was evaded.

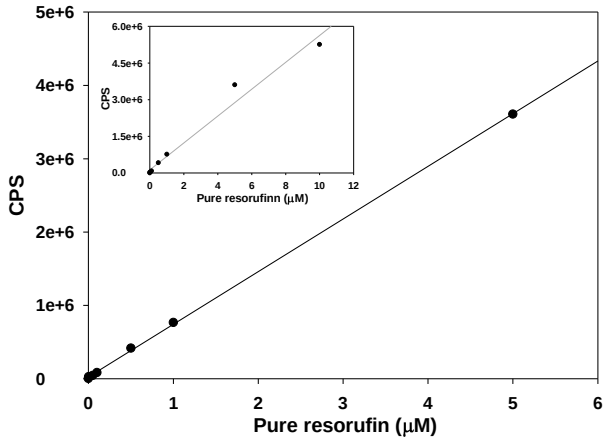


Figure 4.9. Example of a calibration plot of pure resorufin. Linearity was maintained to 5 µM (y= $7.18e^5x + 2.32e^4$; $r^2 = 0.999$). Even though, in box, the fluorescence starts to be quenched at 10 µM (y= $5.50e^5x + 1.30e^5$; $r^2 = 0.974$), this concentration was selected, for subsequent tests. No opportunity to produce this amount of resorufin, so no inner cell effect could be present.

4.3.6. Reproducibility and, Limit of detection (LOD) and Limit of quantification (LOQ)

Repeatability was determined by making eleven measurement of resorufin at two concentrations, 4 µM, the end of the linear range and 1 µM. The Relative Standard Deviation (RSD) in Table 4.7 shows the data varied relative to the mean, but only a variation of 4.1% at 1 µM and 4.9% at 4 µM.

Table 4.7. Statistical variables to determine the reproducibility of the measurements of resorufin under our laboratory conditions. Where $\bar{x}$, is the mean; ***V***, the variance; σ, standard deviation; and **RSD**, the Relative Standard Deviation.

	1 µM	**4 µM**
$\bar{x}$	$1.16e^{6}$	$4.06e^{+6}$
V	$2.31e^{9}$	$3.92e^{10}$
σ	$\pm$ $4.80e^{4}$	$\pm$ $1.98e^{5}$
RSD	4.1%	4.9%

The limits of detection (LOD) and quantification (LOQ), for the spectrofluorometric method were 1.91 and 3.33 nM, respectively. They could not be determined for the spectrophotometric method.

4.3.7. Diaphorase kinetics

It is of primary importance to determine the Michaelis–Menten steady-state enzyme kinetic parameters (K_m and k_{cat}) that characterize the substrate(s) consumed in the reaction. These constants are critical in determining the concentrations needed to perform the enzyme assay. Furthermore, they serve as estimates of the *in vivo* substrate concentrations during times when the cell or organism is operating in a state of standard metabolism.

Unfortunately we were unable to calculate the bi-substrate K_m for diaphorase, since different concentrations of NADH and

NADPH were not low enough to determine the linearity of the reaction (Figure 4.10; 4.11). Despite this, valuable information is obtained concerning the interaction of the substrates during the reaction.

The production of resorufin (resorufin min^{-1}), in the diaphorase reaction, when the NADH concentration varies, and the NADPH stays constant, shows that maximum velocities were attained when the NADPH concentration was zero. It seems that when the NADPH concentration increased the velocity of the reaction decreased, regardless of the NADH concentration (Figure 4.10). This was contrary to what we expected. We had anticipated higher diaphorase activities with increasing concentrations of both NADH and NADPH (cumulative effect of co-substrates), but this occurred only with NADH.

When the activity of the diaphorase (v), was measured as a function of changing NADPH concentrations and keeping NADH constant, we observed, as stated previously, that increasing values of NADPH decreased the velocity of the reaction. Even at low levels of NADPH the reaction velocity decreased to almost 50 % (Figure 4.11). This showed that NADPH somehow inhibits diaphorase activity in the presence of NADH. However when NADH is absent, there is still some activity of the diaphorase, although it is very limited if we compared it to activity levels obtained when there is no NADPH present (Figure 4.10; 4.11).

Thus, diaphorase does react with NADPH, but at much lower rates than with NADH. Additionally, when the two substrates are together, the activity decreases dramatically. Zooplankton also seems to present the same behavior in the presence of NADPH (Figure 4.12, and Osma, Personal communication).

3.3.8. Biological Samples

In order to compare the classic spectrophotometric ETS method (Owens and King, 1975) with this new espectrofluorometric one, two series of experiments were made. The first was with diaphorase, a dehydrogenase capable of oxidizing both pyridine nucleotides (PNs), NADH (Figure 4.1) as well as NADPH. (Shapiro and Silanikove 2010; Silanikove and Shapiro, 2012). For both INT and resazurin, the reaction rate was linear with increasing diaphorase concentrations. Considering the slopes (Figure 4.12a), the resazurin-based detection of ETS activity was ~1.68 times higher than INT-based detection of ETS activity by the spectrophotometric method indicating a clear statistical difference between slopes ($p < 0.01$). In the experiment with zooplankton, done by serial dilution, the slopes were more equal. The spectrofluorometric ratio was only ~1.14 higher than the other one (Figure 4.12). There was no statistical difference between slopes ($p > 0.05$). Even so, one should note that with the INT method, at low biomass, the limit of detection is close to zero while with resazurin the signal remains high (Figure 4.12b).

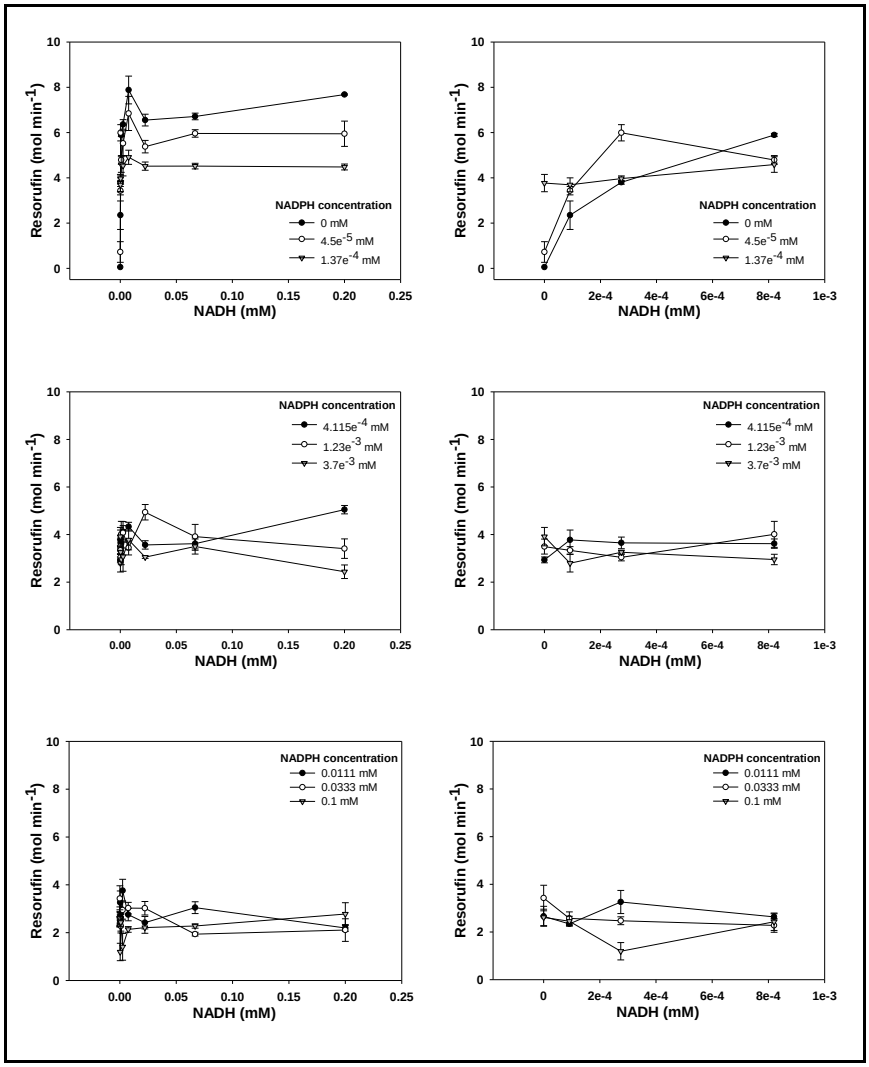


Figure 4.10. Attempt to establish the K_m for diaphorase. The NADH concentrations are maintained while the NADPH changes. The graphics on the left show the entire range of NADH concentrations, while the right one only shows the four lower concentrations of this substrate. It shows that the rate of production of resorufin is proportional to the concentration of NADH. This is clearly evident because as the NADPH concentrations increase, the speed of resorufin production decreases. Notice that the scale on the *y* axis is the same for all plots.

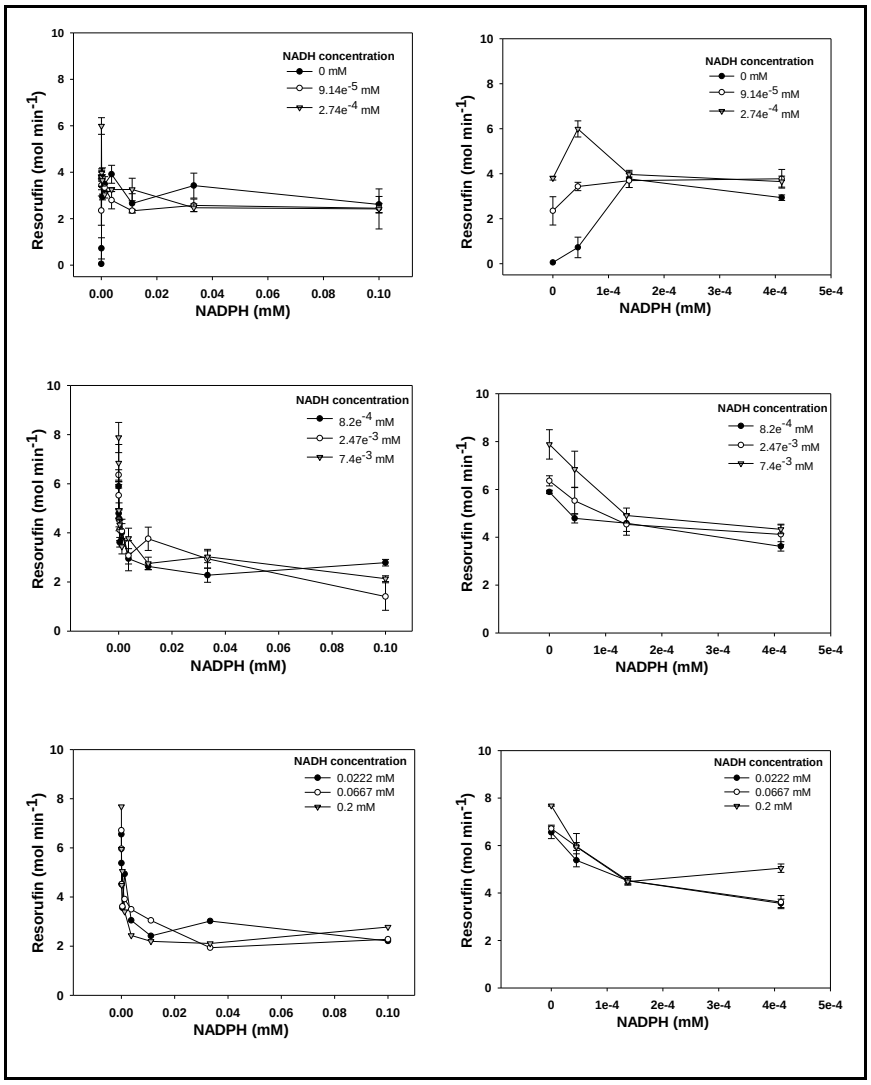


Figure 4.11. Attempt to establish the K_m for diaphorase. The NADPH concentrations are maintained while the NADH changes. The graphics on the left shows the entire range of NADPH concentrations, while the right one only shows the four lower concentrations of this substrate. In the absence of NADPH the resorufin production is proportional to the NADH concentration. But once the NADPH is added, production rates stagnate independently of the concentrations of NADH. Notice that the scale in the *y* axis is the same for all the plots.

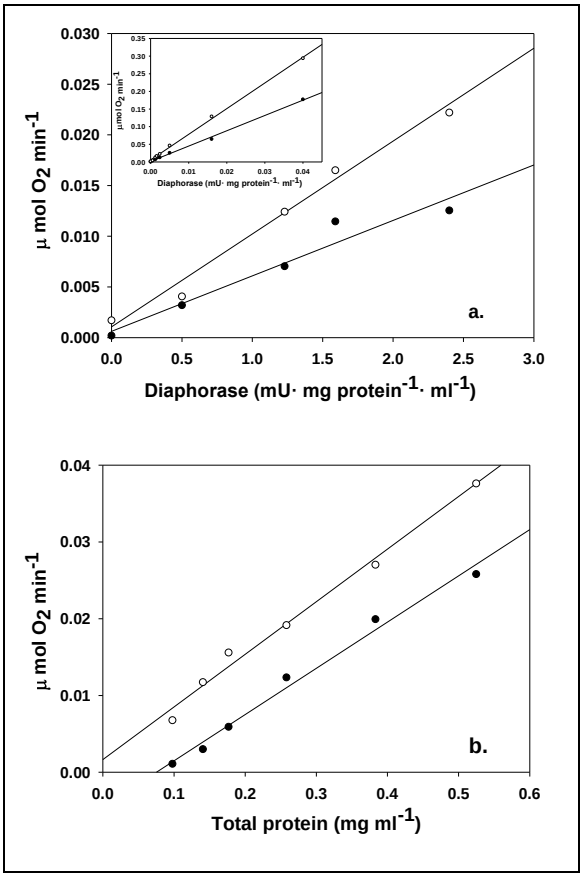


Figure 4.12. Φ comparison using INT (black circles) or resazurin (open circles) as electron acceptors in ETS assays. In **a.**, Φ as a function of different diaphorase concentrations. In both methods linearity with the increasing concentration of diaphorase was observed; r^2 =0.94 and 0.98 for the low concentrations and r^2 =0.997 and 0.995 for the whole range respectively. The resazurin method yields higher Φ and a higher sensibility at all concentrations, in both ranges the ratio between slopes was 1.68. In **b**, as in a, but with zooplankton homogenate dilutions providing the range of enzymatic catalysis instead of diaphorase. Here both methods shared the same correlation with biomass (r^2 =0.987), with slopes of 0.060 for INT and 0.068 for resazurin, this is a ratio Resazurin:INT of 1.14.

The literature suggests that the substrate activities are additive to the total ETS (Packard et al., 1971; Garfield et al., 1979; Hobbie et al., 1972; Christensen and Packard, 1977; Gómez et al., 1996). But, during the previous experiment and before analyzing the K_m results we had some concern about the role of NADPH on the ETS reaction. To investigate this, a simple experiment was developed. NADH and NADPH were tested by themselves with a series of diaphorase and mysid homogenate concentrations. This was done for both methods to assure the result was not a methods artefact (Figure 4.13).

Initially the behavior of the ETS in the two assays was expected to be similar, whatever the combination of substrates with diaphorase or mysid homogenate But it was not the case. There was no difference in the substrate reduction by diaphorase in presence of INT or resazurin (Figure 4.13, left). However, with the Mysids, NADPH stimulated virtually no activity with either of the two methods. Furthermore, when the two substrates were together the signal was not additive. When NADPH was at 0.1 mM the inhibition was higher than when it was at 0.42 µM, this argued that NADPH inhibited the reduction of the electron acceptor in both methods, especially in mysid homogenates (Figure. 4.11). In both methods, the NADPH did not contribute to the reaction, especially in mysids. In any case, the fluorescent method always showed higher ETS activities. The same pattern was found in the bi-substrate K_m determinations (Figure 4.10; 4.11).

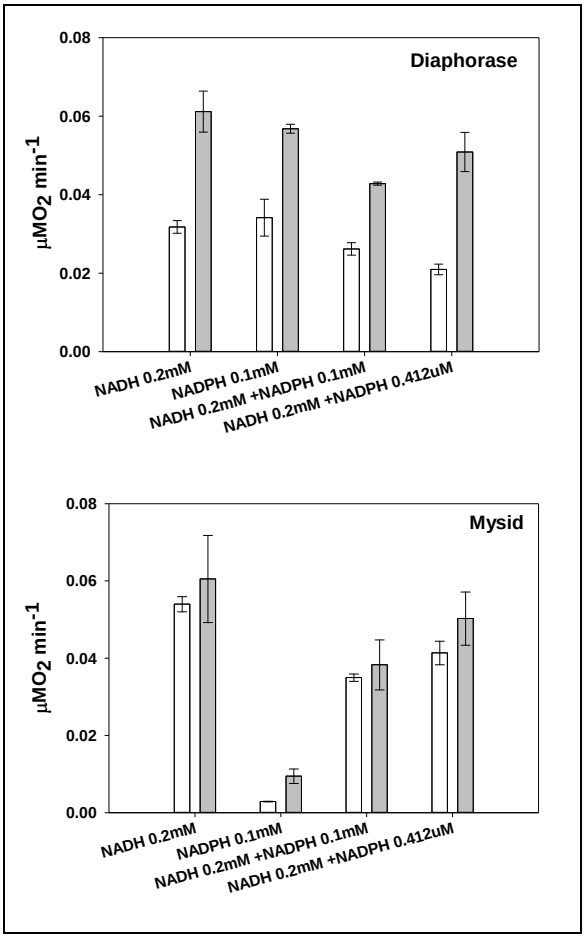


Figure 4.13. Effect of NADPH on the ETS signal from diaphorase-catalyzed reactions and in reactions based on a mysid homogenate. The spectrophotometric assay using, INT (white bars) and the spectrofluorometric assay using resazurin (gray bars) were compared.

4.4 Discussion

4.4.1. Components assembly

There is some inconsistency in literature about the emission and excitation wavelengths of resorufin. According to Sigma-Aldrich, the excitation wavelengths (λ_{ex}) of resorufin in Tris-HCl buffer at pH = 8.0 range from 550 to 572 nm and between 583 and 585 nm for the emission. Researchers found the emission wavelength (λ_{em}) to be around 590 nm (Zhou et al., 1997; Maeda et al., 2000; Rasmussen, 1999; Perrot et al., 2003). In our laboratory the values of λ_{em} and λ_{ex} for Rp and Rr determinations fell between the values reported in the literature. Also, it is important to establish that the background buffered solution, during resazurin reduction, does not interfere with the reaction. This chemical neutrality would guarantee the specific detection of resorufin.

Accordingly, resorufin fluorescence was tested for different concentrations of R_p and R_r with the finding that the relative fluorescence units were almost identical (data not shown). The R_r, was confirmed with R_p, with a high correlation (r^2 =0.999) which demonstrated that both compounds were comparable for the calculation of resorufin production during an enzymatic reaction.

There are many studies that use resazurin as an indicator of cell viability (Table 4.1). A few of these studies do try to use resazurin

reduction as a proxy for oxygen consumption (respiration) like the ones of Abu-Amero and Bosley (2005) who introduced a noninvasive method for detecting oxidative phosphorylation activity (without isolating mitochondrial fractions) in order to detect human mitochondrial respiratory dysfunction in circulating lymphocytes. Zhang et al. (2004) developed a mechanism for measuring *in vitro* metabolic activity of isolated mitochondria from rat liver using resazurin as an indicator of mitochondrial activity. Unfortunately, these studies did not quantify O_2 consumption in mitochondria; they only measured mitochondrial metabolic capacity for producing resorufin.

Oxygen (O_2) has a high redox potential (+820 mV) which gives it the ability to oxidized substances with lower redox potentials. In our resazurin-reduction reaction, oxygen did not compete for the electrons in the reaction mixture, (Figure 4.3). This indicated that we were measuring the maximum velocities of the reduction reaction. In addition it simplified the interpretation of the electron flux during the enzymatic reaction and suggested that cytochrome oxidase was pulling electrons in the direction of resazurin, this idea is developed below.

Succinate is known to contribute reducing equivalents to the ETS, as in *Artemia salina* (Packard and Taylor, 1968). Other studies showed it to be a minor player in the respiration of oceanic plankton (Kenner and Ahmed, 1975; Gómez et al., 1996; Savenkoff et al., 1995). In attempting to determine an optimum succinate

concentration, we found that it had no impact on the overall ETS activity. Furthermore, when succinate was in the substrate buffer with NADH and NADPH, as recommended by Kenner and Ahmed, (1975) for phytoplankton or by Gómez et al., (1996) for zooplankton, the results showed that it did not contribute to the reduction of resazurin via diaphorase catalysis (Figure 4.12). Consequently succinate was eliminated from the substrate mixture for zooplankton.

A broad range of concentrations for NADH and NADPH was examined starting with the midpoint concentrations reported by Owens and King (1975) (Table 4.5). In this range there was no evidence of substrate limitation. Accordingly, for economic reasons, we decided to work with concentration slightly lower. In this way, the cost of an ETS assay was reduced without losing the characteristics of a quality enzymatic reaction.

4.4.2. Biological Samples

When comparing the two methods with two different enzyme sources (diaphorase, and mysids), the espectrofluorometric method was ~1.68 times more sensitive with diaphorase than the spectrophotometric one and ~1.14 times more sensitive with a mysid (Figure 4.12). Although in the mysid, when we examined individual measurements, some curiosities were revealed. At the three higher protein concentrations this ratio was only 1.5; the next two protein concentrations were 3.3; while at the lower one, the

signal difference was 6.24 times higher. Both methods responded clearly to the ETS activity and to its relation with biomass, but the resazurin method was more sensitive than the INT method at low biomass (enzyme) concentrations. This sensitivity became even more apparent by the high intercept on the fluorescence (*Y*) axis (Figure 4.12a). In this case, when we had biomasses less than 0.1 mg prot. ml^{-1}, we found that the INT method failed to detect ETS activity. This was also reflected in the LOD and LOQ values, where in the spectrophotometric method they could not be detected (see results). It was the opposite with the spectrofluorometric method (1.91 and 3.34 nM respectively).

The importance of the redox potentials lies in the fact that the introduction of an artificial electron-acceptor (redox indicator) with redox potential intermediate between any two members of the electron transport chain can interrupt the flow of electrons. Thus, whenever a substrate is oxidized in the presence of a tetrazolium salt (like INT), the released electrons will be trapped, and not transported through the usual sequence of cytochromes. This shuts down the respiratory chain, but records its electron flux. Resazurin is intermediate only between final reduction of O_2 and cytochrome oxidase (Cyt.a3) (Alamar Blue Arcus Biologicals). Resazurin is reduced instead of oxygen and may substitute for O_2 for any of the oxidoreductases that routinely utilize molecular oxygen as an electron acceptor. This could explain the differences in the slopes of both methods. It could be that with zooplankton, the integrated enzymatic apparatus of the ETS (complex I, II, IV, ubiquinone, cytochromes) influences the flow of electrons in the reaction, and

consequently the velocity of reduction of the artificial acceptor, regardless of whether it is resazurin or INT. This would lead to minimal differences between the slopes (p-value 0.3151). While, with diaphorase it could be a direct substrate-electron acceptor reaction via diaphorase, in other words it could be a possible measurement of "redox status" of the solution (Matsumo et al., 1990 Rasmussen, 1999; O'Brien et al., 2000; Candeias et al., 1998).

This is also connected to the susceptibility to redox potentials. According to Twigg (1945) and Rasmussen (1999) resazurin accepts electrons from the ETS only between cytochrome oxidase (Cyt a_3) and O_2. This is because of its high redox potential (E^0 +0.38 V). On the other hand, tetrazolium salts have low redox potentials. For example, MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenylte-tetrazolium bromide) reacts at E^0 = -0.11 V; TTC, at -0.8 V (Barnes, 1956); and INT, at -0.09V (Smith and McFeters, 1997). Slater et al, (1963) found that the point of interaction for INT is Cyt c. Also tetrazolium salts, in general, in histochemical studies, showed low specificity (Hope and Vicent, 1989). Following the previous idea, INT will not be reduced by the cytochromes (Smith and McFeters, 1997) as is resazurin. This means that with tetrazolium salts, such as INT, the electrons will not cover all the ETS components. But in zooplankton, this does not happen because the slopes between methods are the same, possibly indicating that the gatekeeper of the electron transport system is substrates-complex I This difference in the point in which the electron acceptor acts, can explain the difference that exist in the

oxygen consumption signals, between the spectrophometric method and the spectrofluorometric one in the presence of diaphorase (Figure 4.12; 4.13).

When we started this research, we believed that diaphorase, as oxidizer of β-NADH or β-NADPH in the presence of an electron acceptor, as the Sigma-Aldrich company claims, could have been used as a standard for ETS activity measurements. But as we learned, diaphorase (EC: 1.8.1.4) does not exhibit the same reaction characteristics that mysid homogenates exhibit (Figure 4.12; 4.13). Also, in studying the effect of NADPH on the ETS signal, we found that with diaphorase, high levels of activity were present regardless of the pyridine nucleotide or detection method used (Figure 4.13). With mysids, NADPH stimulated virtually no activity in either of the two methods (Figure 4.13). This was also described for zooplankton by Gómez et al, (1996) and Savenkoff et al, (1995). Furthermore, when the two substrates were used together the ETS activity was not additive as suggested by Gómez et al, (1996) and Borgmann (1976). There was inhibition that seems proportional to the addition of NADPH in both methods (Figure 4.10). The same pattern was found when we attempted to calculate the bi-substrate kinetics of the diaphorase (Figure 4.10; 4.11). In any case, the fluorescent method always showed higher ETS activities.

One of the objectives of ETS research is to learn the relationship between enzymatic measurements and the rate of oxygen

consumption (eg. Chance and Williams, 1956; Packard, 1971; Ikeda, 1988; Packard et al., 1996; Madon et al., 1998; Herrera et al., 2011). In this study we found there is no difference between methods (Figure 4.12). The fluorescence-based measurement of ETS activity in zooplankton (mysids) is simple, viable, and promising for the future detection of ϕ in the oceans.

4. 5. Summary

1. Compared to each assay's limit of detection, the resorufin signal was always higher than the formazan signal. The espetrofluorometric method presented here more reliably detects low levels of ETS activity than does the spectrophotometric one.

2. In both assays, NADPH, only weakly, contributes to the ETS signal in mysid homogenates.

3. NADPH in the presence of NADH seems to make no contribution on the totality of the ETS signal. It is inhibitory.

4. ETS activity in mysid homogenates, as measured by either the spectrophotometric or the espectrofluorometric method was not enhanced by succinate.

5. The fluorescent ETS method was not quenched by oxygen.

Chapter 5

Respiratory Electron Transport Activities in oceanic samples and in different marine organisms: Comparison of methods and the influence of different substrates

Maldonado F., Gomez, M., and Packard, T.

Plankton Ecophysiology Group, Instituto de Oceanografía y Cambio Global, Universidad de Las Palmas de Gran Canaria, Canary Islands, Spain

Abstract

Potential respiration measurements vary, depending on the substrates (NADH, NADPH and succinate) present in the *in vitro* reaction and depending on the organism. Here we examine this variability in aquatic organisms. Six different species were analyzed, recently hatch brine shrimp *(Artemia salina),* young seahorse (*Hippocampus guttulatus*), gilt-head bream (*Sparus* aurata)*,* abolone *(Haliotis tuberculata* spp.) and mussels (*Mytilus sp.*). In addition we analyzed oceanic microplankton samples from

the Canary waters between 5 and 600 meters. All ETS analyses were made with two methods, the classic spectrophotometric method using the tetrazolium electron acceptor, INT, and a spectrofluorometric method that uses the fluorescent resazurin as electron acceptor. Both detect electron flux in cell-free homogenates. In all the measurements, the fluorometric results were higher. In the organisms they ranged 1.13 to 5.16 times higher than in analyses by the spectrophotometric method, in the ocean depth-profile samples, they ranged from 1.14 to 12.07 times higher. The large differences were due to the high sensitivity of the fluorometric method at the low biomasses. We determined that multiple ETS substrates were not additive to the ETS signal, and that the V_{max} is dependent on the species or sample as well as on the substrates added to the ETS reaction. In the majority of samples, NADPH and succinate do not contribute to the total *in vitro* enzymatic reaction and in some ocean samples NADPH had an inhibitory effect. This variability can be signals of the metabolic diversity in spite of the universality of the ETS.

5.1. Introduction

From respiration in the ocean water column one can calculate carbon flux, nutrient retention efficiency, deep-ocean carbon sequestration, and benthic respiration in different oceanic ecosystems. Just as it is important to determine respiration as a function of environmental conditions in the planktonic community, it

is also important to conduct similar investigations in select organisms. In this way variations in ecosystem respiration will be better understood. However, the *in vivo* measurement of the plankton community respiration as well as the respiration of select organisms is often difficult and laborious, at best and impossible, at worst. In any case, they can never be made on time and space scales useful for oceanography. Consequently the ETS method (Packard, 1969; Owens and King, 1975; Packard and Christensen, 2004) is used for its ease, reliability, and high data acquisition rate. This *in vitro* biochemical method measures the electron flux, from the metabolism of fats, proteins, carbohydrates, to oxygen (or another environmentally-provided electron-acceptor). This flux is the potential respiration (V_{max}). The term, *in vitro*, is used because the flux is measured in a test-tube under optimal conditions of the ETS. The ETS measurements are made on selected planktonic or benthic organisms, on zooplankton net samples, or on the microplankton and bacterioplankton filtered from seawater samples by glass-fiber filters.

In the previous chapter, we introduced a promising fluorometric variation of the method based on a different electron acceptor, resazurin. Here we compare the original spectrophotometric method with the new fluorometric one in different marine phyla as well as in microplankton-rich seawater ranging from the sea surface to 600 m. In these seawater samples 5 l were filtered, half to eighth times less volume than usual for this type of sample (Packard et al., 1971; Hobbie et al., 1972; Packard and Williams,

1981; Packard et al., 1983b; Arístegui et al., 2005). These experiments were designed to demonstrate the sensitivity of fluorometric analysis over spectrophotometric analysis.

When microplankton-rich seawater or zooplankton samples are taken and analyzed, it is assumed that the ETS activity in all the organisms that comprise the sample, behaves the same way. Although, ETS is a universal enzyme system that is share by the three domains of life share (Tzagoloff, 1982; Packard, 1985a, b; Nelson and Cox, 2008) nevertheless, it is likely that there are some metabolic variations between taxonomic groups. We understand that to perform enzymatic measurements on each group of organisms in a plankton community on board an oceanographic cruise is an unthinkable task. Therefore, it is crucial to make laboratory measurements to help interpret the results from natural samples. Previous results suggested variability caused by different ETS substrates on the ETS reaction kinetics. Here, we investigate this variability by applying the new fluorescent ETS method (F_m) introduced by Maldonado et al, (in preparation) and comparing the results with the classic spectrophotometric ETS method (S_m) (Owens and King, 1975). This will be done with natural seawater samples and with different marine organisms using known ETS substrates (NADH, NADPH and succinate) in the *in vitro* ETS reaction.

5.2. Materials and methods

5.2.1. Samples acquisition

5.2.1.1 Marine organisms

To compare ETS activity among different marine organisms, ETS activity was measured in the brine shrimp, *Artemia salina;* male mysidacean (*Leptomysis* sp.), newly hatched seahorses, *Hippocampus guttulatus*; in the gilt-head bream larvae, *Sparus aurata*; the abolone, *Haliotis tuberculata*; and in the marine mussel, *Mytilus* sp. The artemia were obtained from the EOMAR hatching laboratory (Herrera et al., 2011); the seahorses, the gilt-head bream larvae, the abalone, and the *mussels, from* the GIA-ECOAQUA. Duplicate analyses were made on each sample.

5.2.1.2 Seawater samples

Although transparent to the eye, seawater is rich in microorganisms, viruses, archaea, bacteria, protozoans, green-flagellates, diatoms, coccolithophorids, dinoflagellates, copepods, and nauplii of all types of zooplankton and benthonic organisms. All except the viruses respire and have an electron transport system. When several liters of seawater are filtered through a glass-fiber filter, these minute organisms were caught on the fabric of the filter. Then, when the filter was sonified or macerated in an Elvehjem-Potter teflon-glass tissue-grinder, the respiratory ETS of all these organisms was released into a cell-free homogenate and

easily assayed for its capacity to transfer electrons from the reactions of intermediary metabolism to oxygen. Accordingly, such seawater samples were collected by the German oceanographic research ship, the R/V Poseidon, at a location (station) in Taliarte Bay, south of Las Palmas de Gran Canaria, Spain, during February 22-23 of 2014. The ship was conducting the field part of the German BIOACID Research Project (http://www.bioacid.de). Samples were taken from 5m to 600 m, every hundred meter from depth until 100m and then from there at 75, 50, 25 and 5 m. Five liters were sampled per depth and filtered through Whatman GF/F glass-fiber filters (0.2 µm pore-size) on board the vessel. Filters were folded, dried of excess seawater, and preserved immediately in liquid nitrogen. Once in the laboratory of the "Grupo de Ecofisiología de Organismos Marinos" (EOMAR) at the "Universidad de las Palmas de Gran Canaria" (ULPGC), the samples were stored at -80 °C.

5.2.2. Electron Transport System analysis

All the samples were measured for ETS activity by both the classical spectrophotometric method (S_m) according to Packard (1971), Owens and King (1975), Gómez et al. (1996), and Packard and Christensen (2004) and the new fluorometric one (F_m) according to Maldonaldo et al. (in preparation, Chapter 4 in this Thesis) (Table 5.1). When reported in oxygen units (µM O_2) in contrast to electron units (µM e^-) ETS is called potential respiration (ϕ). The spectrophotometric measurements were done with a

Beckman DU® 650 using 1 cm (light path) disposable plastic spectrophotometer Semi-Micro cuvettes. Fluorescence measurements were made with a Horiba FluoroMax-4 Spectrofluorometer equipped with a xenon lamp. Only the sample signal was taken into account (S). Lamp checks were done periodically to assure the lamp's performance. Helma® GS quartz fluorometer cuvettes (1.5 ml) were used. In both methods temperature was maintained at *in situ* levels by water baths. Reactions were followed kinetically for eight minutes (Packard and Christensen, 2004).

To promote accurate comparison of the two methods, especially at low biomass concentrations, measurements were based on the same homogenate (the sample is homogenized in cold Milli-Q water to avoid interferences with the different type of buffers in each method). In all samples, two separate assays of electron flux were made, one with all the 3 substrates (SB) and the other with only NADH added as a substrate. The first assay is the classic ETS assay made according to the kinetic method of Packard and Christensen (2004). The second assay is a measure of the activity of Complex I of the respiratory ETS; technically it is NADH-INT oxidoreductase. Savenkoff et al. (1996) and Maldonado et al. (2012) refer to it as NADH-dehydrogenase.

Measuring to two types of electron flux enabled us to determine if the activity with the different substrates was additive or if a

Table 5.1. Comparison of reagents used in the spectrophotometric method (S_m) and the fluorometric method (F_m) method. Succinate is present in both methods.

	F_m	S_m
Homogenate buffer		
Phosphate (M)	-	0.1
Tris (M)	0.1	-
pH	8.0	8.5
PVP (mg prot.·ml^{-1})	1.5	1.5
Mg prot.SO_4 (µM)	75	75
Triton X-100 (ml·l^{-1})	-	2
Electron acceptor		
INT (mg prot.·ml^{-1})	-	2
Resazurin (µM)	10	-
Substrate buffer		
NADH (mM)	1.0	1.7
NADPH (mM)	0.15	0.25
Succinate (M)	0.133	0.133

substrate had an inhibitory effect. Note that we are trying to maintain the convention started by Packard and Christensen (2004) in which ETS activity is reported in units of µM electrons while potential respiration is reported in units of µM oxygen. This destinction was not made in the older literature where ETS activity was often reported in units of oxygen. To compare ϕ with ETS activities from the older literature no conversion factor is necessary. At least for the same methodology, these older ETS activities and ϕ are equivalent. If the methodology is different then the conversion factors of Christensen and Packard (1979) must be considered.

5.2.3. Statistical analysis

For each sample, differences in ϕ between methods (F_m versus S_m) and substrate condition (NADH versus SB) were tested by a 2 factor ANOVA. Data was log (x+1) transformed for sea horse samples to achieve homogeneous variances; the rest of the species conformed to the assumption of homogenous variances (Bartlett test, $p > 0.05$). Data was analyzed using R Development Core Team 2010 (R Foundation for Statistical Computing, Vienna, Austria).

5.3. Results

All respiration values reported in this chapter are potential (ϕ). With respect to the different ETS substrates, both the spectrophotometric (S_m) and the fluorometric (F_m) methods showed the same pattern within different marine organisms (Figure 5.1 to 5.6; Table 5.2). The F_m method always gave significantly higher values with respect to the S_m ($p < 0.05$); Figures 5.1 to 5.6; Table 5.2), in all organisms except the mussel, regardless of the substrate condition. Within the same method there was a slight tendency for the electron flux to be higher with NADH, with statistical differences ($p < 0.05$) in the gilt-head bream and abalone. The only exception was in the seahorse, *H. guttulatus*, where the

highest ϕ were found when all three substrates (SB) were present ($p < 0.05$).

For $F_{m,}$ (Table 5.2), with NADH donating electrons, the lowest ϕ was in *H. guttulatus* (1.62 µmols O_2 h^{-1}) while with the SB, it was in *S. aurata* (1.11 µmols O_2 h^{-1}). The highest value for NADH and SB with F_m, was in *H. tuberculata* (19.87 and 6.59 µmols O_2 h^{-1} respectively). For the S_m, the lowest ϕ with NADH and the SB was from *H. guttulatus* (0.55 and 0.59 µmols O_2 h^{-1} respectively) while the highest were from the mussel (4.51 and 4.37 µmols O_2 h^{-1}, respectively). From the point of view of the specific activity, the lowest values with F_m were from *S. aurata* ($6.7x10^{-3}$ and $4.3x10^{-3}$ µmols O_2 µg protein^{-1} h^{-1}, respectively) and the highest were from *H. tuberculata* oxidizing NADH ($4.9x10^{-2}$ µmols O_2 µg protein^{-1} h^{-1}) and from the mussel oxidizing the SB ($1.7x10^{-2}$ µmols O_2 µg protein^{-1} h^{-1}). For the S_m, the lowest specific ϕ values were from *S. aurata* ($3.4x10^{-3}$ and $3.8x10^{-3}$ µmols O_2 µg protein^{-1} h^{-1}, respectively) and the highest were from the mussel ($1.5x10^{-2}$ µmols O_2 µg protein^{-1} h^{-1} both) (Table 5.2).

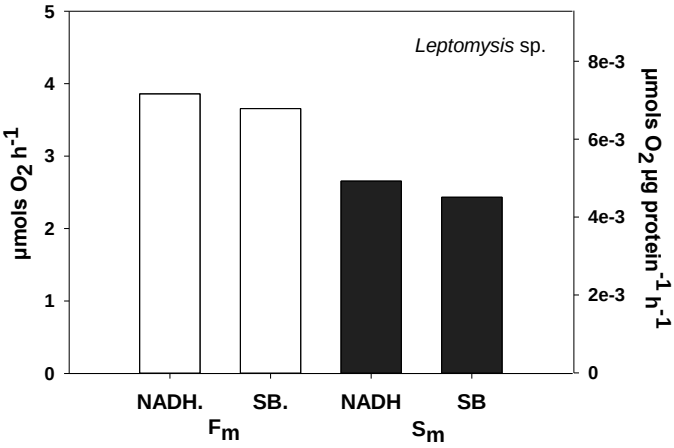


Figure 5.1. ϕ (µmols O_2 h^{-1}) and specific ϕ (µmols O_2 µg protein^{-1} h^{-1}) of four male mysids. Even when there was no statistical differences ($p > 0.05$), the ϕ values were slightly higher with NADH in both methods. The F_m values for ϕ were 1.5 higher ($p < 0.05$).than those from the S_m in both substrate conditions.

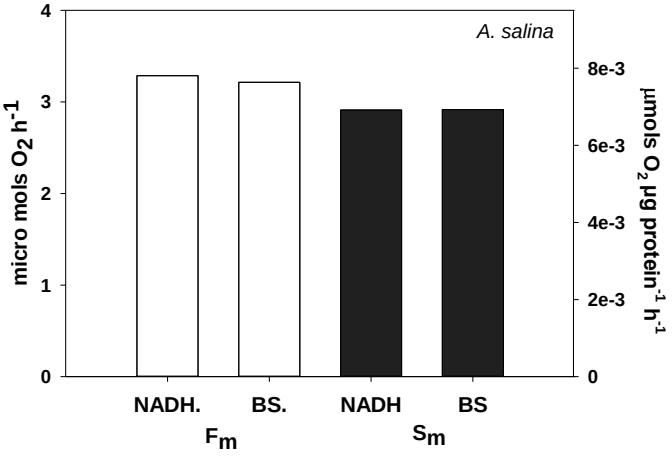


Figure 5.2. ϕ (µmols O_2 h^{-1}) and specific ϕ (µmols O_2 µg protein^{-1} h^{-1}) of *A. salina*. There was no difference in both methods in relation to the substrate scenarios ($p > 0.05$). Indicating no contribution of NADPH or succinate to the reaction. The F_m signals were approximately 1.1 higher than those from the S_m ($p < 0.05$).

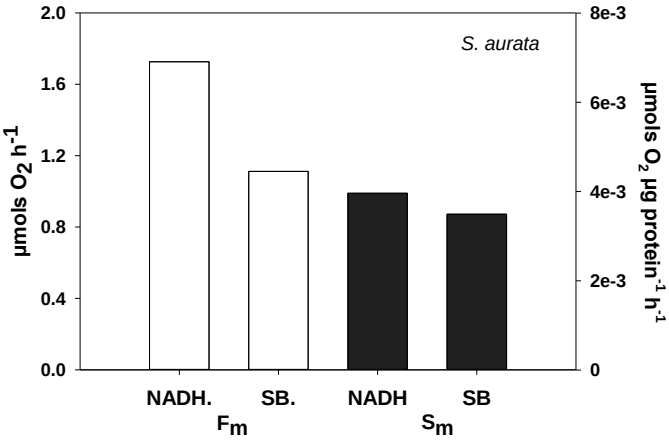


Figure 5.3. ϕ (µmols O_2 h^{-1}) and specific ϕ (µmols O_2 µg $protein^{-1}$ h^{-1}) of the fish larvae *S. auratus*. Within the F_m and S_m there was a statistical difference ($p < 0.05$) between substrate conditions. With F_m there were higher ϕ rates with NADH, 1.55 times higher than SB ($p > 0.05$). The F_m signals were approximately 1.74 higher with NADH and 1.28 with SB respect S_m ($p < 0.05$).

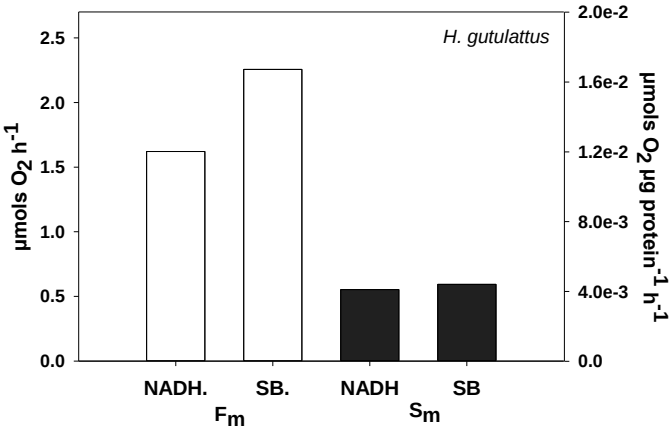


Figure 5.4. ϕ (µmols O_2 h^{-1}) and specific ϕ (µmols O_2 µg $protein^{-1}$ h^{-1}) of the seahorse *H. gutulatus*. With S_m the difference was lower than with ($p > 0.05$). With F_m the signal was 1.39 times higher with SB than with NADH alone ($p < 0.05$). The F_m signals were approximately 2.93 higher with NADH and 3.8 higher with SB as compared to the S_m results($p < 0.05$).

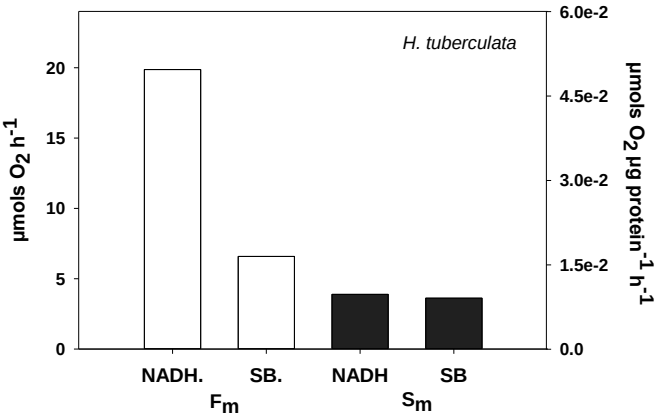


Figure 5.5. ϕ (µmoles O_2 h^{-1}) and specific ϕ (µmols O_2 µg $protein^{-1}$ h^{-1}) of the abalone *H. tuberculata*. In the S_m there is a small difference ($p < 0.05$). Between substrates however, with the Fm, the values for ϕ with NADH were 3 times higher than with SB ($p < 0.05$). There is a considerable difference between methods and substrate conditions ($p < 0.05$).

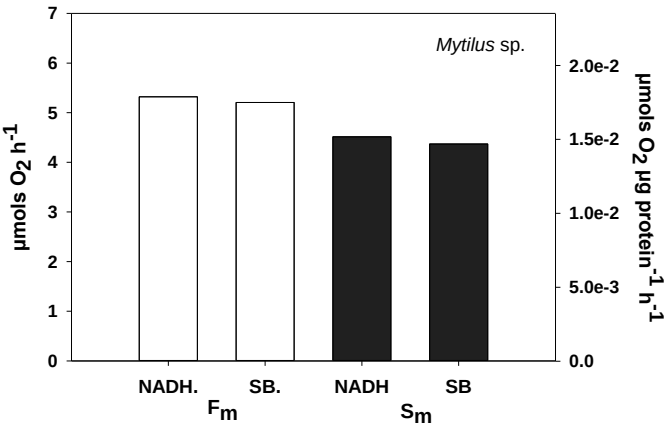


Figure 5.6. ϕ (µmols O_2 h^{-1}) and specific ϕ (µmols O_2 µg $protein^{-1}$ h^{-1}) of the mussel, *Mytilus* sp. The F_m values for ϕ were approximately 1.19 higher than with S_m, under both substrate conditions. Even so, there was no statistical difference between substrate scenarios inside each method and between methods ($p > 0.05$).

In order to detect the impact on ϕ caused by differences in the methods and in the substrates, especially in oceanic waters where biomass is scarce, we filtered 5 l of seawater for each sample. This is much less seawater than is used for the usual seawater ETS measurements (Packard et al., 1971; Hobbie et al., 1972; Packard and Williams, 1981; Packard et al., 1983b; Arístegui et al., 2005) and is a challenge to the sensitivity of both methods (Undenfriend, 1962; Cook and Self, 1993; Passonneau and Lowry 1993; Bisswanger, 2011).

Eight measurements were made in the first 100 m of the seawater column. With the S_m, all signals were very low, but the NADH dehydrogenase activity was higher ($p < 0.05$) than the total ϕ activity, although both did follow the same behavior (Table 5.2; Figure 5.8). Specific ϕ based on the complete substrate mix (SB) ranged randomly from 2.17×10^{-4} to 4.08×10^{-4} µmoles O_2 h^{-1} μg^{-1} l^{-1} while NADH-dh ranged between 2.57×10^{-4} to 5.36×10^{-4} µmoles O_2 h^{-1} μg^{-1} l^{-1}. Two peaks were present, one at 100, the other at 300 m (Figure 5.8). With the F_m, values based on the catalytic action of NADH-dh oscillated between 4.24×10^{-4} to 4.48×10^{-3} µmoles O_2 h^{-1} μg^{-1} l^{-1} while for ϕ, the results ranged between 8.67×10^{-4} and 4.37×10^{-3} µmoles O_2 h^{-1} μg^{-1} l^{-1}; both enzyme activity profiles peaked at 100 m. When the two methods were compared the F_m ϕ signal was one order of magnitude higher ($p < 0.05$) than with the S_m (Table 5.2 and 5.3; Figure 5.8).

The NADH:SB ratio, can give routing information about the electron flux between the different complexes of the ETS. When this ratio is close to one it can be inferred that NADPH and succinate are not contributing to the reaction. This means that Complex 2 (SDH) of the mitochondrial ETS, the eukaryotic microsomal ETS, and the NADPH:NADH exchange shuttles are not being used. This is especially evident for the organisms and the depth profiles measured with the S_m. Values less than one indicate that NADPH and/or succinate have contributed, in some degree, to the reaction. This was evident with F_m in *H. guttulatus* and in the more near-surface as well as in the deepest seawater samples from the ocean profile. In the opposite case, values higher than one indicated that NADH, by itself, was more effective in detecting ϕ. This was manifested in *H. tuberculata* (Table 5.3; Figure 5.9).

5.4. Discussion

Unfortunately, at the moment of analisis the ULPGC decided to close the laboratory with short notice. I had to make the decision between doing more individual measurements per organisms, or make pseudo-replicas of all available organisms. I decided to take the second scenario because it adjusts more to the objective of the study. This is brings a delicate situation with the stadistic employed, since as noted above, working from a single sample,

Table 5.2. ϕ and specific ϕ comparisson between substrate conditions with fluorometric (F_m) and spectrophotometric (S_m) method.

	µmols O_2 h^{-1}				**µmols O_2 µg protein^{-1} h^{-1}**			
	F_m		**S_m**		**F_m**		**S_m**	
	NADH	**SB**	**NADH**	**SB**	**NADH**	**SB**	**NADH**	**SB**
Leptomysis sp.	3.858	3.655	2.627	2.432	$7.8x10^{-3}$	$6.7x10^{-3}$	$4.9x10^{-3}$	$4.5x10^{-3}$
A. Salina	3.287	3.2143	2.912	2.917	$7.8x10^{-3}$	$7.6x10^{-3}$	$6.9x10^{-3}$	$6.9x10^{-3}$
S. aurata	1.726	1.112	0.989	0.872	$6.7x10^{-3}$	$4.3x10^{-3}$	$3.8x10^{-3}$	$3.4\ x10^{-3}$
H. guttulatus	1.621	2.256	0.553	0.593	$1.2x10^{-2}$	$1.6x10^{-2}$	$4.0\ x10^{-3}$	$4.3\ x10^{-3}$
H. tuberculata	19.871	6.585	3.890	3.621	$4.9x10^{-2}$	$1.6x10^{-2}$	$9.5x10^{-3}$	$8.9x10^{-3}$
Mytilus sp	5.321	5.208	4.514	4.369	$1.8x10^{-2}$	$1.7x10^{-2}$	$1.5x10^{-2}$	$1.5x10^{-2}$

Continuance of table 5.2

	µmols O_2 h^{-1}				µmols O_2 µg protein^{-1} h^{-1}			
	F_m		S_m		F_m		S_m	
	NADH	SB	NADH	SB	NADH	SB	NADH	SB
Depht profile (m)								
5	0.047	0.097	0.041	0.039	$4.24x10^{-4}$	$8.67x10^{-4}$	$3.72x10^{-4}$	$3.45x10^{-4}$
	0.048	0.096	-	0.020	$7.34x10^{-4}$	$1.47x10^{-3}$	-	$3.09x10^{-4}$
25	0.338	0.241	0.043	0.031	$3.89x10^{-3}$	$2.78x10^{-3}$	$4.96x10^{-4}$	$3.57x10^{-4}$
	0.095	0.145	0.025	0.024	$9.69x10^{-4}$	$1.40x10^{-3}$	$2.57x10^{-4}$	$2.43x10^{-4}$
50	0.234	0.232	0.040	0.032	$2.05x10^{-3}$	$2.03x10^{-3}$	$3.48x10^{-4}$	$2.83x10^{-4}$
75	0.344	0.266	0.052	0.033	$3.56x10^{-3}$	$2.75x10^{-3}$	$5.36x10^{-4}$	$3.43x10^{-4}$
	0.290	0.311	0.038	0.029	$4.08x10^{-3}$	$4.37x10^{-3}$	$5.40x10^{-4}$	$4.08x10^{-4}$
100	0.434	0.406	0.040	0.034	$4.48x10^{-3}$	$4.20x10^{-3}$	$4.18x10^{-4}$	$3.48x10^{-4}$
200	0.263	0.509	0.036	0.029	$3.13x10^{-3}$	$3.20x10^{-3}$	$4.28x10^{-4}$	$3.42x10^{-4}$
300	0.202	0.439	0.030	0.021	$3.49x10^{-3}$	$2.65x10^{-3}$	$5.13x10^{-4}$	$3.66x10^{-4}$
400	0.277	0.488	0.032	0.026	$2.94x10^{-3}$	$2.62x10^{-3}$	$3.40x10^{-4}$	$2.73x10^{-4}$
500	0.139	0.393	0.027	0.021	$1.41x10^{-3}$	$2.25x10^{-3}$	$2.79x10^{-4}$	$2.17x10^{-4}$
600	0.105	0.347	0.024	0.019	$1.34x10^{-3}$	$2.37x10^{-3}$	$2.97x10^{-4}$	$2.29x10^{-4}$

meaning that duplicates are pseudoreplicas. Even so the tests were performed to get an idea of the behavior of the data.

The fluorometric method was more sensitive than the spectrophotometric method. In marine organisms the average difference was approximately 1.75 under both substrate conditions (excluding the NADH-dh difference in *H. tuberculate* in F_m). In the natural samples is 6.3 times with NADH and 8.1 times with SB.

For both substrate conditions, this relationship shifted dramatically with the natural samples. With NADH-dh, ϕ ratios ranged from 1.14 to 10.72 and with SB they ranged from 2.51 to 12.07 (Figure 5.9; Table 5.3). This resulted from the low S_m sensitivity at low biomass. The F_m data (Figure 5.8; Table 5.2) were more consistent with the surface ϕ values reported by Packard et al. (1985) for waters from the Peruvian upwelling, and by Hernandez-León et al. (1999) for the Northeastern Central Atlantic microplankton. ϕ values for the upper 150 m of the Canary Islands region according to Arístegui and Montero (2005) and Barton et al. (1998) were more related to the ones done by the S_m in the present study (Table 5.2). This was likely due to similarity in the seawater volumes that they filtered and because these investigators used similar methodology. They followed Kenner and Ahmed, 1975a. With the low biomass normally found in the open ocean, if one used the S_m, details of the ϕ in the water column could be lost, If one were to use the F_m, details of the ϕ in the water column would be detected.

Table 5.3. ϕ specific ratios, between substrate conditions and between methods. The NADH:SB substrate condition ratio indicates the degree of dependence of NADH on the reaction. Values higher than one indicate that the maximum velocity is reached only with NADH, lower than one with the SB and close to one indicate that there is no contribution or inhibition to the reaction due NADPH or succinate.

	Resazurin	**INT**	**Resazurin:INT**	
	NADH:SB	**NADH:SB**	**NADH**	**SB**
Leptomysis sp.	1.16	1.00	1.59	1.49
A. salina	1.03	1.00	1.13	1.10
S. aurata	1.56	1.12	1.76	1.26
H. guttulatus	0.75	0.93	3.00	3.72
H. tuberculata	3.06	1.07	5.16	1.80
Mytilus sp.	1.06	1.00	1.20	1.13
Depht profile (m)				
5	0.49	1.08	1.14	2.51
	0.50	-	-	4.76
25	1.40	1.39	7.84	7.79
	0.69	1.06	3.77	5.76
50	1.01	1.23	5.89	7.17
75	1.29	1.56	6.64	8.02
	0.93	1.32	7.56	10.71
100	1.07	1.20	10.72	12.07
200	0.98	1.25	7.31	9.36
300	1.32	1.40	6.85	7.24
400	1.12	1.25	8.66	9.60
500	0.63	1.29	5.05	10.37
600	0.57	1.30	4.51	10.35

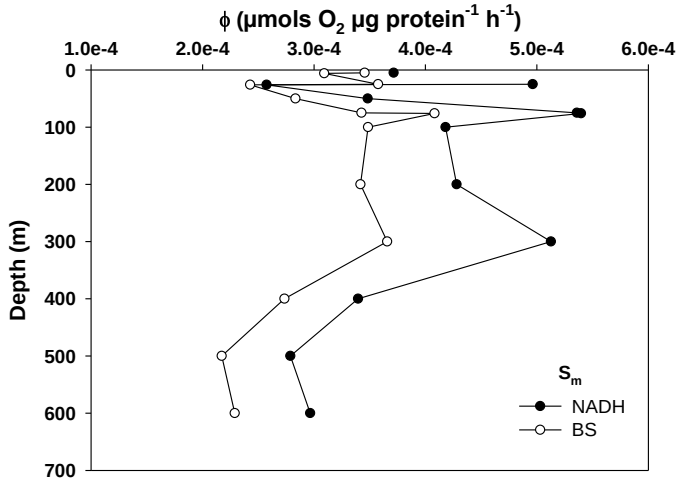


Figure 5.7. Profile of the specific ϕ with the spectrofluorometric method (S_m). The specific ϕ followed the same pattern in both substrate scenarios. With NADH, ϕ was always higher ($p < 0.05$) at all depths except at 25 m.

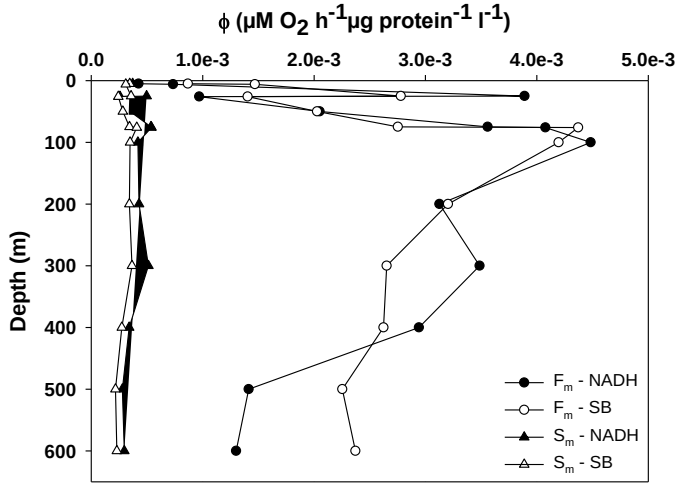


Figure 5.8. Comparison of the profile of ϕ between the spectrophotometric method (triangles) and spectrofluorometric (circles). There was a difference between both methods of one order of magnitude ($p<0.05$). With the F_m, ϕ with NADH, as with SB, followed the same pattern until 200 m ($p> 0.05$), from there ϕ with SB stayed stable while ϕ with NADH declined untill 600m. All differences were statistical significant ($p <0.05$) between substrate conditions, except at 400 m.

As described in the results, depending of sample depth or the organism, the contribution to ϕ by any substrate was relative (Figure 5.1 to 5.8; Table 5.2, 5.3). Such discrepancy can be found also in literature. Toth et al. (1995) found that the principal donor in embryonic stages of *Oryzias latipes (*teleost fish) was NADH and to a lower proportion, NADPH. Savenkoff et al. (1995) found that NADH by itself, contributed more than 85% to ϕ in *Sacaromices cerevisiae* (yeast), *Crypthecodinium cohnii* (dinoflagellate), *Thalassiosira pseudonana* (diatom) and *Praunus flexuosus* (mysid); on the other hand in *Dunaliella tertiolecta* (clrophyta), *P. perfectomarinus* and *P. nautica* (bacteria) NADPH contributed between 56.8 and 68.1% of the total ϕ. In the other organisms, the NADPH contribution fluctuated between 11.8 to 26.5%. Finlay et al. (1983) observed higher contributions to ϕ by NADH in wild protozoa and ciliate *Tetrahymena pyriformis* (77 and 68% respectively) with relatively high NADPH-dh activities (40 and 57% respectively). Kenner and Ahmed (1975b) established that in the marine diatom, *Skeletonema costatum,* tetrazolium reduction (ϕ was higher with NADH while with another marine diatom, *D. tertiolecta* there was more reduction with NADPH.

Christensen and Packard (1979) compared some ETS methods developed at the University of Washington (Seattle). Among the methods compared were the Owens and King (1975) ETS assay developed for zooplankton and the Kenner and Ahmed (1975a) ETS assay developed for phytoplankton. These authors reported their ETS measurements in oxygen units and so they are, effectively, potential respiration measurements. Here, we consider

Owens and King's (1975) results in which the substrate mixture contains only NADH and NADPH and the results from Kenner and Ahmed (1975a) where all three original substrates were used. In the algae, *Chaetoceros debilis,* as well as in the bacteria, *Serratia marinorubra, Pseudomonas denitrificans,* and *Vibrio anguillarum*, there was practically no difference in the ETS activities. In the marine diatom, *Thalassiosira fluvialitis* and in the marine bacterium, *Pseudomonas perfectomarinus,* the activities were slightly higher when three substrates are present. This is concordant with the findings of Savenkoff et al. (1995), but with discrepancies in the percentages. Also, as reported in that study, the ETS values were smaller in the marine diatom, s*keletonema costatum*, and in the marine bacteria, *S. marinorubra* and *V. anguillarum*, when NADPH or succinate were present. Christensen and Packard (1979) reported similar measurements on two natural samples. They measured ϕ with the Kenner and Ahmed (1975) S_m and the combined activities of NADH-dh and NADPH-dh by the S_m of Owens and King (1975). This later method, using only the pyridine nucleotides, measured ϕ in zooplankton because the SDH activity was negligible. Their first measurement was from Puget Sound (Shilshole Bay) where there was no difference between the two different measures of ETS activity. Their second measurement was in Lake Washington (Portage Bay) where the presence of succinate reduced the level of tetrazolium reduction in the samples by 20%. These findings agree with some of our measurements especially in those oceanic samples with zooplankton where the presence of all substrates inhibits potential respiration (eg. Figure 5.1, 5.3, 5.7, 5.8; Table 5.2).

Despite disparities in the literature about what substrates should be in the *in vitro* reaction (Table 5.4), in theory all substrates should be additive for the maximum enzymatic velocity (Packard 1985b; Packard et al. 1996a; Gómez et al., 1996; Savenkoff et al., 1995). The disagreement argues for future chemical and biochemical investigation as in Smith and McFeter (1996). Such research would help interpret the ecological and physiological investigations that have been done in the past. Results from the previous chapter on mysids as well as the other results rom this study suggest that depending of the type of sample, the presence of certain substrates in the reaction mixture is crucial to obtaining the maximum velocity of the reaction. The general reaction mechanism of the ETS is known. However, the reaction *in vitro* does not always give the V_{max} in presence of all substrates (Table 5.2). We conclude that substrate activities are not additive. This same observation has been made by Kenner and Ahmed (1975b) and Toth et al. (1995).

In our studies, here, succinate has little to no contribution in the ETS activity. The same behavior was found by Finlay et al., 1983; Savenkoff et al., 1995; and Toth et al., 1995. Also, in some organisms, NADPH or succinate in some degree inhibits the reaction. In those cases these substrates should be omitted. In others like *H. guttulatus* and in some ocean samples, this combination of substrates is essential. The disparity is still not resolved.

In general, the NADH:SB (the ratio of ϕ µmols O_2 µg protein^{-1} h^{-1} in presence of NADH, to ϕ µmols O_2 µg protein^{-1} h^{-1}based on all three substrates, Table 5.2 and 5.3) explains to what degree the organism or natural samples–are more dependent on NADH or require a combination of substrates. If the ratio is greater than one, the maximum reaction rate is attained with only NADH. Any addition of the other substrates exerts an inhibitory effect on the reaction. On the other hand, if the ratio is less than one, NADPH and/or succinate also contribute to the total reaction. If this ratio is close to one, the data must be interpreted with more detail since it can be assumed that NADPH and/or the succinate do not contribute to the reaction or, on the contrary, have some inhibitory effect. In our case all the scenarios are present (Table 5.2, 5.3; Figure 5.4), making a strong assessment that the maximum reaction velocity depends on the type of sample and the substrates added. So we suggest in order to achieve this V_{max}, a previous test should be done. This conclusion is shared by Madon et al. (1998), who eliminated NADPH because it had so little contribution to the overall ETS activity (≤ 10%). Such exclusions also simplify the analysis and make it less expensive.

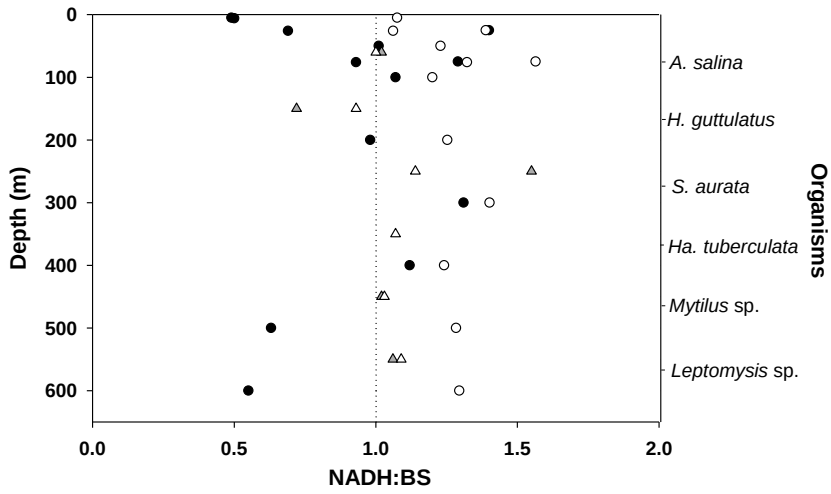


Figure 5.9. Activity ratio between NADH-dh and ETS. Depth profiles based on F_m (●) or S_m (○). Organism metabolism detected by F_m (▲) or by S_m (△). A ratio of one signifies that NADPH or succinate does not contribute to the ETS. Ratios slightly higher than one mean that there is some kind of inhibition of the reaction by NADPH or succinate. Ratios slightly lower mean that NADPH or succinate contribute to the reaction. Ratios much higher than one mean that NADH-dh dominates the reaction; lower than one, mean that NADPH and/or succinate are contributing to the reaction.

A possible explication for the high ϕ result with the abalone *H. tuberculata*, is that the organisms were well-fed. These mollusks are herbivores. At the moment of the homogenization, the suspension turned dark green-brown. Since the F_m is much more sensitive than the S_m, it is possible that the measurement was detecting a combination of the abalone ETS as well as the ETS of the algae in its digestive tract. If these cells were more NADH dependent, it could explain the elevated measurements that we observed. In the case of *H. guttulatus* and with the fluorometric analyses of the deepest seawater samples, we observed something similar. In these cases, the metabolism could have

been more influenced by microsomal metabolism (Strittmatter, 1968) than is normally observed. On the other hand, some NADPH could have been donating electrons to the ETS via transhydrogenase activity (Hatefi, 1973).

There is little doubt about the universality of the ETS biochemistry (Tzagoloff, 1982; Packard, 1985a, b; Nelson and Cox, 2008). However, there are different sites of electron entry that depend on taxonomic differences. The NADH dehydrogenase or complex 1 receives the electrons from the NADH. Almost identical forms of this complex are present in the archaea, bacteria and eukaryotes (Friedrich and Scheide, 2000; Lane, 2015); it is universal. Succinate is normally oxidized at complex II by succinate dehydrogenase. Both complexes I and II pass their electrons to complex III within the matrix of the lipoprotein membrane via ubiquinone. Then Complex III passes them on to cytochrome c that normally sits just outside the inner mitochondrial membrane about 14 angstroms away from complex IV, cytochrome oxidase (Nelson and Cox, 2008; Lane, 2015). On the other hand, NADPH as described by Kenner and Ahmed (1975a) can donate electrons to the ETS in two ways:

> "(a) NADPH can reduce NAD^+ to NADH in the presence of an active transhydrogenase enzyme; the NADH produced in (this) manner will serve as a substrate for mitochondrial (and microsomal) ETS. (b) NADPH may participate directly as a substrate in the mitochondrial system. To a limited extent, NADPH may be able to

substitute for NADH in the ETS. The second site of entry of electrons into the ETS is probably microsomal."

The diversity in the living world (as we see above) can also be represented by different metabolic pathways and variable enzymatic activities. Alternative behavior of the ϕ between different groups of organisms can depend on the environmental, on physiological conditions, or on both. Organisms have to control their own respiratory chain, despite the universality of ETS. This is especially true in bacteria, as an example in the denitrifying bacteria. When anoxia is present these bacteria change their final electron acceptor in the ETS, passing electrons to nitrate instead of to O_2. This requires a shift from cytochrome oxidase to dissimilatory nitrate reductase (Payne, 1976; Packard et al., 1983a; Packard, 1985). Also Marrs and Gest (1973) conclude that electrons tend to go through alternative routes, depending on their metabolic origins. Furthermore, cells can maintain separate pools of NADPH and NADH, with different redox potentials. In general, NADH transfers electrons in aerobic-catabolic reactions to the ETS and NADPH generally supplies electrons to anabolic reactions (Nelson and Cox, 2008). This can explain the shift of ETS activity with the F_m in the deepest ocean profile samples (Figure 5.8). The change in the tendency of the ϕ-depth profile can be due to a shift in the metabolism of the deep plankton community, since as we have seen, the *in vitro* ϕ activity depends partially on the substrates added and on the type of organism. But still, we know so little about what is the specific roll of the substrates and biochemistry in the ETS in each group of plankton.

Table 5.4. ETS substrates used in the analysis of different organisms and samples.

Organism	NADH	NADPH	Succinate	Author
Phytoplankton	+	+	+	Kenner and Ahmed, 1975a, b; Packard, 1971
Diatoms[+]	+	+	+	Blasco et al., 1982
Zooplankton	+	+	-	Owens and King, 1975; Ikeda et al., 2004, 2006
	+	+	+	Hernandez-León, 1988; Gómez et al., 1996; Hernandez-León and Gómez, 1996; Minutoli and Guglielmo, 2009; Yebra et al., 2009; Devol, 1981; Postel et al., 1995
L. lingvura	+	+	+	Herrera et al., 2011
Artemia salina	+	+	+	Martínez et al., 2010
	-	-	+	Packard and Taylor, 1968
Calonoids	+	+	+	Teuber et al., 2013; Bode et al., 2013
	+	+	-	Båmstedt, 1980;
Plankton	+	+	+	Packard and Williams, 1981; Packard, 1985b, Packard et al., 1971; Garfield et al., 1979; Hernandez-León et al., 1999, Arístegui and Montero, 2005; Arístegui et al., 2005; Schalk, 1988[*+], Ramírez et al., 2006
	+	+	-	Packard and Christensen, 2004
Microplankton	+	+	+	Arístegui and Montero, 1995; Hobbie et al., 1972; Packard et al., 1983b; Reinthaler et al., 2006; Martínez et al., 1990; Garfield et al., 1983; Devol and Packard, 1978; Packard, 1985a
Micronekton	+	+	-	Schalk, 1988

Continuation of table 5.4.

Organism	NADH	NADPH	Succinate	Author
Bacterias*	+	+	+	Christensen et al., 1980
P. nautica	+	+	-	Packard et al., 1996a; Roy et al., 1999
P. perfectomarinus	+	+	+	Packard et al., 1983a; Christensen et al., 1980
Dhapnia	+	+	-	Simĉiĉ and Brancelj, 2004
Benthic macrofauna	+	+	-	Cammen et al., 1990
Marine sediments	+	+	+	Relexans, 1996
Mussel (*Dreissena polymorpha*)	+	-	-	Madon et al., 1998
	+	+	-	Fanslow et al., 2001
Aquatic plants**	+	+	-	Peñuelas et al., 1988
Ictioplankton	+	+	-	Ikeda, 1989
	+	+	+	Schalk, 1988
Fish	+	+	-	Toth et al., 1995
Coral reefs***	+	+	+	Agostini et al., 2013
Biofilms	+	+	+	Blenkinsopp and Lock, 1990
Sediments	+	+	-	Christensen and Packard, 1977
Aquatic plants	+	+	-	Peñuelas et al., 1988

**Vibrio sp.* SA774*, V. adaptatus, V. anguillarum, Serratia marinorubra and P. perfectomarinus*.
** *Elodea canadensis, Myriophyllum spicatum* L., *Potamogeton crispus* L., *Po.pectinatus* L.*, Ruppia cirrhosa, Rhynchostegium riparioides, Fontinalis antipyretica, Cambomba caroliniana.*
****Galaxea fascicularis, Porites cyclindrica, Pocillopora damicornis, Montipora digitata* and *Porites sp.*
[+] *Thalassiosira pseudonona, T. weissflogii, Skeletonema costalum, Ditylum brightwellii and Coscinodiscus sp.*
[*+] *Include organism from* Cephalopoda, Chaetognatha, Tunicata, Siphonophora and medusa.

The differences in what is the predominant electron donor to the ETS, is subject to much discussion. It can vary depending on the organism or physiological state. We are aware that this topic needs much more research in both the field and in the laboratory. This research is necessary if we are to achieve a better understanding of ocean physiological processes and their associated organisms and if we want to build more accurate ocean models.

5.5. Summary

1. A new fluorescent method for measuring the ETS was evaluated showing that it was more sensitive to low ETS levels than the spectrophotometric method.

2. The three ETS substrates (NADH, NADPH, and succinate) were not additive to the total ETS activity.

3. NADPH, in some degree, inhibited the enzymatic INT and resazurin reduction in some samples and in some organisms.

4. In applying the ETS assay to new organisms or new unusual field samples, it would be useful to include analysis with the different substrates.

5. The ratio of the reductase activities based on NADH-and NADPH, can give an indication of the type of metabolism inherent in new samples.

6. Discusión general

El método ETS ha demostrado que las mediciones enzimáticas están correlacionadas con el consumo de oxígeno (eg. Chance y Williams, 1955; Packard, 1971; Ikeda, 1988; Packard et al., 1996a; Madon et al., 1998; Herrera et al., 2011). Pero esta relación entre la respiración (R) y la respiración potencial (ϕ) puede a su vez dar información del estado fisiológico del zooplancton (Christensen et al., 1980 Hernández-León y Gómez, 1996). Nuestros resultados indican que la comunidad zooplactónica presenta un estado fisiológico relativamente bueno (R/ϕ ≤0.5) en las tres zonas geográficas estudiadas. Aunque en Perth-Sidney y Cartagena-Cartagena las relaciones R/ϕ son bajas (0.37). En la segunda región, para el tamaño predominante (100-500 µm) la ϕ tiene valores altos en relación a R (Tabla 2.1). Si en todos estos casos, tomamos el cociente R/ϕ de la división directa de estas mediciones se obtienen valores superiores a 0.5 lo que supondría un buen estado fisiológico (Tabla 2.1). Haciendo este mismo análisis desde el mismo punto de las fracciones de tamaño, tanto la talla de 500-1000 µm y >1000 µm presentan relaciones mayores a 0.5, mientras que las de 100-500 µm es menor (0.41) pero de este valor no se puede inferir que se encuentren en un estado fisiológico precario, ya que precisamente en esta talla la dispersión es la más alta, lo que puede estar influyendo en este resultado (Tabla 2.2, Figura 2.2).En general, de forma independiente a si los datos se agrupan por la zona geográfica o por las clases de talla, la relación R/Φ era igual o superior a 0.5, concluyendo que este zooplancton en nuestras muestras se

encontraba en un estado fisiológico relativamente bueno, por lo que al parecer en entornos oligotróficos, el zooplancton logra mantener un aceptable estado fisiológico.

También es determinante entender como varia la relación R/ϕ, en función de la disponibilidad de alimentos. La teoría sostiene que la relación R/Φ debería disminuir con el tiempo, ya que la inanición reduce los niveles de intermedios del ciclo de Krebs y otros donantes esenciales para el ETS (NADH y NADPH). Esto a su vez tendría un efecto sobre la respiración. Sin embargo, en nuestros experimentos esto parece no ocurrir (Figura 2.5 y 2.6) aunque realmente no existieron diferencias significativas ($p > 0.05$) entre el R/Φ a las 0h y 24h (Figura 2.6). Herrera et al. (2011) encontraron el mismo fenómeno en misidáceos cultivados. Aún así La respiración puede verse afectada (Hernández-León y Ikeda, 2004) y puede responder inmediatamente a los estímulos externos, como pudiera ser el efecto botella, el aumento de las densidades y la manipulación de los organismos. Éste aumento de R a las 24h fue tal que se presentó una diferencia significativa ($p < 0.05$) en relación a las mediciones iniciales (0h) (Figura 2.5). En cambio el ϕ es más conservativo, lo que impide que sea alterado en tan poco tiempo, esto se evidencia en que no existen diferencias ($p > 0.05$) entre las mediciones de 0h y 24h (Figura 2.5)

Una de las principales aportaciones de esta tesis, es la profundización en uno de los métodos más empleados para calcular la respiración celular, el ETS. Además de presentar una evolución del mismo remplazando el aceptor final de electrones

clásico por uno fluorescente, lo que implica un gran avance en la sensibilidad del método, que se traduce en menores biomasas requeridas, menores tiempos de manipulación de muestras y menor utilización de reactivos lo que podría tener repercusión en la disminución en los costes.

Otra importante aportación es revindicar que la medición de la respiración celular potencial a través de procesos enzimáticos es un proceso que necesita cumplir con unos requisitos básicos de la enzimología. Debe incluir algún sustrato o sustratos especifico(s) de la enzima, de lo contrario el ensayo no es específico (Cornish-Bowden, 2004; Fruton y Simmonds, 1958; Segel, 1993). Realizar una prueba enzimática sin sustratos sería el equivalente a medir cualquier número de reacciones que pueden reaccionar con el detector, en este caso el INT.

Para corroborar esta hipótesis, el INT se mezcló, sin ningún tipo de enzima, con algunas sustancias que suelen estar presentes en las células. La señal obtenida utilizando el método de Båmstedt está al mismo nivel que los blancos del método enzimático (Figura 3.4) lo que demuestra que la señal observada es debida a la medición de glutatión, cisteína, y ácido ascórbico, sustancias que se encuentran universalmente en todos los eucariotas y procariotas (Mercante et al., 1995; Nelson y Cox, 2008; Newton et al., 1996). Además, es probable que la medición de la vitamina B_{12}, mercaptanos y otros compuestos orgánicos tengan también capacidad reductora.

En consecuencia, no se recomienda el uso de esta metodología propuesta por Båmstedt (2000) o cualquiera derivada de esta para estimar la respiración, ya que durante el proceso se diluyen los sutratos a un nivel desconocido generando que las enzimas trabajen por debajo de sus K_m, no mide lo que pretende medir, no es específica, es insensible y no puede ser reproducida.

En nuestro equipo, quisimos dar otro paso más en el desarrollo de este importante método. Para ello propusimos reemplazar el aceptor artificial de electrones INT, y utilizar por primera vez en estudios bioquímicos oceanográficos, el resazurin. Este compuesto al ser reducido, se transforma en un compuesto altamente fluorescente. Esta propiedad física, tiene como gran ventaja su especificidad y alta sensibilidad. En la mayoría de los estudios consultados, se utiliza el resazurin como simple indicador de la viabilidad de las células.

La fluorescencia, en este caso del resorufin, puede estar afectada por algunas condiciones, entre estas se encuentra el efecto del oxígeno y la fuerza iónica. El oxígeno tiene un potencial redox alto (+820 mV). En nuestras condiciones experimentales no se presenta competencia del oxígeno por los electrones en la reacción, asegurando que se estan midiendo las velocidades máximas de la reacción. Esto sugiere que la citocromo oxidasa de alguna manera ayuda a guiar los electrones hacia el resazurin. Los resultados de la fuerza iónica no son concluyentes, por consecuencia no se tendrán en cuenta estas variables en futuras mediciones.

Los resultados aquí obtenidos muestran que el succinato no parece tener ninguna contribución en la reducción del resazurin a través de la catálisis de la diaforasa. También se determinó que en presencia de NADH y NADPH no aporta ninguna actividad, ni con la diaforasa ni con zooplancton (Figura 4.13). En consecuencia proponemos la eliminación del succinato de los sustratos, al menos para el zooplancton.

Otra variable para considerar en el momento de establecer las condiciones del nuevo método, es asegurarnos que los sustratos específicos se encuentren en condiciones de saturación, garantizando de esta manera, que no exista ningún tipo de limitación durante la reacción. Para esto se probó una amplia escala de concentraciones de NADH y NADPH (Tabla 4.5). En dicho rango no se presentó evidencia alguna de limitación. Se decidió trabajar con concentración para el NADH de 1.0 mM y del NADPH de 0.15mM (Figura 4.6) estas concentraciones son ligeramente inferiores a las del método tradicional, lo que posibilitaría que la prueba pudiera ser más económica, sin perder su eficacia.

Al comparar los dos métodos con dos fuentes diferentes de enzimas (diaforasa, y misidáceos) las pendientes del método espetrofluorométrico son ~1,68 veces más sensibles con la diaforasa y ~1,14 veces con los misidáceos (Figura 4.12).Dentro de estas relaciones, se presenta la particularidad que en las tres concentraciones más altas esta relación es sólo del 1.5; las concentraciones intermedias son del 3.3; mientras que en los

valores más bajos, la diferencia de la señal es 6.24. Ambos métodos responden claramente a la actividad ETS mitocondrial y su relación con la biomasa, pero el F_m mantiene la sensibilidad a bajas concentraciones de biomasa (enzimas) caso contrario con el S_m. En este caso si tenemos biomasas menores a 0,1 mg prot. ml^{-1}, nos encontraremos que con el método espectrofotométrico no tendrá la sensibilidad suficiente para detectar actividad alguna (Figura 4.12). Esto también se refleja en los valores de LOD y LOQ (1.91 y 3.34 nM de respectivamente) del F_m, caso contrario del S_m, donde el ruido de fondo es mayor que el analito, haciéndolo imposible de determinar. Hay que destacar también que la desviación estándar relativa (RSD) que determina la repetitividad de las mediciones en el F_m, es tan sólo del 4% (Tabla 4.7).

Otra diferencia entre los dos métodos, y que podría explicar por qué aunque miden la misma actividad no lo hacen con la misma intensidad, es por lo que el potencial redox de cada uno de los aceptores finales de electrones. El resazurin que por su alto potencial redox (E^0 +0.38 V) es intermediario sólo entre el citocromo oxidasa (Cyt.a3) y el punto final de reducción, el O_2. Por otro lado, el INT presenta un potencial redox (-0.09V) (Hope y Vicent, 1989; Smith y McFeters, 1997), lo que implica que el punto de interacción del INT es hasta el Cyt c. (Slater et al., 1963). La introducción de un aceptor artificial de electrones con un potencial redox intermedio (eg, INT) entre cualquiera de los dos componentes de la cadena de transporte de electrones, puede interrumpir el flujo de electrones. Por lo tanto, cada vez que un

sustrato se oxida en presencia del INT, los electrones liberados quedarán atrapados y puede que no sean transportados a través de la secuencia habitual de los citocromos (Figura 1.1), generando un corte en la cadena respiratoria.

Por todo lo expuesto parece ser que el INT no será reducido por los citocromos como ocurre con el resazurin. Esto significa que con el INT, los electrones no cubren todo sistema del ETS. Aunque en el zooplancton, hay indicios de que el regulador del sistema de transporte de electrones es el complejo I, que estaría funcionando a modo de un regulador del flujo en las primeras etapas de la cadena, esto se pone de manifiesto ya que no existen diferencias ($p > 0.05$) entre las pendientes de los dos métodos (Figura 4.12). Esta diferencia en el punto en el que actúa el aceptor de electrones, puede explicar la diferencia que existe en las señales de consumo de oxígeno en presencia de zooplancton y la diaforasa. Con esta última, podría estar ocurriendo una reacción directa (sin reguladores como por ejemplo el complejo I o los citocromos) en otras palabras una posible medición del "estado redox" de la solución (Matsumo et al., 1990; Rasmussen, 1999; O'Brien et al., 2000; Candeias et al., 1998).

La no diferencia entre métodos ($p > 0.05$) nos indica también que son comparables, lo que hace promisorio la implementación del método espectrofluorométrico.

En el momento del ensamblaje del método, se creía que la diaforasa como oxidante de β-NADH o β-NADPH en presencia de un aceptor de electrones, como afirma el fabricante Sigma, se puede utilizar como unidad estándar para las actividades de medición del ETS. A medida que avanzamos en las pruebas se observó que la diaforasa (EC: 1.8.1.4) no sigue la misma tendencia que en el homogeneizado de zooplancton (Figura 4.13). Además, la señal de ETS con la diaforasa, es alta en presencia de cualquier piridin nucleótido, mientras que con los misidáceos, el NADPH prácticamente no tiene ninguna actividad (Figura 4.13). Este resultado también se describe para el zooplancton (Gómez et al., 1996; Zavenkoff et al., 1995) y para la nitrato reductasa en macroalgas (Hurd et al., 1995). Sin embargo, cuando los dos sustratos están juntos la señal no es aditiva según lo sugerido por Gómez et al. (1996) y Borgmann (1976). La inhibición de la señal parece ser proporcional a la adición de NADPH en ambos métodos (Figura 4.5; 4.13). El mismo patrón se encuentra cuando se intentó calcular la cinética bi-sustrato con la diaforasa (Figura 4.10; 4.11). Por lo tanto, no se puede realizar una calibración estándar para la actividad ETS bajo las condiciones empleadas.

Una vez implementada la línea base para la introducción de la nueva metodología espectrofluorométrica, es necesario corroborarla con diferentes tipos de muestras, y probarlo a bajas condiciones de biomasa, además se hace indispensable resolver la pregunta del efecto real de los sustratos en la velocidades de reacción del ETS *in vitro*, al mismo tiempo que se compara con el método espectrofotométrico

El primer escenario se realizó con organismos de diferentes phyllums. En estos organismos la diferencia promedio, en ambas condiciones de sustratos, entre el F_m y el S_m fue de aproximadamente 1.7, con excepción de *H. tuberculata* con NADH (5.16) (Tabla 5.2). Esta relación cambia drásticamente con las muestras naturales. Con solo el NADH como sustrato esta diferencia oscila entre 1.14 a 10.72; con SB entre 2.51 y 12.04. Las altas diferencias son como resultado de la falta de sensibilidad del S_m a biomasas bajas (Tabla 5.3).

Comparando los valores obtenidos, los datos del F_m son más consistentes con los valores de ϕ superficiales encontrados por Packard et al. (1985) para aguas de afloramiento en Perú, también para el microplancton del Nor-Atlántico Central (Hernández-León et al., 1999). Por otra parte los valores de ϕ obtenidos con S_m, se asemejan a los encontrados en los primeros 150 m de la región de Canarias por Arístegui y Montero (2005) y Barton et al. (1998). Este hecho podría estar relacionado con que los autores señalados filtran la misma cantidad de agua que en el presente estudio y además utilizan el método de Kenner y Ahmed (1975a). Cuando las biomasas son tan bajas, (habituales en el océano abierto), con el S_m se puede estar perdiendo resolución en los procesos respiratorios en la columna de agua.

Dependiendo de la biomasa de la muestra o bien del tipo de organismo, la contribución del NADH o la combinación de los diferentes sustratos al ETS, es relativa (Tabla 5.2). Este tipo de discrepancia se puede encontrar también en la literatura. Toth et

al. (1995) describen que el NADH es el principal donante de electrones en etapas embrionarias de *Latipes oryzias* (pez teleósteo) y en una menor proporción el NADPH. Savenkoff et al. (1995) describen que el NADH por sí mismo contribuye con más del 85% de la actividad ETS en *Sacaromices cerevisiae* (levadura), *Crypthecodinium cohnii* (dinoflagelados), *Thalassiosira pseudonana* (diatomeas) y *Praunus flexuosus* (mísidaceo); por el contrario en *Dunaliella tertiolecta* (clorophyta), *P. perfectomarinus, P. nautica* (bacteriums) el NADPH tiene contribuciones entre 56,8 y 68,1%,mientras que en los primeros la contribución del NADPH fluctúan entre 11,8 y 26,5%. Finlay et al. (1983) establecen que la mayor contribución al ETS es debida al NADH en protozoos y en el ciliado *Tetrahymena pyriformis* (77 y 68%, respectivamente), con actividades relativamente altas de NADPH (40 y 57% respectivamente). Kenner y Ahmed (1975b) establecen que en *Skeletonema costatum* tiene mayor actividad con NADH mientras *Dunaliella tertiolecta* con NADPH. Christensen y Packard (1979) hicieron una comparación de algunas metodologías diferentes del ETS, aquí queremos considerar los métodos utilizados por Owens y King (1975), donde lo sustratos empleados son el NADH y NADPH; y por Kenner y Ahmed (1975a) donde los tres sustratos están presentes. En ese trabajo, Christensen y Packard (1979), encontraron que prácticamente no existe diferencias en los valores de ETS, en el alga *Chaetoceros debilis* así como en la bacteria *Serratia marinorubra*, *P. denitrificans* y *Vibrio anguillarum*; en *T. fluvialitis* y *P. perfectomarinus* la actividad es ligeramente mayor cuando los tres sustratos están presentes, esto es, de alguna manera concordante con los resultados de Savenkoff et al. (1995), pero con diferencias en los porcentajes. También, como se

informa en este estudio, *S. costatum, S. marinorubra* y *V. anguillarum,* presentan valores de ETS menores cuando el NADPH o succinato están presentes. Christensen y Packard (1979) hacen la misma prueba en muestras naturales, en Shilshole Bay donde no encontraron diferencia entre los métodos; por otro lado, las muestras de Portage Bay sufrieron una disminución del ETS en la presencia de succinato. Esto puede estar en la misma línea de lo argumentado en el presente trabajo, especialmente porque esas muestras oceánicas pueden presentar organismos multicelulares como el zooplancton, que en algunas ocasiones en presencia de todos los sustratos pueden presentar una reducción de la respiración potencial (Figura 4.13, 5.1). Sumado a esto, los resultados del ETS en los organismos multicelulares pueden varían dependiendo del órgano o tejido y a su vez estos variar la dependencia para cada sustrato, como Borgmann (1977) determina en *Orconectes propinquus*.

A pesar de que en la literatura no hay unanimidad sobre qué sustratos deben estar en la reacción *in vitro* (Tabla 5.4), algunos autores afirman que la actividad de los sustratos por separado es aditiva, adquiriendo así la velocidad máxima enzimática (Packard, 1985b; Packard et al., 1996a; Gómez et al., 1996; Savenkoff et al., 1995). Esto tiene un sentido bioquímico y biológico, ya que estos sustratos son participes del sistema de transporte de electrones mitocondrial, pero en algún punto de la reacción *in vitro*, este concepto falla al no obtener siempre la V_{max}. Los resultados del capítulo 4 y 5 sugieren que, dependiendo del tipo de muestra la presencia de ciertos sustratos en la reacción es crucial para

obtener la velocidad máxima de reacción (Figura 4.13, 5.2 a 5.6, 5.8), esta misma observación la hacen Kenner y Ahmed (1975b) y Toth et al. (1995).

Aunque Packard y Taylor (1968) describen la actividad de la succinato deshidrogenasa en *A. salina,* y esta se correlaciona con la respiración, en el presente estudio el succinato parece tener poca o ninguna contribución en la actividad ETS. Por otro lado otros autores en la literatura (Finlay et al., 1983; Savenkoff et al., 1995; Toth et al., 1995) encuentran resultados similares a los nuestros. Más aún, en algunos organismos el NADPH o el succinato parecen inhibir la reacción, por lo que en estos casos esos sustratos deben ser omitidos (Figura 5.2, 5.3, 5.5, 5.6; Tabla 5.2). En otros organismos como *H. guttulatus* y en algunas muestras de microplancton, esta combinación de sustratos parece ser esencial (Figura 5.4, 5.8; Tabla 5.2).

La relación NADH:SB puede explicar en qué grado los distintos organismo o las muestras de microplancton naturales son más dependientes de NADH por sí mismo o de una combinación de los diferentes sustratos. Si la relación es mayor que uno, indica que la V_{max} se adquiere solamente con el NADH. Por el contrario, si la relación es inferior a uno, indica que el NADPH y/o el succinato también contribuyen a la reacción total. Si esta relación es cercana a uno, los datos debieran ser interpretados con un mayor detalle ya que puede suponerse que el NADPH y/o el succinato no contribuyen a la reacción o por el contrario están teniendo algún grado de efecto inhibitorio (Figura 5.9; Tabla 5.3). En nuestro caso

todos los escenarios están presentes, así que se sugiere para pruebas futuras realizar un ensayo previo con el fin de lograr el V_{max}. Esta pequeña prueba podría simplificar el trabajo y hacerlo menos costoso.

No hay duda acerca de la universalidad de la bioquímica del ETS (Tzagoloff, 1982; Packard, 1985a, b; Nelson y Cox, 2008). Sin embargo, hay diferentes sitios donde los electrones fluyen en el ETS. La NADH deshidrogenasa o complejo I, recibe los electrones del NADH; este complejo está presente en las Archeas, bacterias y eucariotas (Friedrich y Scheide, 2000). Junto con el Complejo II o succinato deshidrogenasa, pasan los electrones al complejo III o ubiquinona:citocromo c oxidorreductasa (Nelson y Cox, 2008) (Figura 1.1). Por otro lado, el NADPH como lo describen Kenner y Ahmed (1975a) puede donar electrones al ETS de dos maneras: 1, el NADPH puede reducir NAD^+ a NADH en presencia de una transhidrogenasa activa; el NADH producido servirá como un sustrato para el ETS mitocondrial y microsomal. 2, el NADPH puede participar directamente como sustrato en el sistema mitocondrial y hasta cierto punto el NADPH puede ser capaz de sustituir al NADH en el ETS. El segundo sitio de entrada de los electrones al ETS es probablemente microsomal.

La diversidad de los seres vivos puede estar también presente en las diferentes rutas metabólicas, actividades enzimáticas, comportamientos alternativos de la respiración, incluso entre organismos de una misma especie, y puede depender de las condiciones fisiológicas o ambientales. Los organismos podrían controlar su propio sistema regulatorio. Esto es especialmente

cierto en las bacterias. Como por ejemplo en las nitrificantes, que en condiciones de anoxia, cambian su aceptor final de electrones, pasando de O_2 al nitrato, esto implica un cambio en los citocromos (Payne, 1976; Packard et al., 1983a; Packard, 1985). También Marrs y Gest (1973) llegan a la conclusión de que los electrones tienden a ir a través de rutas alternativas, en función de sus orígenes metabólicos. Además las células pueden mantener porciones separadas de NADPH y NADH, con diferentes potenciales redox. En general, el NADH transfiere electrones en las reacciones aeróbicas-catabólicas al ETS y el NADPH generalmente suministra electrones para las reacciones anabólicas (Nelson y Cox, 2008). Esto puede explicar el cambio de la actividad ETS en el F_m en las muestras naturales. El cambio en la tendencia del perfil ETS puede ser debido al cambio en la comunidad planctónica en cada profundidad, ya que como hemos visto, la actividad *in vitro* ETS depende de los sustratos añadidos (relación NADH:SB) y el tipo de organismo(Figura 5.2 a 5.9).

La discusión sobre quien es el donante principal de electrones en el ETS, es aún objeto de discusión, ya que puede variar dependiendo del organismo y probablemente del estado fisiológico. Somos conscientes de que acoplar el trabajo de campo y el de laboratorio requiere un gran esfuerzo. Pero es necesario tener una mejor comprensión de los procesos fisiológico de los diferentes organismos y de las muestras oceánicas para realizar modelos más precisos, además de tratar de mantener las metodologías a la par de los desarrollos tecnológicos y de los conocimiento que se vayan desarrollando.

7. Futuras líneas de investigación

Todos los métodos de trabajo, como en este caso el ETS, tienen que seguir evolucionando con los constantes avances en la tecnología y el conocimiento. Con la presente tesis se pretende fortalecer esta útil metodología, reafirmar la importancia de seguir las bases enzimáticas e incrementar en lo posible su precisión.

El método ETS fluorescente abre una nueva ventana a mediciones más precisas y al ser más sensible, se puede trabajar a bajas concentraciones de biomasa, lo que por ejemplo disminuye los tiempos de filtración al necesitar menos volumen de agua. Esto a su vez repercute en la calidad de la señal, ya que el sistema enzimático puede bajar su actividad rápidamente una vez que el organismo muere y aún más cuando esto ocurre a temperatura ambiente.

La adición de todos los sustratos que están involucrados en el sistema de transporte de electrones, debe asegurar la medición de la velocidad máxima de la reacción. Pero teniendo en cuenta los resultados del presente trabajo y de la literatura (eg: Ryan y King, 1962; Packard y Taylor, 1968; Borgmann, 1977; Savenkoff et al., 1995; Gómez et al, 1996) parece necesario realizar estudios previos con cada tipo de muestras para determinar si es el NADH por si solo o es la actividad conjunta de todos los sustratos, lo que resulta estar más relacionada con la bioquímica de la comunidad u organismo para así asegurar la determinación del V_{max}.

El desarrollo de una curva estándar para la actividad de ETS, a través de diaforasa u otro mecanismo no debe ser desechado. Esto supondría un gran avance para hacer comparable todas las futuras mediciones de ETS. Diferentes deshidrogenasas están implicadas en muchos procesos catabólicos y anabólicos (Liu, 1986). Las diaforasas están ampliamente distribuidas, encontrándose en muchas especies desde las bacterias hasta los mamíferos (O'Brien et al., 2000). A la luz de los resultados presentados en este trabajo, sería necesario la construcción de esta tarea con las enzimas más precisas de los sustratos implicados en la reacción del ETS, como el NADH deshidrogenasa (EC 1.6.99.3), NADPH deshidrogenasa (EC 1.6.99.1), o succinato deshidrogenasa (SDH) (EC 1.3.5.1).

Uno de los grandes avances del presente trabajo es medir el ETS por un método espectrofluorométrico, que puede llegar a ser de 10 a 100 veces más sensible que el tradicional espectrofotométrico. A pesar de este gran avance, se podría incrementar la sensibilidad aún más, usando cubetas con un mayor paso de luz, o de sistemas tipo LWCC (Liquid Waveguide Capillary Cells). Como estos sistemas son empleados en detectar metales traza incluso a niveles nano-molares (González et al., 2014 and Santana-Casiano et al., 2000).

Un gran desafío del método ETS, es demostrar que las mediciones enzimáticas están altamente correlacionados con el consumo *in vivo* de oxígeno (eg, Chance y Williams, 1955; Packard, 1971; Ikeda, 1988; Packard et al., 1996a; Madon et al.,

1998; Herrera et al., 2011). También otros autores han encontrado una correlación entre la reducción de resazurin y el consumo de oxígeno, sin añadir sustratos (Liu, 1983. McNicholl et al., 2007; Haggerty et al., 2009; González-Pinzón et al., 2012). El uso del complejo resazurin-resorufin, gracias a sus características no tóxicas, alta sensibilidad y gran afinidad por las actividades metabólica propias de la actividad mitocondrial, abre la posibilidad de desarrollar mediciones reales *in vivo* en sistemas oceánicos.

Los coeficientes R/ETS reflejan el grado de acoplamiento entre estos dos procesos Es necesario realizar medidas de respiración con las nuevas tecnologías más sensibles, como los optodos y acoplarla al ETS fluorescente.

Las reacciones redox son muy dependientes del pH, y aunque se usen soluciones tampón (PO_4 o Tris) sería necesario realizar estudios sobre la variación del pH durante la reacción, lo que dará una idea de cómo obtener valores más precisos de ETS.

Se deben realizar pruebas previas con las diferentes condiciones de sustratos, para poder obtener la velocidad máxima de la reacción (V_{max}).

Sería interesante poder determinar las variaciones del ETS en componentes del plancton multicelular (eg. La fracción más grande del zooplancton, ictioplancton) ya que hay estudios

(Borgmann, 1977) donde sugiere que dependiendo del tejido, la actividad ETS varia así como su dependencia de los sustratos y determinar una posible relación con su estado de desarrollo.

Conclusiones generales

1. Los valores específicos para R y Φ, en la Gran Bahía Australiana fueron de 1.23 ±0.60 y 1.97 ±0.56 µmol O_2 h^{-1} mg^{-1}, respectivamente; en el sur del Océano Índico fueron de 1.41 ±0.65 y 1.77 ±0.64 µmol O_2 h^{-1} mg^{-1}, respectivamente y durante la travesía del Atlántico de 0.94 ±0.69 y 1.54 ±1.02 µmol O_2 h^{-1} mg^{-1}, respectivamente. No se encontraron diferencias entre ninguna de las regiones ($p > 0.05$).

2. A pesar de la variabilidad en los datos, el coeficiente de 0.75 de la ley de Kleiber, que establece una relación exponencial entre ϕ o R con la biomasa y la relación R/ϕ, demuestran que la comunidad de zooplancton de las tres regiones muestreadas se encuentran en condiciones fisiológicas óptimas.

3. No parece haber un efecto de la inanición en el comportamiento metabólico del zooplancton obtenido en el Océano Índico y el Océano Atlántico, lo que podría sugerir una adaptación a las condiciones oligotróficas del medio ambiente o bien que 24h de inanición no fue tiempo suficiente para ver cualquier efecto en la disminución de la respiración.

4. La correspondencia entre el log R/log B y log ϕ/log B muestras una relación con más tendencia hacia la isométria que alométria.

5. Tres de los seis compuestos bioquímicos intracelulares probado (glutatión, cisteína y ácido ascórbico) reaccionaron no enzimáticamente con el INT.

6. La señal de la producción de formazan en la ausencia de sustratos específicos, es menor en un factor de diez que la señal de la producción de formazan utilizando la metodología empleada por Owens y King (1975) para el zooplancton, método basado en la adicción de los sustratos al ensayo ETS.

7. El ensayo de Båmstedt (2000), mide la capacidad natural de la muestra de zooplancton para reducir el INT. No está midiendo la respiración potencial o la actividad ETS.

8. El método espectrofluorimétrico es robusto y totalmente comparable con el clásico, con la ventaja de que es más sensible inclusive a bajas biomasas y potencialmente más económico. La señal del método espectrofluorométrico fue siempre mayor que el método espectrofotométrico llegando a presentar diferencias de un orden de magnitud.

9. Se determinó el límite de detección (LOD) y cuantificación (LOQ) del método espectrofluorométrico que fue de 1.91 y 3.33 nM, respectivamente así como una reproducibilidad del 96%. Para el método espectrofotométrico no pudo determinarse.

10. El NADPH tiene una baja contribución a la señal del ETS en muestras de misidáceos.

11. En presencia de NADH, el NADPH y/o el succinato parece no tener ninguna contribución a la totalidad de la señal de ETS, presentando inclusive un efecto inhibidor en algunas muestras u organismos.

12. En la reacción con la diaforasa, el succinanto no sirve como un donante de electrones, ni tiene un efecto sinérgico cuando se añade con los otros dos piridin nucleótidos. La reducción de la diaforasa y la reducción del ETS por el resazurin son reacciones diferentes, lo que imposibilita la utilización de la diaforasa en la calibración de la actividad ETS.

13. La relación NADH: NADPH puede dar una idea del tipo de metabolismo que presentan los organismos.

14. El Oxígeno no inhibe la reducción del resazurin.

References

Abou-Sleiman, P. M., Muqit, M. M. K., and Wood, N. W. 2006. Expanding insights of mitochondrial dysfunction in Parkinson's disease. Nat. Neurosci. 7:207-219.

Abu-Amero, K. K., Alzahrani, A.S., Zou M., and Shi, Y. 2006. Association of mitochondrial DNA transversion mutations with familial medullary thyroid carcinoma/multiple endocrine neoplasia type 2 syndrome. *Oncogene*. 25(5):677-684.

Abu-Amero, K. K., and Bosley, T. M. 2005. Detection of mitochondrial respiratory dysfunction in circulating lymphocytes using resazurin. *Arch. Pathol. Lab. Med*. 129(10):1295-1298.

Agostini, S., Fujimura, H., Fujita, K., Suzuki, Y., and Nakano, Y. 2013. Respiratory electron transport system activity in symbiotic corals and its link to calcification. *Aquat. Biol.* 18:125-139.

Aguiar-González, B., Packard, T. T., Berdalet, E., Roy, S., and Gómez, M. 2012. Respiration predicted from an enzyme kinetic model and the metabolic theory of ecology in two species of marine bacteria. *J. Exp. Mar. Biol. Ecol*. 412:1–12.

Aleem, A. A. 1955. Measurement of plankton populations by triphenyltetrazolium chloride. *Kiel. Meeresforsch*. 11(2):160–173.

Ali-Vehmas, T., Louhi, M., and Sandholm, M. 1991. Automation of the resazurin reduction test using fluorometry of microtitration trays. *J. Vet. Med. B*. 38(1-10):358-372.

Ali-Vehmas, T., Conere, S, T., Sisk, G. D., and Allshire, A. 2009. Mitochondrial modulation of oxygen-dependent radiosensitivity in some human tumour cell lines. *Brit. J. Radiol.* 82(982):847-854.

Allan, A. P., and Gillooly, J. F. 2007. The mechanistic basis of the metabolic theory of ecology. *Oikos*. 116:1073-1077.

Alley, M. C., Uhl, C. B., and Lieber, M. M. 1982. Improved detection of drug cytotoxicity in the soft agar colony formation assay through use of a metabolizable tetrazolium salt. *Life. Sci*. 31(4):3071–3078.

Anoopkumar-Dukie, S., Carey J. B., Conere, T., O'Sullivan, E., Van Pelt, F. N., and Allshire, A.2005. Resazurin assay of radiation response in cultured cells. *Brit. J. Radiol*. 78(934):945–947.

Argerich, A., Haggerty, R., Martí, E., Sabater, F., and Zarnetske, J. 2011. Quantification of metabolically active transient storage (mats) in two reaches with contrasting transient storage and ecosystem respiration. *J. Geophys. Res.* 116:G03034.

Arístegui, J., Duarte, C. M., Gasol, J. M. and Alonso-Sáez, L. 2005. Active mesopelagic prokaryotes support high respiration in the subtropical northeast Atlantic Ocean. *Geophys. Res.Lett.* 32(3):L03608.

Arístegui, J. and Montero, M. F. 1995. The relationship between community respiration and ETS activity in the ocean. *J. Plankton. Res*. 17(7):1563-1571.

Arístegui, J. and Montero, M. F. 2005. Temporal and spatial changes in plankton respiration and biomass in the Canary Islands region: the effect of mesoscale variability. *J. Mar. Sys.* 54(1):65-82.

Båmstedt, U. 1979. Seasonal variation in the respiratory rate and ETS activity of deep-water zooplankton from the Swedish west coast. *Cyclic phenomena in marine plants and animals.* Pergamon Press, Oxford. 267-274p.

Båmstedt, U. 1980. ETS activity as an estimator of respiratory rate of zooplankton populations. The significance of variations in environmental factors. *J. Exp. Mar. Biol. Ecol.* 42(3):267-283.

Båmstedt, U. 2000. A new method to estimate respiration rate of biological material based on the reduction of tetrazolium violet. *J. Exp. Mar. Biol. Ecol*. 251(2):239–263.

Babich, H., and Borenfreund, E. 1987. Cultured fish cells for the ecotoxicity testing of aquatic pollutants. *Toxic. Assess.* 2(2):119-133.

Babson, A.L., and Babson, S.R., 1973. Kinetic colorimetric measurement of serum lactate dehydrogenase activity. *Clin. Chem*. 19(7):766–769.

Bakermans, C., Tsapin, A. I., Souza-Egipsy, V., Gilichinsky, D. A., and Nealson, K. H. 2003. Reproduction and metabolism at -10°C of bacteria isolated from Siberian permafrost. *Environ. Microbiol*. 5(4):321-326.

Barnes, C. A., and Collias, E. E. 1961. Some considerations of oxygen utilization rates in Puget Sound. *J. Mar. Res.* 17:68–80.

Barnes, E. M. 1956. Tetrazolium reduction as a means of differentiating *Streptococcus faecalis* from *Streptococcus faecium*. *J. Gen. Microbiol*. 14(1):57–68.

Barnes, S. P., Bradbrook, S. D., Cragg, B. A., Marchesi, J. R., Weightman, A. J., Fry, J. C., and Parkes, R. J. 1998. Isolation of sulfate-reducing bacteria from deep sediment layers of the Pacific Ocean. *Geomicrobiol. J.* 15:67-83.

Barnes, S., and Spenney, J. G. 1980. Stoichiometry of the NADH-Oxidoreductase reaction for dehydrogenase determinations. *Clin. Chim. Acta*. 107(3): 149-154.

Barton, E. D., Arístegui, J., Tett, P., Cantón, M., Garcıa-Braun, J., Hernández-León, S., Nykjaer, L., Almeida, C., Almunia, J.,

Ballesteros, S., Basterretxea, G., Escánez, J., García-Weill, L., Hernández-Guerra, A., López-Laatzen, F., Molina, R., Montero, M. F., Navarro-Pérez, E., Rodríguez, J. M., van Lenning, K., Vélez, H. and Wild, K. 1998. The transition zone of the Canary Current upwelling region. *Prog. Oceanogr*. 41(4):455-504.

Bell, M. S. 2005. Lavoisier in the year one: The birth of a new science in an age of revolution. Norton, New York.

Bertoldo, C., and Antranikian, G. 2006. The order Thermococcales. In: *The prokaryotes.* Springer New York. pp. 69-81.

Bigger, S. W., and Verity, B. 2003. A fluorometric approach to studying the effects of ionic strength on reaction rates: An undergraduate steady-state fluorescence laboratory experiment. *J. Chem. Educ*. 80(10):1191-1193.

Bindoff, N. L., and Wunsch, C. 1992. Comparison of synoptic and climatologically mapped sections in the South Pacific Ocean. J*. Climate*. 5:631-645.

Bisswanger, H. 2011. Practical Enzimology. Second edition. Weinheim, Germany. 360p.

Bitton, G., and Dutka, B. J. 1983. Bacterial and biochemical tests for assessing chemical toxicity in the aquatic environment: A review*. Crit. Rev. Env. Sci. Tec*. 13(1):51-67.

Blasco, D., Packard, T. T. and Garfield, P. C. 1982. Size dependence of growth rate, respiratory electron transport system activity, and chemical composition in marine diatoms in the laboratory. *J. Phycol. 18*(1):58-63.

Blenkinsopp, S. A. and Lock, M. A. 1990. The measurement of electron transport system activity in river biofilms. *Water. Res*. 24(4):441-445.

Bode, M., Schukat, A., Hagen, W. and Auel, H. 2013. Predicting metabolic rates of calanoid copepods. *J. Exp. Mar. Biol. Ecol.* 444:1-7.

Borgmann, U. 1977. Electron transport system activity in *Daphnia* and crayfish. *Can. J. Zoo.* 55(5):847-854.

Braydich-Stolle, L., Hussain, S., Schlager, J. J., and Hofmann, M. C. 2005. *In vitro* cytotoxicity of nanoparticles in mammalian germline stem cells. *Toxicol. Sci*. 88(2):412–419.

Brown, J. H., Gillooly, J. F., Allen, A. P., Savage, V. M., and West, G. 2004. Toward a metabolic theory of ecology. *Ecol*. 85:1771-1789.

Brown, M., and Miller, K. 1992. The ascorbic acid content of eleven species of microalgae used in mariculture. *J. Appl. Phycol*. 4(3):205–215.

Bryan, J. R., Riley, J. P., and P. J. Leb, Williams. 1976. A Winkler procedure for making precise measurements of oxygen concentration for productivity and related studies. *J. Exp. Mar. Biol. Ecol*. 21(3):191–197.

Bueno, C., Villegas, M. L., Bertolotti, S. G., Previtali, C. M., Neumann, M. G., and Encinas, M. V. 2002. The excited-state interaction of resazurin and resorufin with aminesin aqueous solutions. Photophysics and photochemical reaction¶. *Photochem. Photobiol.* 76(4):385-390.

Cammen, L. M., Corwin, S. and Christensen, J. P. 1990. Electron transport system (ETS) activity as a measure of benthic macrofaunal metabolism. *Mar. Ecol. Prog. Ser*. 65:171-182.

Candeias, L. P., MacFarlane, D. P. S., McWhinnie, S. L. W., Maidwell, N. L., Roeschlaub, C. A., Sammes, P. G., and Whittlesey, R. 1998. The catalyzed NADH reduction of resazurin to resorufin. *J. Chem. Soc*. Perkin Trans. 2(11):2333-2334.

Castillo, G. M., and Thibert, R. J. 1988. Fluorometric cyclic assays for pyridine nucleotides reduced or oxidized in enzyme-coupled assays. *Microchem. J*. 38(2):191-205.

Carrit, D. E., and Carpenter, J. H. 1966. Comparison and evaluation of currently employed modifications of theWinklermethod for determining dissolved oxygen in seawater; a NASCO Report. *J. Mar. Res*. 24(3):286–318.

Catomeris, P. and Thibert, R. J. 1988. Study and optimization of the resazurin/diaphorase system. *Microchem. J.* 38(3):390-398.

Chance, B., and Williams, G. R. 1955. Respiratory enzymes in oxidative phosphorylation IV. The respiratory chain. *J. Biolog. Chem.* 217(1):429-438.

Chance, B., and Williams, G. R. 1956. Respiratory enzymes in oxidative phosphorylation VI the effects of adenosine diphosphate on azide treated mitochondria. *J. Biol. Chem*. 221(1):477–489.

Chandrasekaran, D., Venkatesan, P., Tirumurugaan, K. G., Nambi, A. P., Thirunavukkarasu, P. S., Kumanan, K., and Vairamuthu, S. 2014. A study on Methicillin resistant *Staphylococcus aureus* mastitis in dairy cows. *J. Appl. Nat. Sci.* 6(2):356-361.

Chen, A. R., and Koren, H. S. 1985. Impaired oxidative burst does not affect human monocyte tumoricidal activity. *J. Immunol.* 134(3):1909-1913.

Cheronis, N. D., and Stein, H. 1956. Tetrazolium salts as chemical reagents. *J. Chem. Educ*. 33(3):120–125.

Christensen, J. P., Owens, T. G., Devol, A. H. and Packard, T. T. 1980. Respiration and physiological state in marine bacteria. *Mar. Biol.* 55(4):267-276.

Christensen, J. P. and Packard, T. T. 1977. Sediment metabolism from the northwest African upwelling system. *Deep-Sea Res*. 24(4):331-343.

Christensen J. P., and Packard, T. T. 1979. Respiratory electron transport activities in phytoplankton and bacteria: Comparison of methods. *Limnol. Oceanogr*. 24(3):576-583.

Christensen, J.P., Packard, T. T., Dortch, F. Q., Minas, H. J., Gascard, J. C., Richez, C., and Garfield, P. C. 1989. Carbon oxidation in the deep Mediterranean Sea: evidence for dissolved organic carbon source. *Global Biogeochem. Cycles*. 3(4):315–335.

Connolly, M., Fernández-Cruz, M. L., Quesada-Garcia, A., Alte, L., Segner, H., and Navas, J. M. 2015. Comparative cytotoxicity study of silver nanoparticles (AgNPs) in a variety of rainbow trout cell lines (RTL-W1, RTH-149, RTG-2) and primary hepatocytes. *Int. J. Environ. Res. Public. Health*. 12(5): 5386-5405.

Cook, D. B., and Self, C. H. 1993. Determination of one thousandth of an attomole (1 zeptomole) of alkaline phosphatase: Application in an immunoassay of proinsulin. *Chem. Chem*. 39(6): 965-971.

Cornish-Bowden, A. 2004. Fundamentals of enzyme kinetics. 3rd edition. Portland Press, London.

Curl Jr., H. C., and Sandberg, J. 1961. The measurement of dehydrogenase activity in marine organisms. *J. Mar. Res*. 19:123–138.

Dart, M. G., Mesta, J., Crenshaw, C., and Ericsson, S. A. 1994. Modified resazurin reduction test for determining the fertility potential of bovine spermatozoa. *Syst. Biol. Reprod. Med*. 33(2):71-75.

Davey, E. A. 1964. The enzyme measurement of primary production. M.S. Thesis, Oregon State University, Corvallis.

De Fries, R., and Mistuhash, M. 1995 Quantification of mitogen induced human lymphocyte proliferation: comparison of Alamar Blue assay to 3H-thymidine incorporation assay. *J. Clin. Lab. Anal*. 9(2):89-95.

De Roeck-Holtzhauer, Y., Quere, I., and Claire, C. 1991. Vitamin analysis of five planktonic microalgae and one macroalga. *J. Appl. Phycol*. 3(3):259–264.

del Giorgio, P.A., 1992. The relationship between ETS (electron transport system) activity and oxygen consumption in lake plankton: a cross-system calibration. *J. Plankton Res*. 14(12):1723–1741.

del Giorgio, P. A., and Duarte, C. M. 2002. Respiration in the open ocean. *Nature*. 420(6914):379–384.

Devol, A. H. 1975. Biological oxidations in oxic and anoxic marine environments: rates and processes. Ph.D Thesis, University of Washington.

Devol, A. H. 1981. Vertical distribution of zooplankton respiration in relation to the intense oxygen minimum zones in two British Columbia fjords. *J. Plankton. Res.* 3(4): 593-602.

Devol, A. H. and T. T. Packard. 1978. Seasonal changes in respiratory enzyme activity and productivity in Lake Washington microplankton. *Limnol. Oceanogr.* 23(1): 104-111.

Dienstknecht, T., Ehehalt, K., Jenei-Lanzl, Z., Zellner, J., Müller, M., Berner, A., Nerlich, M., and Angele, P. 2010. Resazurin dye as a reliable tool for determination of cell number and viability in mesenchymal stem cell culture. *Bull. Expe. Biol. Med.* 150(1):157-159.

Du, H., Fuh, R. A., Li, J., Corkan, A., and Lindsey, J. S. 1998. PhotochemCAD: a computer-aided design and research tool in photochemistry. *Photochem. Photobiol*. 68(2):141–142.

Eker, S., and Kargi, F. 2006. Biological treatment of para-chlorophenol containing synthetic wastewater using rotating brush biofilm reactor. *J. Hazard. Mater.* 135(1):365-371.

Eker, S., and Kargi, F. 2009. Biological treatment of 2, 4, 6-trichlorophenol (TCP) containing wastewater in a hybrid bioreactor system with effluent recycle. *J. Environ. Manage.* 90(2):692-698.

Erb, R. E., and Ehlers, M. H. 1950. Resazurin reducing time as an indicator of bovine semen fertilizing capacity. *J. Dairy. Sci*. 33(12):853-864.

Erb, R. E., Ehlers, M. H., and Flerchinger, F. H. 1952. Modified resazurin reduction test for estimating fertilizing capacity of bull semen. *J. Dairy. Sci.* 35(10):881-888.

Falkowski, P. G., Algeo, T., Codispoti, L., Deutsch, C., Emerson, S., Hales, B., Huey, R. B., Jenkins, W. J., Kump, L. R., Levin, L. A., Lyons, T. W., Nelson, N. B., Schofield, O. S., Summons, R., Talley, L. D., Thomas, E., Whitney, F., and Pilcher, C. B. 2011. Oxygen, deoxygenation: past, present, and future. EOS Trans. *Am. Geophys. Union*. 92(48):409–410.

Fanslow, D. L., Nalepa, T. F., and Johengen, T. H. 2001. Seasonal changes in the respiratory electron transport system (ETS) and respiration of the zebra mussel, *Dreissena polymorpha* in Saginaw Bay, Lake Huron. *Hidrobiologia* 48(1–3):61–70.

Fernández-Urruzola, I., Packard, T. T., and Gómez, M. 2011. GDH activity and ammonium excretion in the marine mysid, Leptomysis lingvura: effect of age and starvation. *J. Exp. Mar. Biol. Ecol*. 409(1–2):21–29.

Finlay, B. J., Span, A. and Ochsenbein-Gattlen, C. 1983. Influence of physiological state on indices of respiration rate in protozoa. *Comp. Biochem. Phys A*. 74(2):211-219.

Friedrich, T. and Scheide, D. 2000. The respiratory complex I of bacteria, archaea and eukarya and its module common with membrane-bound multisubunit hydrogenases. *FEBS let.* 479(1):1-5.

Fruton, J. S., and Simmonds, S. 1958. General Biochemistry. John Wiley and Sons, Inc., New York.

Fuse, H., Ohta, S., and Katayama, T. 1993. Varicocelectomy: changes in resazurin reduction test. *Sys. Biol. Reprod. Med.* 31(1):49-54.

Gabrielson, J., Hart, M., Jarelöv, A., Kühn, I., McKenzie, D., and Möllby, R. 2002. Evaluation of redox indicators and the use of digital scanners and spectrophotometer for quantification of microbial growth in microplates. *J. Microbiol. Method*. 50(1):63–73.

Garfield, P. C., Packard, T. T. and Codispoti, L. A. 1979. Particulate protein in the Peru upwelling system. *Deep-Sea. Res Part A.* 26(6):623-639.

Garrison, T. 2012. Essentials of Oceanography. Sixth edition. Brooks/Cole. CENGAGE Learning. Belmont, USA. p.460.

Gillooly, J.; Brown, J.; West, G.; Savage, V.; and Charnov, E. 2001. Effects of size and temperatura on metabolic rate. *Science.* 293:2248–2251.

Glass, R. H., Ericsson, S. A., Ericsson, R. J., Drouin, M. T., Marcoux, L. J., and Sullivan, H. 1991. The resazurin reduction test provides an assessment of sperm activity. *Fertil. Steril*. 56(4):743-746.

Glazier, D. 2005. Beyond the “3/4-power law”: variation in the intra- and interespecific scaling of metabolic rate in animals. *Biol. Rev*. 80:611–661.

Glazier, D., 2006. The 3/4-power law is not universal: evolution of isometric, ontogenetic metabolic scaling in pelagic animals. *BioScience* 56:325–332.

Gnaiger, E. 2009a. Capacity of oxidative phosphorylation in human skeletal muscle. New perspectives of mitochondrial physiology. *Int. J. Biochem. Cell. B*. 41(10):1837-1845.

Gnaiger, E., 2009b. Mitochondrial Pathways and Respiratory Control. **In**: Gnaiger, E. (Ed.), 2nd ed. OROBOROS MiPNet Publications, Innsbruck.

Gómez, M., Torres, S., and 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.

González, A. G., Santana-Casiano, J. M., González-Dávila, M., Pérez-Almeida, N., and Suárez de Tangil, M. 2014. Effect of *Dunaliella tertiolecta* organic exudates on the Fe(II) oxidation kinetics in seawater*. Environ. Sci. Technol*. 48(14):7933−7941.

González, R. J., and Tarloff, J. B. 2001. Evaluation of hepatic subcellular fractions for Alamar blue and MTT reductase activity. *Toxicol. in vitro*. 15(3):257-259.

González-Pinzón, R., Haggerty, R. and Myrold, D. D. 2012. Measuring aerobic respiration in stream ecosystems using the resazurin-resorufin system. *J. Geophys. Res: Biogeo*. 17(G3):2005-2012.

Gray, H. B., and Ellis Jr, W. R. 1994. Electron transfer. *Bioinorganic Chemistry. Sausalito, CA: University Science Books*, 315-363.

Guilbault, G. G. Practical fluorescence theory, methods, and techniques. 1973. Marcel Dekker, Inc. New York. pp 994.

Guilbault, G. G., and Kramer, D. N. 1965. Fluorometric procedure for measuring the activity of dehydrogenases. *Anal. Chem*. 37(10):1219-1221

Gumprecht, R., Gerlach, H., and Nehrkorn, A. 1995. FDA hydrolysis and resazurin reduction as a measure of microbial activity in sediments from the south-east Atlantic. *Helgoländer. Meeresun.* 49(1-4):189-199.

Gundemir, S., and Johnson, G. V. 2009. Intracellular localization and conformational state of transglutaminase 2: Implications for cell death. *PLos ONE*. 4:e6123.

Haggerty, R., Argerich, A., and Martí, E. 2008. Development of a ‘‘smart’’ tracer for the assessment of microbiological activity and sediment-water interaction in natural waters: The resazurin-resorufin system. *Water. Resour. Res.* 44(4):W00D01.

Haggerty, R., Martí, E., Argerich, A., von Schiller, D., and Grimm, N. B. 2009. Resazurin as a ‘‘smart’’ tracer for quantifying metabolically active transient storage in stream ecosystems. *J. Geophys. Res*. 114:G03014.

Hatefi, Y. (1973). Oxidation of reduced triphosphopyridine nucleotide by submitochondrial particles from beef heart. *Biochem. Bioph. Res. Co.* 50(4):978-984.

Hemmingsen, A. M. 1960. Energy metabolism as related to body size and respiratory surfaces, and its evolution. Reports of the Steno Memorial Hospital and the Nordisk Insulin Laboratorium (Copenhagen). 9:1-110.

Hernández-León, S. 1988. Gradients of mesozooplankton biomass and ETS activity in the wind-shear area as evidence of an island mass effect in the Canary Island waters. *J. Plankton Res. 10*(6):1141-1154.

Hernández-León. S., and Gómez, M. 1996. Factors affecting the respiration/ETS ratio in marine zooplankton. *J. Plankton Res. 18*(2):239-255.

Hernández-León, S., Gómez, M., Pagazaurtundua, M., Portillo-Hahnefeld, A., Montero, I., and Almeida, C. 2001. Vertical distribution of zooplankton in Canary Islands: implications for export flux. *Deep-Sea. Res. Pt I*. 48(4):1071–1092.

Hernández-León, S., and Ikeda, T. (2005). Zooplankton respiration. Chapter 5. In: *Respiration in Aquatic Systems. Ed.* Del Giorgio and Williams. Oxford University Press, New York. p57-82.

Hernández-León, S., Postel, L., Arístegui, J., Gómez, M., Montero, M. F., Torres, S., Almeida, C., Kühner, K., Brenning, U. and Hagen, E. 1999. Large-scale and mesoscale distribution of plankton biomass and metabolic activity in the Northeastern Central Atlantic. *J. Oceanogr. 55*(4):471-482.

Herrera, A., Gómez, M., Molina, L., and Packard, T. T. 2011. Rearing techniques and nutritional quality of two mysids from Gran Canaria (Spain). *Aquac. Res*. 42(5):677–683.

Herrera, A., Packard, T. T., Santana, S. and Gómez, M. 2011. Effect of starvation and feeding on respiratory metabolism in *Leptomysis lingvura* (GO Sars, 1866). *J. Exp. Mar. Biol. Ecol.* 409(1):154-159.

Herrmann, R. 1991. Dyes in diagnostics. *CHIMIA. Int. J. Chem.* 45(10):317-318.

Hill, J. A., Pratt, J. M., and Williams, R. J. P. 1964. 987 The chemistry of vitamin B12. Part I. The valency and spectrum of the coenzyme. *J. Chem. Soc*. 0:5149–5153.

Hobbie, J. E., Holm-Hansen, O., Packard, T. T., Pomeroy, L. R., Sheldon, R. W., Thomas, J. P. and Wiebe, W. J. 1972. A study of the distribution and activity of microorganisms in ocean water. *Limnol. Oceanogr.* 17(4):544-555.

Hope, B. T., and Vincent, S. R. 1989. Histochemical characterization of neuronal NADPH-diaphorase. *J. Histochem. Cytochem*. 37(5):653-611.

Hurd, C. L., Berges, J. A., Osborne, J., and Harrison, P. J. 1995. An *in vitro* nitrate reductase assay for marine macroalgae: optimization and characterization of the enzyme for *Fucus gardneri* (Phaeophyta). *J. Phycol.* 31(5):835-843.

Ikeda, T. 1970. Relationship between respiration rate and body size in marine plankton animals as a function of the temperature of habitat. *Bull. Facul.Fishe. Hokkaido University*. 21(2):91-112.

Ikeda, T. 1989. Estimated respiration rate of myctophid fish from the enzyme activity of the electron-transport-system. *J. Oceanograp. Soc. Jap*. 45(3):167-173.

Ikeda, T., Sano, F., and Yamaguchi, A. 2004. Metabolism and body composition of a copepod (*Neocalanus cristatus*: Crustacea) from the bathypelagic zone of the Oyashio region, western subarctic Pacific. *Mar. Biol.*145(6):1181-1190.

Ikeda, T., Sano, F., Yamaguchi, A., and Matsuishi, T. 2006. Metabolism of mesopelagic and bathypelagic copepods in the western North Pacific Ocean. *Mar. Ecol. Progr. Ser. 322*:199-211.

Ikeda, T., and Takahashi, T. 2012. Synthesis towards a global-bathymetric model of metabolism and chemical composition of marine pelagic chaetognaths. *J. Exp. Mar. Biol. Ecol*. 424:78–88.

Inczedy, J., Lengyel, T., Ure, A. M., Gelencsér, A., and Hulanicki, A. 1998. Compendium of analytical nomenclature. *Blackwell, Oxford*.

Irving, H. M. N. H., Freiser, H., and West, T. S. 2013. Compendium of analytical nomenclature: definitive rules 1977. Elsevier.

Jannasch, H. W., Wirsen, C. O., Molyneaux, S. J., and Langworthy, T. A. 1988. Extremely thermophilic fermentative archaebacteria of the genus *Desulfurococcus* from deep-sea hydrothermal vents. *Appl. Environ. Microb*. 54(5):1203-1209.

Jenneman, G. E., McInerney, M. J., and Knapp, R. M. 1986. Effect of nitrate on biogenic sulfide production. *App. Environ. Microbiol.* 51(6):1205-1211.

Jiang, L. M., Hoogenkamp, M. A., van der Sluis, L. W., Wesselink, P. R., Crielaard, W., and Deng, D. M. 2011. Resazurin metabolism assay for root canal disinfectant evaluation on dual-species biofilms. *J. Endodont.* 37(1):31-35.

Johns, C. K., and Howson, R. K. 1940. Potentiometric studies with resazurin and Methylene Blue in milk. *J. Dairy. Sci*. 23(4): 295-302.

Kalathil, S., Lee, J., and Cho, M. H. 2012. Efficient decolorization of real dye wastewater and bioelectricity generation using a novel single chamber biocathode-microbial fuel cell. *Bioresource. Technol.* 119:22-27.

Keilin, D. 1966. The history of cell respiration and cytochrome. Cambridge University Press, London.

Kennedy, K. J., and Droste, R. L. 1987. Diffusional limitations of anaerobic biofilms. *Can. J. Civil. Eng.* 14(5):631-637.

Kenner, R. and Ahmed, S. 1975**a**. Measurements of electron transport activities in marine phytoplankton. *Mar. Biol*. 33(2):119–127.

Kenner, R. and Ahmed, S. 1975**b**. Correlation between oxygen utilization and electron transport activity in marine phytoplankton. *Mar. Biol.* 33(2): 129-133.

King, F. D., and Packard, T. T. 1975a. The effect of hydrostatic pressure on respiratory electron transport system activity in marine zooplankton. *Deep-Sea Res*. 22(2):99–105.

King, F. D., and Packard, T. T. 1975b. Respiration and the activity of the respiratory electron transport system in marine zooplankton. *Limnol. Oceanogr*. 20(5):849–854.

King, L. K., Parker, B. C. 1988. Enumerating total viable and metabolically active bacteria in groundwater. *Appl. Environ. Microbiol*. 54(6):1630–1631.

Kleiber, M., 1932. Body size and metabolism. *Hilgardia* 6:315–332.

Kleiber, M., 1961. The fire of life: an introduction to animal energetics. John Wiley and Sons Inc, New York. 454 pp.

Kolokotrones, T., Savage, V., Deeds, E. J., and Fontana, W. 2010. Curvature in metabolic scaling. *Nature*. 464:753-756.

Koontongkaew, S., Thaweboon, B., and Tungtrongchitr, R. 1986. The toxicity of betel nut extract determined by inhibition of microbial dehydrogenase activity. *Tox. Assess.* 1(4):419-429.

Kornberg, A. 1989. For the love of enzymes: The odyssey of a biochemist. Harvard University Press, Cambridge 0-674-30776-3.

Kumar, A., Zhang, M., Zhu, L., Liao, R. P., Mutai, C., Hafsat, S., Sherman, D., and Wang, M-W. 2012. High-throughput screening and sensitized bacteria identify an M. tuberculosis dihydrofolate reductase inhibitor with whole cell activity. *PLoS ONE*. 7(6):e39961.

La Ferla, R., and Azzaro, M. 2004. Metabolic CO_2 production in the Mediterranean Sea: a study basin for estimating carbon budget in the sea. *Sci. Mar*. 68(Suppl. 1):57–64.

Lakowicz, J. R. 1983. Quenching of fluorescence. In: Principles of fluorescence spectroscopy. Springer US. P. 257-301.

Lane, N., 2002. Oxygen: The Molecule that Made the World. Oxford University Press, New York.

Lane, N., 2005. Power, Sex, and Suicide. Oxford University Press, New York.

Lane, N. 2010. Life Ascending. Oxford University Press, New York.

Lane, N. 2015. *The vital question: energy, evolution, and the origins of complex life*. WW Norton & Company.

Larsen, T., Larsen, M. K., and Friggens, N. C. 2011. Enzymatic and fluorometric determination of triacylglycerols in cow milk and other opaque matrices. *Food. Chem*. 125(3): 1110–1115.

Lemke, D., Liao, Z., Wöhling, T., Osenbrück, K., and Cirpka, O. A. 2013. Concurrent conservative and reactive tracer tests in a stream undergoing hyporheic exchange. *Water. Resour. Res.* 49(5):3024–3037.

Liao, Z., Lemke, D., Osenbrück, K., and Cirpka, O. A. 2013. Modeling and inverting reactive stream tracers undergoing two-site sorption and decay in the hyporheic zone. *Water. Resour. Res.* 49(6):3406–3422.

Liu, D. 1983. Resazurin reduction method for activated sludge process control. *Envir. Sci. Tech.* 17(7):407-411.

Liu, D. 1986. Resazurin reduction method for toxicity assessment of water soluble and insoluble chemicals. *Tox. Assess.* 1(2):253-258.

Liu, D., and Strachan, W. M. J. 1979. Characterization of microbial activity in sediment by resazurin reduction. *Arch. Hydrobiol. Beih. Ergebn. Limnol*. 12:24–31.

Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. 1951. Protein measurement with a folin-phenol reagent. *J. Biol. Chem*. 193(1):265–275.

Madon, S. P., Schneider, D.W. and Stoeckel, J. A. 1998. In-situ estimation of zebra mussel metabolic rates using the electron transport system (ETS) assay. *J. Shellfish Res.* 17(1): 195-204.

Maeda, H., Matsu-ura, S., Senba, T., Yamasaki, S., Takai, H., Yamauchi, Y., and Ohmori, H. 2000. Resorufin as an electron acceptor in glucose oxidase-catalyzed oxidation of glucose. *Chem. Pharm. Bull*. 48(7):897-902.

Mæland, A., Rønnestad, I., Fyhn, H. J., Berg, L., and Waagbø, R. 2000. Water-soluble vitamins in natural plankton (copepods) during two consecutive spring blooms compared to vitamins in *Artemia franciscana* nauplii and metanauplii. *Mar. Biol*. 136(5):765–772.

Marrs, B., and Gest, H. 1973. Genetic mutations affecting the respiratory electron-transport system of the photosynthetic bacterium *Rhodopseudomonas capsulata*. *J. Bacteriol.* 114(3):1045-1051.

Martinez, I., Gómez, M., and Packard, T. T. 2010. Potential respiration is a better respiratory predictor than biomass in young *Artemia salina*. *J. Exp. Mar. Biol. Ecol.* 390(2):78-83.

McNicholl, B. P., McGrath, J. W., and Quinn, J. P. 2007. Development and application of a resazurin-based biomass activity test for activated sludge plant management. *Water. Res.* 41(1):127-133.

Mahler, H. R., and Cordes, E. H. 1971. Biological Chemistry, 2nd ed. Harper and Row Publishers, New York. pp 1009.

Maldonado, F. 2009. Evaluación de la actividad del sistema de transporte de electrones en zooplancton en presencia o ausencia de sustratos específicos. 978-84-693-0652-9.

Maldonado, F., Packard, T. T., and Gómez, M. 2012. Understanding tetrazolium reduction and the importance of substrates in measuring respiratory electron transport activity. *J. Exp. Mar. Biol. Ecol*. 434:110-118.

Mariscal, A., Lopez-Gigosos, R. M., Carnero-Varo, M., and Fernandez-Crehuet, J. 2009. Fluorescent assay based on resazurin for detection of activity of disinfectants against bacterial biofilm. *App. Microbiol. Biot.* 82(4):773-783.

Martinez, R., and Estrada, M. 1992. Respiratory electron transport activity of microplankton in the Weddell Sea during early spring: influence of the ice cover and the ice edge. *Polar Biol*. 12(2):275–282.

Martínez, I., Gómez, M. and Packard, T. T. 2010. Potential respiration is a better respiratory predictor than biomass in young *Artemia salina*. *J. Exp. Mar. Biol. Ecol.* 390(2):78-83.

Martínez, R. 1997. Enhancement of microplankton respiratory activity in the Almeria-Oran front (Western Mediterranean Sea). *J. Plankton. Res.19*(12):2051-2065.

Martínez-García, S., Fernández, E., Aranguren-Gassis, M., and Teira, E. 2009. *In vivo* electron transport system activity: a method to estimate respiration in natural marine microbial planktonic communities. *Limnol. Oceanogr.-Method.* 7:459–469.

Matsumoto, K., Yamada, Y., Takahashi, M., Todoroki, T., Mizoguchi, K., Misaki, H., and Yuki, H. 1990 Fluorometric determination of carnitine in serum with immobilized carnitine dehydrogenase and diaphorase. *Clin. Chem*. 36(12):2072-2076.

Merchie, G., Lavens, P., Dhert, P., Dehasque, M., Nelis, H., De Leenheer, A., Sorgeloos, P. 1995. Variation of ascorbic acid content in different live food organisms. *Aquaculture*. 134(3–4):325–337.

Minutoli, R. and Guglielmo, L. 2009. Zooplankton respiratory Electron Transport System (ETS) activity in the Mediterranean Sea: spatial and diel variability. *Mar. Ecol. Prog. Ser.* 381: 199-211.

Morita, R. Y., and ZoBell, C. E. 1955. Occurrence of bacteria in pelagic sediments collected during the mid-Pacific expedition. *Deep. Sea. Res*. 3(1):66-73.

Moyer, R. H., and Campbell, J. J. R. 1963. Mechanism of resazurin reduction in milk of low bacterial content. *J. Dairy. Scienc*. 46(9):897-906.

Nachlas, M. M., Margulies, S. I., Goldberg, J. D., and Seligman, A. M. 1960. The determination of lactic dehydrogenase with a tetrazolium salt. *Anal. Biochem*. 1(4–5):317–326.

Namavar, F. E. R. R. Y., Verweij, A. M., Bal, M. A. R. T. I. N., Van Steenbergen, T. J., De Graaff, J., and MacLaren, D. M. 1983. Effect of anaerobic bacteria on killing of *Proteus mirabilis* by human polymorphonuclear leukocytes. *Infect. Immune.* 40(3):930-935.

Nelson, D. L., M. M., Cox. 2008. Lehninger Principles of Biochemistry. W. H. Freeman and Company, New York.

Newton, G. L., Arnold, K., Price, M. S., Sherrill, C., Delcardayre, S. B., Aharonowitz, Y., Cohen, G., Davies, J., Fahey, R. C., and Davis, C. 1996. Distribution of thiols in microorganisms: mycothiol is a major thiol in most actinomycetes*. J. Bacteriol*. 178(7):1990–1995.

Nixon, M. C., and Lamb, A. B. 1945. Resazurin test for grading raw milk. *Can. J. Comp. Med. Vet. Sci.* 9(1):18.

O'Brien, J., Wilson, I., Orton, T., and Pogna, F. 2000. Investigation of the Alamar Blue (resazurin) fluorescent dye for the assessment of mammalian cell cytotoxicity. *Eur. J. Biochem*. 267(17):5421-5426.

Oremland, R. S. 1979. Methanogenic activity in plankton samples and fish intestines A mechanism for *in situ* methanogenesis in oceanic surface waters. *Limnol. Oceanogr.* 24(6):1136-1141.

Osma, N., Fernández-Urruzola, I., Packard, T. T., Postel, L., Gómez, M., and Pollehne, F. 2014. Short-term patterns of vertical particle flux in northern Benguela: a comparison between sinking POC and respiratory carbon consumption. *J. Marine. Syst.* 140: 150-162.

Owens, T., and F. King. 1975. The measurement of respiratory electron transport system activity in marine zooplankton. *Mar. Biol*. 30(1): 27–36.

Oxtoby, D., Gillis, H., and Butler, L. (2015). Principles of modern chemistry. Sixth edition. Cengage Learning. Saunders College Publishing, Fort Worth, Texas, USA. 876 pp.

Packard, T. T. 1969. Estimation of the oxygen utilization rate in seawater from the activity of the respiratory electron transport system in plankton. PhD thesis. University of Washington. 129p.

Packard, T. T. 1971. The measurement of respiratory electron-transport activity in marine phytoplankton. *J. Mar. Res*. 29(3):235–244.

Packard, T. T. 1985a. Measurement of electron transport activity of microplankton. *Adv. Aquat. Microb.* 3:207-261.

Packard, T. T. 1985b. Oxygen consumption in the ocean: measuring and mapping with enzyme analysis. A*dv. Chem. Ser*. 209:177-209.

Packard, T. T., Berdalet, E., Blasco, D., Roy, S. O., St-Amand, L., Lagacé, B., Lee, K. and Gagné, J. P. 1996a. Oxygen consumption in the marine bacterium *Pseudomonas nautica* predicted from ETS activity and bisubstrate enyzme kinetics. *J. Plankton. Res*. 18(10):1819-1835.

Packard, T. T., Berdalet, E., Blasco, D., Roy, S., St-Amand, L., Lagacé, B., Lee, K., and Gagné, J. 1996b. CO_2 production predicted from isocitrate dehydrogenase activity and bisubstrate enzyme kinetics in the marine bacterium *Pseudomonas nautical*. *Aquat. Microb. Ecol*. 11:11–19.

Packard, T. T. and Christensen, J. P. 2004. Respiration and vertical carbon flux in the Gulf of Maine water column. *J. Mar. Res.* 62(1):93-115.

Packard, T. T., and Codispoti, L. A. 2007. Respiration, mineralization, and biochemical properties of the particulate matter in the southern Nansen Basin water column in April 1981. *Deep-Sea Res. Pt. I*. 54(3):403–414.

Packard, T. T., Devol, A. H., and King, F. D. 1975. The effect of temperature on the respiratory electron transport system in marine plankton. *Deep-Sea Res*. 22(4): 237-249.

Packard, T. T., Denis, M., Rodier, M., and Garfield, P. 1988. Deep-ocean metabolic CO_2 production: calculations from ETS activity. *Deep-Sea Res*. 35(3):371-382.

Packard, T. T., Garfield, P. C. and Martinez, R. 1983**a**. Respiration and respiratory enzyme activity in aerobic and anaerobic cultures of the marine denitrifying bacterium, *Pseudomonas perfectomarinus*. *Deep-Sea Res. Part A*. *30*(3):227-243.

Packard, T. T., P. C. Garfield, L. A. Codispoti. 1983b. Oxygen consumption and denitrification below the Peruvian upwelling. **In**: Suess, E., Thiede, J. (Eds.), Coastal Upwelling: Its Sediment Record. Plenum Press, New York, pp. 147–173.

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

Packard, T. T., and Gómez, M. 2013. Modeling vertical carbon flux from zooplankton respiration. *Progr.. Oceanogr*.110:59-68.

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

Packard, T. T., Healy, M. L. and Richards, F. A. 1971. Vertical distribution of the activity of the respiratory electron transport system in marine plankton. *Limnol. Oceanogr.* 16(1):60-70.

Packard, T. T., Osma, N., Fernández-Urruzola, I., Codispoti, L. A., Christensen, J. P., and Gómez, M. 2015. Peru upwelling plankton respiration: calculations of carbon flux, nutrient retention efficiency and heterotrophic energy production. *Biogeosciences. Discuss*. 12:2641-2654.

Packard, T. T., and Taylor, P. B. 1968. The relationship between succinate dehydrogenase activity and oxygen consumption in the brine shrimp *Artemia salina*. *Limnol. Oceanogr.* 13(3):552-555.

Packard, T. T., and Williams, P. J. L. 1981. Rates of respiratory oxygen-consumption and electron-transport in surface seawater from the northwest Atlantic. *Oceanol. Acta*. 4(3):351-358.

Palomino, J. C., Martin, A., Camacho, M., Guerra, H., Swings, J., and Portaels, F. 2002. Resazurin microtiter assay plate: simple and inexpensive method for detection of drug resistance in *Mycobacterium tuberculosis*. *Antimicrob. Agents. Chemoth.* 46(8):2720-2722.

Parry, G. 1960. Excretion. *Physiol. Crust*. 1:341–366.

Passonneau, J. V. and Lowry, O. H. 1993. *Enzymatic analysis: a practical guide*. Springer Science and Business Media. pp402.

Pawlak, K., Skrzypczak, A., and Bialek-Bylka, G. E. 2011. Inner filter effect in the fluorescence emission spectra of room temperature ionic liquids with-β-Carotene. Applications of Ionic Liquids in Science and Technology. Chapter 19. Prof. Scott Handy (Ed.).

Payne, W. J. 1976, Denitrification. Trends. Biochem. Sci. 1:220-222.

Pearre, S. Jr. 1964. Metabolic activity as an indicator of zooplankton abundance. M.s. thesis. Dalhousie University, Halifax. 78p.

Peeters, E., Nelis, H., and Coenye, T. 2008. Resistance of planktonic and biofilm-grown *Burkholderia cepacia* complex isolates to the transition metal gallium. *J. Antimicrob. Chemoth.* 61(5):1062-1065.

Peñuelas, J., Murillo, J., and Azcón-Bieto, J. 1988. Actual and potential dark respiration rates and different electron transport pathways in freshwater aquatic plants. *Aquat. Bot.* 30(4):353–362.

Peroni, C., and Rossi, G. 1986. Determination of microbial activity in marine sediments by resazurin reduction. *Chem. Ecol.* 2(3):205-218.

Perrot, S., Dutertre-Catella, H., Martin, C., Rat, P., and Warnet, J. M. 2003. Resazurin metabolism assay is a new sensitive alternative test in isolated pig cornea. *Toxicol. Sci.* 72(1):122-129.

Pesch, K. L., and Simmert, V. 1929. Untersuchungen über den Frischheitszustand der Milch. Bestimmung der Wasserstoffionenkonzentration und der Reduktionszeit. Milchwirtsch. Forsch. 8:551.

Postel, L., Mumm, N. and Krajewska-Sołtys, A. 1995. Metazooplankton distribution in the Pomeranian Bay, (southern Baltic) –species composition, biomass and respiration. *Bull. Sea. Fish. Inst*. *3*(136):61-73.

Poulsen, C., Mehalick, L. A., Fischer, C. L., Lanzel, E. A, Bates, A. M., Walters, K. S., Cavanaugh, J. E., Guthmiller, J. M., Johnson, J. K., Wertz, P. W., and Brogden, K. A. 2015. Differential cytotoxicity of long-chain bases for human oral gingival epithelial keratinocytes, oral fibroblasts, and dendritic cells. *Toxicol. Lett*. 237(1):21-29.

Proctor, M. 1997. Nitrogen-fixing, photosynthetic, anaerobic bacteria associated with pelagic copepods. *Aquat. Microb. Ecol*. 12(2):105-113.

Ramsdell, G. A., Johnson Jr, W. M. T, and Evans, F. R. 1935. Investigation of resazurin as an indicator of the sanitary condition of milk. *J. Dairy. Scienc.* 18(11):705–717.

Rabalais, N. N., Diaz, R. J., Levin, L. A., Turner, R. E., Gilbert, D., and Zhang, J. 2010. Dynamics and distribution of natural and human-caused coastal hypoxia. *Biogeosciences.* 7:585–619.

Ramírez, T., Liger, E., Mercado, J. M., Cortés, D., Vargas-Yañez, M., Sebastián, M., Reul, A.; Aguilera, J. and Bautista, B. 2006. Respiratory ETS activity of plankton in the northwestern Alboran Sea: seasonal variability and relationship with hydrological and biological features. *J. Plankton. Res*. 28(7):629-641.

Rasmussen, E. S. 1999. Use of fluorescent redox indicators to evaluate cell proliferation and viability. *In vitro. Mol. Toxicol*. 12(1):47-58.

Relexans, J. C. 1996. Measurement of the respiratory electron transport system (ETS) activity in marine sediments: state of the art and interpretation. I Methodology and review of literature data. *Mar. Ecol. Prog. Ser. 136*:277-287.

Rein, M. F., Sullivan, J. A., and Mandell, G. L. 1980. Trichomonacidal activity of human polymorphonuclear neutrophils: killing by disruption and fragmentation. *J. Infect. Dis*. 142(2):575-585.

Rijstenbil, J., and Wijnholds, J. 1996. HPLC analysis of nonprotein thiols in planktonic diatoms: pool size, redox state and response to copper and cadmium exposure. *Mar. Biol*. 127(1):45–54.

Ricciardi, A., Ianniello, R. C., Tramutola, A., Parente, E., and Zotta, T. 2014. Rapid detection assay for oxygen consumption in the *Lactobacillus casei* group. *Anna. Microbiol*. 64(4):1861-1864.

Rogers, G. E. 1953. The localization of dehydrogenase activity and sulfhydryl groups in wool and hair follicles by the use of tetrazolium salts. *Q. J. Microsc. Sci*. 3(27):253–268.

Roy, S. O., Packard, T. T., Berdalet, E. and St-Amand, L. 1999. Impact of acetate, pyruvate, and physiological state on respiration and respiratory quotients in *Pseudomonas nautica*. *Aquat. Microb. Ecol.* 17(1):105-110.

Rutter, W. 1967. Protein determinations in embryos. In: Wilt, F.H., Wessels, N.V. (Eds.), Methods in Developmental Biology. Academic Press, London. pp. 671–684.

Salcedo, F., Pereyra, C. M., Di Palma, A. A., Lamattina, L., and Creus, C. M. 2015. Methods for studying biofilms in *Azospirillum* and Other PGPRs. **In**: *Handbook for Azospirillum.* pp. 199-229. Springer International Publishing.

Sandberg, M. E., Schellmann, D., Brunhofer, G., Erker, T., Busygin, I., Leino, R., and Fallarero, A. 2009. Pros and cons of using resazurin staining for quantification of viable *Staphylococcus aureus* biofilms in a screening assay. *J. Microbial. Methods*: 78(1):104-106.

Santana-Casiano, J. M., González-Dávila, M., Rodríguez, Ma. J., and Millero, F. J. 2000. The effect of organic compounds in the oxidation kinetics of Fe (II*). Mar. Chem*. 70(1):211-222.

Sarrus; Rameaux. 1939. Bulletin de l'académie royale de médicine*.* 3:1094.

Satoh, M., Hirachi, Y., Yoshioka, A., Kobayashi, M., and Oyama, Y. 2002. Determination of cellular levels of nonprotein thiols in phytoplankton and their correlations with susceptibility to mercury. *J. Phycol*. 38(5):983–990.

Savage, V. M., Gillooly, J. F. Woodruff, W. H., West, G .B., Allen, A. P., Enquist, B. J., and Brown, J. H. 2004. The predominance of quarter-power scaling biology. *Funct. Ecol*. 18:257-282.

Savenkoff, C., Packard, T. T., Rodier, M., Gérinno, M., Lefèvre, D. and Denis M. 1995. Relative contribution of dehydrogenases to overall respiratory ETS activity in some marine organisms. *J. Plankton. Res*. 17(8):1593-1604.

Schalk, P. H. 1988. Respiratory electron transport system (ETS) activities in zooplankton and micronekton of the Indo-Pacific region. *Mar. Ecol. Prog. Ser*. 44(1):25-35.

Scheffler, I. 2008. Basic molecular biology of mitochondrial replication. Drug-induced mitochondrial dysfunction. JA Dykens and Y. Will, eds. 37-70.

Schropp, S. J., Scranton, M. I., and Schwarz, J. R. 1987. Dissolved hydrogen, facultatively anaerobic, hydrogen-producing bacteria, and potential hydrogen production rates in the western North Atlantic Ocean and Gulf of Mexico. *Limnol. Oceanogr.* 32(2):396-402.

Schuurmans, D. M., Duncan, D. T., and Olson, B. H. 1964. A bioautographic system employing mammalian cell strains, and its application to antitumor antibiotics. *Cancer. Research*. 24(1 part 1):83-89.

Segel, I. H. 1976. Biochemical Calculations: How to solve mathematical problems in general biochemistry. John Wiley and Sons, New York.

Segel, I. H. 1993. Enzyme kinetics: behavior and analysis of rapid equilibrium and steady state enzyme systems. Wiley Classics Library. Wiley-Interscience, New York.

Seidler, E., 1991. The tetrazolium-formazan system: design and histochemistry. *Prog. Histochem. Cytochem*. 24(1):1–86.

Seiwell, H. R. 1937. Consumption of oxygen in seawater under controlled laboratory conditions. *Nature*. 140(1937):506–507.

Shahani, K. M., and Badami, M. C. 1958. A Resazurin disk assay method for detecting antibiotics and natural starter inhibitory activity in milk. *J. Dairy. Sci.* 41(1958):1510-1517.

Shapiro, F., and Silanikove, N., 2010. Rapid and accurate determination of D and L-lactate, lactose and galactose by enzymatic reactions coupled to formation of a fluorochromophore: Applications in food quality control. *Food. Chem.* 119(2):829–833.

Shapiro, S. S.; and Wilk, M. B. 1965. An analysis of variance test for normality (complete samples). *Biometrika*. 52 (3-4):591–611.

Shen, Y. J., Shoichet, M. S., and Radisic, M. 2008. Vascular endothelial growth factor immobilized in collagen scaffold promotes penetration and proliferation of endothelial cells. *Acta. Biomater*. 4(3):477–489.

Sigma-Aldrich Catalogue, 2011. http://www.sigmaaldrich.com/analytical-chromatography/ analytical-reagents/analytical-reagents-explorer.html.25/07/2012.

Silanikove, N., and Shapiro, F. 2012. Combined assay for lactose and galactose by enzymatic reactions. *Dietary Sugars: Chemistry, Analysis Function and Effects*, Chapter 23:395-404.

Simčič, T. and Brancelj, A. 2004. Respiratory electron transport system (ETS) activity as an estimator of the thermal tolerance of two Daphnia hybrids. *J. Plankton. Res*. 26(5):525-534.

Slater, T. F., Sawyer, B, and Sträuli, U. 1963. Studies on succinate-tetrazolium reductase systems: III. Points of coupling of four different tetrazolium salts III. Points of coupling of four different tetrazolium salts. *Bioch. Biophys.acta*. 77:383-393.

Smith, J. J., and McFeters, G. A. 1997. Mechanisms of INT (2-(4-iodophenyl)-3-(4-nitrophenyl)-5-phenyl tetrazolium chloride), and CTC (5-cyano-2,3-ditolyl tetrazolium chloride) reduction in *Escherichia coli* K-12. *J. Microbiol. Meth.* 29(3):161-175.

Snedecor, G. W. 1934. Calculation and interpretation of analysis of variance and covariance. Collegiate Press, Inc. Iowa.

Stanaway, D., Haggerty, R., Benner, S., Flores, A., and Feris, K. 2012. Persistent metal contamination limits lotic ecosystem heterotrophic metabolism after more than 100 years of exposure: A novel application of the resazurin resorufin smart tracer. Envir. Sci. Tech. 46(18):9862-9871.

Stark, P. L., and Lee, A. 1982. The microbial ecology of the large bowel of breastfed and formula-fed infants during the first year of life. *J. Med. Microbiol.* 15(2):189-203.

Steven, B., Leveille, R., Pollard, W. H., and Whyte, L. G. 2006. Microbial ecology and biodiversity in permafrost. *Extremophiles*. 10(4):259-267.

Strittmatter, P. 1968.Microsomal electron transport. *In:* Biological oxidations. Singer. New York: Wiley Interscience. pp 171-192.

Strzeżek, R., Filipowicz, K., Stańczak, M., and Kordan, W. 2013. Spectrophotometric analysis of the resazurin reduction test as a tool for assessing canine semen quality. B. Vet. I. Pulawy. 57(2):281-285.

Sverdrup, H. U., Johnson, M. W., and Fleming, R. H. 1942. The oceans their physics, chemistry, and general biology. Prentice-Hall, New York.

Teuber, L., Schukat, A., Hagen, W. and Auel, H. 2013. Distribution and ecophysiology of calanoid copepods in relation to the oxygen minimum zone in the eastern tropical Atlantic. *PLoS ONE*. 8(11): e77590.

Thies, F., and Grimme, L. H. 1995. O-Dealkylation of coumarin and resorufin ethers by unicellular green algae: kinetic properties of *Chlorella fusca* and *Chlorella sorokiniana.* Arch. Microbiol. 164(3):203-211.

Thomsom, K., Liu, D., and Kaiser, K. L. E. 1986. A direct resazurin test for measuring chemical toxicity. *Toxic. Assess*. 1(4):407-418.

Toth, L., Szabo, M. and Webb, D. J. 1995. Adaptation of the tetrazolium reduction test for the measurement of the electron transport system

(ETS) activity during embryonic development of medaka. *J. Fish. Biol.* 46(5):835-844.

Treeck, O., Wackwitz, B., Haus, U., and Ortmann, O. 2006. Effects of a combined treatment with mTOR inhibitor RAD001 and tamoxifen *in vitro* on growth and apoptosis of human cancer cells. *Gynecol. Oncol*. 102(2):292-299.

Tzagoloff, A. 1982. Mitochondria. Plenum Press, New York. Pp 342.

Uglow, J. S. 2002. The Lunar Men: Five friends whose curiosity changed the world. Farrar Straus Giroux, New York.

Undenfriend, S. 1962 Fluorescence Assay in Biology and Medicine. Academic Press, New York, N. Y. p. 312.

UNESCO 1968. Zooplankton sampling. Unesco Press, Paris.174pp.

Van den Driessche, F., Rigole, P., Brackman, G., and Coenye, T. 2014. Optimization of resazurin-based viability staining for quantification of microbial biofilms. *J. Microbiol. Methods*. 98:31-34.

Villegas-Mendoza, J., Cajal-Medrano, R., and Maske, H. 2015. INT (2-(4-Iodophenyl)-3-(4-Nitrophenyl)-5-(Phenyl) Tetrazolium Chloride) is toxic to prokaryote cells precluding its use with whole cells as a proxy for *in vivo* respiration. *Microb. Ecol.* 70(4): 1004-1011.

Wang, L., Yan, B., Liu, N., Li, Y., and Wang, Q. 2008. Effects of cadmium on glutathione synthesis in hepatopancreas of freshwater crab, *Sinopotamon yangtsekiense*. *Chemosphere*. 74(1):51–56.

Wattenberg, L. W., and Leong, J. L. 1960. Effects of coenzyme Q10 and menadione on succinic dehydrogenase activity as measured by tetrazolium salt reduction. *J. Histochem. Cytochem*. 8(4):296–303.

Webb, K. L., and Johannes, R. E. 1967. Studies of the release of dissolved free amino acids by marine zooplankton. *Limnol. Oceanogr*. 12(3):376–382.

Wei, L., Hongying, H., Xin, G., Yuling, C., Hui, W., Yufeng, G., and Yudong, S. 2006. Effects of micronutrient niacin on treatment efficiency of textile wastewater. *Wuhan. U. J. Nat. Sci.* 11(3):737-741.

Wellsbury, P., Mather, I., and Parkes, R. J. 2002. Geomicrobiology of deep, low organic carbon sediments in the Woodlark Basin, Pacific Ocean. *FEMS. Microbiol. Ecol*. 42(1):59-70.

Weselsky, P. 1871, Ueber die Azoverbindungen des Resorcins. *Ber. Dtsch. Chem. Ges*. 4:613–619.

Weymouth, F. W., Crismon, J. M., Hall, V. E., Belding H. S.,and Field II, J. 1944. Total and tissue respiration in relation to body weight a comparison of the kelp crab with other crustaceans and with mammals. *Phy. Zoo*. 17(1):50-71.

Wong, J. Y., Huggins, G. S., Debidda, M., Munshi, N. C., and De Vivo, I. 2008. Dichloroacetate induces apoptosis in endometrial cancer cells. *Gynecol. Oncol.* 109(3):394-402.

Yebra, L., Hernández-León, S., Almeida Peña, C. and Bécognée, P. 2009. Metabolism and biomass vertical distribution of zooplankton in the Bransfield Strait during the austral summer of 2000. *Polar. Res*. 28:415-425.Zalata, A. A., Lammertijn, N., Christophe, A., and Comhaire, F. H. 1998. The correlates and alleged biochemical background of the resazurin reduction test in semen*. Int. J. Androl*. 21(5):289-294.

Zare, M. R., Amin, M. M., Nikaeen, M., Zare, M., Bina, B., Fatehizadeh, A., Rahmani, A., and Ghasemian, M. 2015. Simplification and sensitivity study of Alamar Blue bioassay for toxicity assessment in liquid media. *Desalination. Wat. Treatm.*(ahead-of-print).

Yurista, P. M. 1999. A model for temperature correction of size specific respiration in *Bythothrephes cederstroemi* and *Daphnia middendorffiana*. *J. Plankton Res*. 21(4):721–734.

Zeuthen, E. 1953. Oxygen uptake as related to body size in organisms. *Quart. Rev. Biol.* 28:1-12.

Zhang, H. X., Du, G. H., and Zhang, J. T. 2004. Assay of mitochondrial functions by resazurin *in vitro*. *Acta Pharmacol Sin*. 25(3): 385–389.

Zhou, M., Diwu, Z., Pachuk-Voloshina, N., and Haugland, R. P. 1997. A stable nonfluorescent derivative of resorufin for the fluorometric determination of trace hydrogen peroxide: applications in detecting the activity of phagocyte NADPH oxidase and other oxidases. *Anal. Biochem*. 253(2):162-168.

Agradecer al Gobierno de Canarias a través de la Agencia Canaria de Investigación, Innovación y Sociedad de la Información, por concederme la beca de investigacion y hacer possible este trabajo