Planktonic potential CO_2 emission calculation: Preliminary results by applying an adapted enzymatic methodology to marine ecosystems.

Calculando la emisión potencial de CO_2 de la comunidad planctónica: Resultados preliminares de la aplicación de una metodología enzimática adaptada a los ecosistemas marinos.

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Abstract: The first steps in developing an enzyme assay for Isocitrate Dehydrogenase (IDH) activity in seawater and marine plankton are presented here. This enzyme plays a key role in the Krebs cycle, being responsible for the emission of one of the three CO_2 molecules related to this central phase of cellular respiration. The methodology that we have adapted from the literature allows the calculation of the potential CO_2 emission linked to the planktonic community (between 0.7µm and 2000µm). It will improve the estimation of the impact of plankton on the Carbon flux and the actual Carbon sink capacity of the ocean. This assay has been applied to the 0.7µm -50µm fraction of the Canary Island coastal plankton community, to the 50µm -2000µm fraction of this community, and also, to sediment-trap samples. Results show different relationships between potential CO_2 emission and potential O_2 consumption during cellular respiration in the different samples. Likely, the different proportions of autotrophs, heterotrophs and mixotrophs in those fractions and the variability in the activity of their metabolic pathways, leads to this behaviour. More experiments need to be made. Nevertheless, this methodology is leading to a better understanding of cellular respiration in marine samples and new knowledge about the role of the food chain, vertical carbon flux and the current sequestering capacity for anthropogenic CO_2 in these plankton communities.

Key words: *Isocitrate dehydrogenase (IDH), Krebs cycle, Potential Respiration, Electron Transport System (ETS), CO_2 emission, Marine plankton community.*

1. INTRODUCTION:

Isocitrate Dehydrogenase (IDH) is one of the enzymes in the Krebs cycle where sugars, fatty acids and amino acids are oxidized. It produces CO_2 and energy-rich pyridine nucleotides, molecules essential to energy generation. The respiratory Electron Transport System (ETS) uses these molecules in order to synthesize ATP, another energy-rich molecule, while simultaneously reducing O_2 to H_2O.

Several studies about the different types of IDH have been done, but most of them are based on higher organisms, usually mammals. Nevertheless, some have been based on marine organisms, such as fishes (Munilla-Morán & Stark, 1989; Munilla-Morán, 1994), mussels (Lima *et al*., 2007) or marine bacteria (Berdalet *et al*., 1995). As far as we know, none of them are based on marine planktonic communities in the size-range of 0.7µm to 2000µm.

These communities are essential in the Carbon cycle, having a key role in the effectiveness of the oceanic biological pump. They are the base of the food chain, and the first stage in oxidizing the organic carbon synthesized (from CO_2) by the phytoplankton. Furthermore, these communities are ubiquitous through the water column, impacting differently in the carbon-flux transfer efficiency at different depths.

Successful calculations of respiratory CO_2 emission from plankton using the enzyme proxy, ETS, has recently been done (Packard *et al*., 2015). Mathematical models of respiration incorporate the Respiration Quotient (RQ), the ratio between CO_2 emission about the physiological O_2 consumption (Romero-Kutzner *et al*., 2015). This step can be eliminated if a proxy for CO_2 emission can be developed. Here, we develop such a proxy using measurements of IDH activity in different size fractions (0.7µm - 2000µm) of marine plankton.

2. ADAPTATION OF THE METHOD:

Our IDH assay is based on the methodology of Berdalet *et al.* (1995) for bacteria and Munilla-Morán & Stark (1989) for fish. Here we modify and optimize a combination of these two methods for marine plankton.

2.1 Buffer selection: An IDH assay is performed in a buffered reaction mixture containing inorganic cationic cofactors. Different buffers and cationic cofactors have been used by various investigators. Here we test, experimentally, the efficiency of Tris, phosphate, and MOPS buffers as well as the cofactors, Mn^{2+} and Mg^{2+} in yielding high IDH activities. Activity was measured spectrophotometrically by following the production of NADPH as the increase in absorbance at 340nm at 18.0 ºC. To determine the best combination between buffer and the cationic cofactor, ten experiments were done. Preliminary test were run with purified $NADP^+$-dependent isocitrate dehydrogenase (SIGMA I2002). Five different IDH concentrations were tested on six different extraction buffers (MOPS 0.025M, MOPS 0.025M with lysozyme (SIGMA L6876), Tris 0.1M, Tris 0.1M with lysozyme, Phosphate buffer 0.1M, Phosphate buffer 0.1M with lysozyme), all at pH 8.5.

The experiments with Mg^{2+} as a cation cofactor were done in the following way: 100 µl of standard were added to a mixture of 300µl of 3.3mM DL-Sodium-isocitrate (SIGMA I1252) and 3.3mM $MgCl_2$ (PANREAC 131396.1210) in the respective buffer. Then, to start the reaction, 100 µl of 0.4mM β-$NADP^+$ (SIGMA N0505), in the same respective buffer were added. The final volume was 500 µl. The increase in absorbance at 340nm was recorded for 350 seconds. Only the linear part was taken in account. The experiments with Mn^{2+} as the cation cofactor were done in the same way, but using $MnCl_2$ (PANREAC 131410.1210). Note that PO_4 buffer cannot be used with $MnCl_2$ because they react with each other.

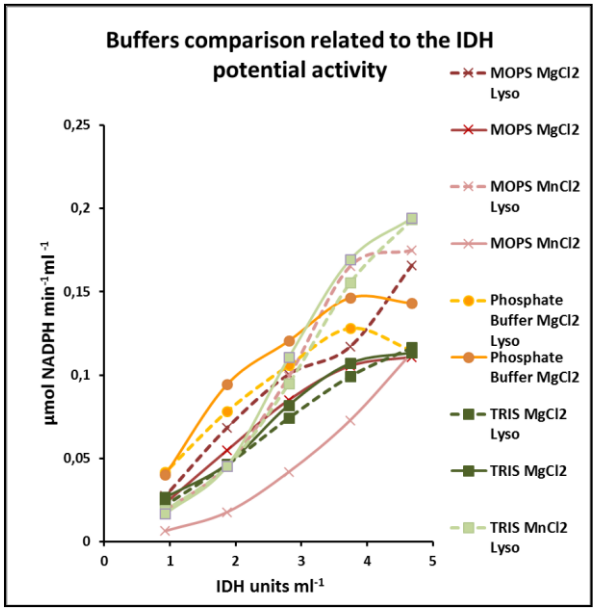

Fig. 1.Buffer comparison related to the IDH potential activity.

IDH activity is low in marine plankton, so choosing the best buffer and cationic cofactor combination is important to produce the highest signal possible. In this case, the Mg^{2+} and PO_4 buffer with or without lysozyme seemed to be the most appropriate (Fig. 1).

2.2 pH and Temperature pH between 7.8 and 9 yield optimum IDH activity (Munilla-Morán & Stark, 1989). We chose pH 8.5, but verification is needed. The IDH temperature (T) optimum occurred at 40ºC (Munilla-Morán, 1994), however, to combine the IDH and ETS methodologies, T = 18ºC was selected. The Arrhenius equation needs to be applied to calculate these activities for insitu T.

2.3 NADPH extinction coefficient (A_{340}): To verify A_{340} under current conditions (0.1M PO_4 buffer, pH 8.5, 18ºC) as well as to determine the effect of isocitrate and $MgCl_2$ on A_{340}, three extinction coefficients were determined (Fig. 2).

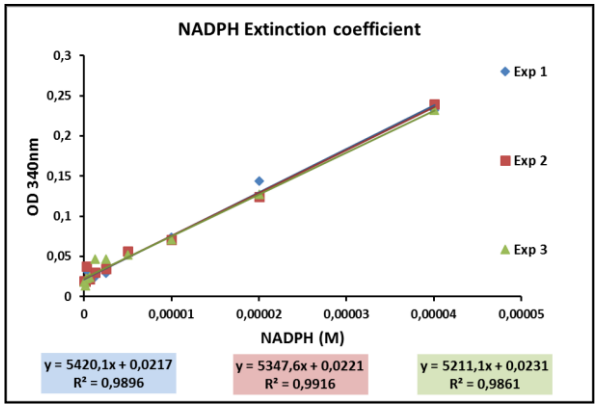

Fig. 2.NADPH A_{430} measured at pH 8.5 and 18ºC. Exp 1: different concentrations of NADPH in 3.3mM sodium isocitrate and 3.3mM $MgCl_2$ in 0.1M Phosphate buffer. In Exp 2, these concentrations were prepared in 3.3mM $MgCl_2$ and 0.1M Phosphate buffer. In Exp 3, they were prepared in 0.1M Phosphate buffer, only.

NADPH concentrations were followed at 340nm (Fig. 2). In our hands, the absorbance increase related to NADPH concentrations evidenced an A_{430} of 5.42. Note that as the solution becomes more complex the A_{340} increases from 5.21 to 5.42.

2.4 $NADP^+$ and Isocitrate Kinetics (K_m and V_{max}): Specific substrate and cofactor concentrations need to be calculated to optimize the new enzyme assay. To do this, IDH kinetics constants were calculated from Michaelis-Menten plots (Fig. 3 and 4) and their Hanes-Woolf equivalents (Suelter, C.H., 1985). Atlantic seawater samples were used for the experiments. Mysid samples of *Siriella armata* from Risco Verde (Gran Canaria) were taken by SCUBA diving and stored alive. Zooplankton size fractions (50µm-200µm and 200µm-2000µm) were also taken from Sardina del Norte (Gran Canaria) using a WP2 net dragged by 3 SCUBA divers. Samples were fractionated through 2000µm, 200µm and 50µm nets. Finally, samples were taken from Alcaravaneras Beach (Las Palmas de Gran Canaria), and passed through a 50µm net and a 0.7µm Glass Fiber Filter (GF-F Whatman) to obtain the small size fraction

(0.7µm-50µm). All plankton samples were stored at -80ºC until analysis. Isocitrate K_ms were calculated from Michaelis - Menten plots (Fig. 3). $NADP^+$ was held at 0.24mM during the different experiments. Hanes-Woolf plots yielded a mean K_m of 0.167mM for the Isocitrate in these samples.

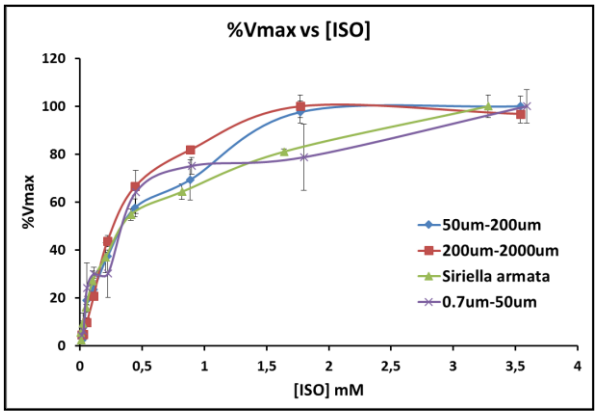

Fig.3: Michaelis-Menten plot to determine the IDH K_m for isocitrate from different plankton community size fractions as well as for the mysid, S.armata. The size fraction above 200µm represents zooplankton. The smaller fractions will also contain phytoplankton and protozoans, and the smallest will contain, in addition, bacteria and archea.

$NADP^+$ K_ms were calculated as before. Here, isocitrate was held constant at 3.3mM during the different experiments (Fig. 4). Hanes-Woolf plots show a mean $NADP^+$-K_m of 0.096mM.

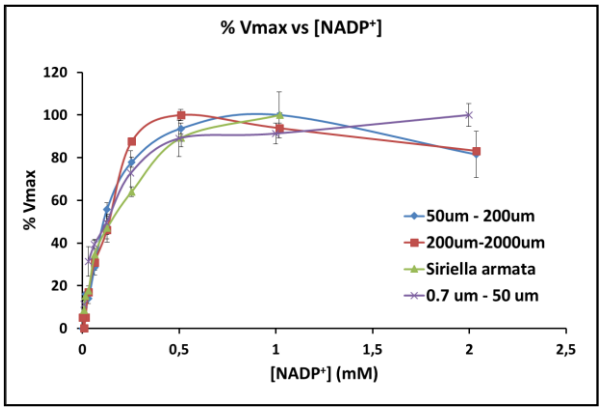

Fig.4: Michaelis-Menten plot to determine the IDH K_m for $NADP^+$ from different plankton community size fractions as well as for the mysid, S. armata.

Isocitrate at 3.5mM and $NADP^+$ at 1mM will ensure IDH optimal activity in the size range: 0.7 to 2000µm.

3. APPLICATION OF THE METHOD

3.1 Samples: This method was applied to the 50µm-2000µm fraction samples taken during KOSMOS GC 1.0, a German funded research project (BIOACID), carried out during the spring, 2014 in 17 m mesocosms suspended in Melenara Bay (Gran Canaria). In addition, 0.7µm-50µm samples were taken during KOSMOS GC 2.0 campaign during the fall 2014 in Gando Bay. Sediment samples from the bottom of the mesocosms were also taken during KOSMOS GC 2.0 and passed through 0.7µm GF-F. All these samples were frozen -196ºC and stored at -80ºC until analysis. Size fractions larger than 50µm were sonicated in 1mL of 0.1M PO_4 buffer (pH 8.5) and centrifuged at 4000rpm and 0ºC for 10 min. The supernatant fluid was then tested for IDH activity. GF-F samples were homogenated in a teflon tissue grinder in 1.5mL of 0.1M PO_4 buffer (pH 8.5) and centrifuged as above.

3.2 Enzymatic activity: IDH activity ($\mu lCO_2 \cdot h^{-1}$) was measured spectrophotometrically by following NADPH production as the absorbance increase at 340nm at 18.0 ºC. In a final volume of 500 µl, 300µl of 3.5mM sodium isocitrate and 3.3mM $MgCl_2$ in 0.1M PO_4 buffer (pH 8.5) solution was added. Then 100 µl of the supernatant of the sample was added. Finally, 100 µl of 1mM β-NADP in 0.1 M PO_4 buffer (pH8.5) was added and mixed, to start the reaction. The absorbance increase was recorded for up to 400 seconds. Only the linear part was used.

Potential respiration (Φ) ($\mu lO_2 \cdot h^{-1}$) for the 0.7µm-50 µm fraction and for the sediment matter was determined spectrophotometrically by Packard *et al*. (1971) as modified by Kenner & Ahmed (1975). For the 50µm-2000µm fraction, the Owens & King (1975) and Gómez *et al.* (1996) ETS methods were used.

3.3 Results: Φ and IDH activity were measured for every sample to determine the relationship between the Krebs cycle part of the respiration process, linked to the CO_2 emission, and the ETS part of the respiration process, linked to the O_2 consumption.

Natural samples were grouped in three categories (Fig. 5): 1. A 50µm-2000µm fraction was a mixed, largely heterotrophic community. 2. A 0.7µm -50 µm fraction was a mixed community combining autotrophs and mixotrophs. 3. A sediment trap sample from 17m representing a mixed community, predominated by heterotrophic bacteria.

Φ in the largest plankton fraction is 2.24 (r^2 = 0.79) times higher than the IDH activity. This ratio is larger in the phytoplankton dominated fraction, reaching 5.7, but is variable leading to a low coefficient of determination (r^2 = 0.39). The highest ratio occurs in the microbial-dominated community from the sediment trap samples (10.61, r^2 = 0.87).

4. DISCUSSION:

These communities have mixed trophic metabolic pathways. The 50µm-2000µm fraction is clearly a heterotrophic and eukaryotic community. The IDH activity in the mitochondria is linked to the emission of one of the three CO_2 molecules that are produced during respiration. During glucose metabolism the potential emission of CO_2 would be 3 times the

measured IDH activity. During lipid metabolism it would be twice.

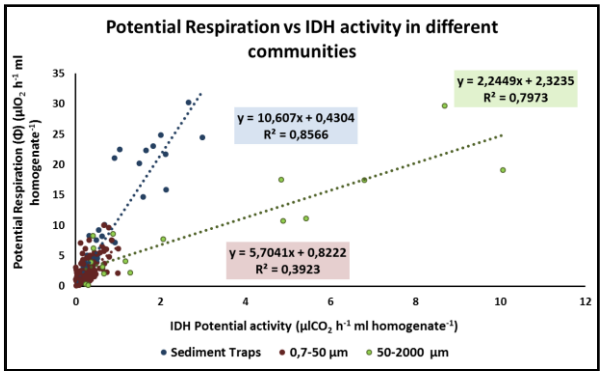

Fig.5: Potential respiration vs potential CO_2 production calculated from ETS and IDH activities in different natural samples (sediment matter, 0.5µm-50µm and 50µm-2000µm fraction).

From the comparison between Φ and this potential CO_2 production from the 50µm-2000µm fraction, the O_2 consumption would be 0.75 times the CO_2 emission. Thus, we can hypothesize that the Krebs cycle enzymatic structure could be 1.33 times the ETS activity. Following this idea, the mainly phytoplankton community (0.7µm-50µm) would have a weaker Krebs cycle contribution than the zooplankton´s. Autotrophs have other pathways for developing biomolecule precursors and reducing molecules. Something similar may occur in the bacterial community from the sediment trap samples, where the photoautotrophy and the chemoautotrophy could generate a high portion of the energy needs for this community. Much more work needs to be done to confirm these hypothesis.

5. CONCLUSIONS:

These are the first IDH activity measurements in marine plankton. To accomplish them we adapted existing IDH methods and made comparisons with ETS activity measurements. Thus, we note that the different communities shows different relationships between IDH and ETS activity. This is likely due to different pyridine nucleotide requirements linked to different metabolic pathways. In addition to using $NADP^+$ as an IDH substrate, these marine communities may also need NAD^+.

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REFERENCES:

Berdalet, E.; Packard, T.T.; Lagacé, B.; Roy, S.; St-Amand, S.; Gagné, J.-P. (1995) CO2 production, O2 consumption and isocitrate dehydrogrenase in the marine bacterium Vibrio natriegens. Aquatic microbial ecology 9: 211-217.

Gómez, M.; Torres, S.; Hernández-León, S. (1996) Modification of the electron transport system (ETS) method for routine measurements of respiratory rates of zooplankton. *South African Journal of Marine Science* 17(1): 15-20.

Kenner, R.A. and Ahmed, I. (1975). Measurements of Electron Transport Activities in Marine Phytoplankton. *Marine Biology* 33:119-127.

Lima, I.; Moreira, S.M.; Rendón-Von Osten, J.; Soares, A.M.V.M. and Guilhermino L. (2007). Biochemical responses of the marine mussel *Mytilus galloprovincialis* to petrochemical environmental contamination along the North-western coast of Portugal. *Chemosphere* 66: 1230-1242.

Munilla-Morán, R. and Stark, J.R (1989). Biochemical studies in marine species – I. $NADP^+$-dependent isocitrate dehydrogenase from turbot liver (*Scophthalmus maximus L.*). *Comparative Biochemistry and Physiology* 93B (4):823-828.

Munilla-Morán, R. (1994) Biochemical studies in marine species - II. $NADP^+$-dependent isocitrate dehydrogenase from turbot (*Scophthalmus maximus L.*) larvae. *Comparative Biochemistry and Physiology* 107B (1):61-68.

Owens, T.G. and King, F.D. (1975).The measurement of Respiratory Electron-Transport System Activity in Marine Zooplankton. *Marine Biology* 30:27-36.

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. Limnology and Oceanography, 16: 60-70.

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

Romero-Kutzner, V.; Packard, T.T.; Berdalet, E.; Roy, S.O.; Gagné, J.-P.; Gómez, M. (2015) Respiration quotient variability: bacterial evidence. *Marine Ecology Progress Series*, 519: 47-59

Suelter, C.H. (1985). Protein-Ligand Complexes and Enzyme Kinetics. *In*: Suelter, C.H. and Meister, A. *A practical guide to enzymology*. *Biochemistry, a series of monographs*. A Wiley Intersciences publication. Wiley & Sons, New York, 203-264.