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A NEW FLUORESCENT METHOD FOR THE MEASUREMENT OF RESPIRATION IN MARINE PLANKTON

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INTRODUCTION

Respiration consists of the consumption of oxygen, the production of CO_2, and the biological consumption of organic carbon (Lane, 2010). It is a key physiological process in all marine organisms and as such it is fundamental in assessing the metabolic balance in the ocean (del Giorgio and Duarte, 2002). 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). This is because the origin of most oceanic respiration is the microscopic-sized plankton and because obtaining physiologically acceptable samples is so difficult and costly. Furthermore, because the respiration measurement itself, is almost impossible to make except in eutrophic surface waters, where the respiration signal is very strong the ETS technique was developed as a proxy for the respiration of all the organisms in a parcel of seawater (Packard, 1971; Packard et al., 1971 and Packard et al., 1974). 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; Maldonado et al., 2012). The nearly universal electron transfer molecule NADH dehydrogenase, facilitates the transfer of 2 electrons ($NADH+H^+ \rightarrow NAD^{+}+2e^-+2H^+$). This in vitro redox reaction has been detected and its activity measured by the reduction of 2-(ρ-iodophenyl)-3-(ρ-nitrophenyl)-5phenyl tetrazolium chloride salt (INT), whose reduction is detected by the absorbance in a spectrophotometer. But to register an acceptable signal it is necessary to concentrate (nets or filtering) the plankton, since it is so dispersed in the ocean that oxygen consumption rate cannot be detected. During the process the organisms 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.

The main objective is to measure the oxygen consumption on biological oceanographic samples (ie, picoplankton and zooplankton) using an improve enzymatic technic; In this case using the fluorescent dye resorufin as result of the reduction of the resazurin as the artificial electron acceptor of the ETS, that can be detected in a spectrofluorometer. The advantage of this technique is that can be 100 times more sensitive, this causes in the reduction of the biomass need to acquire a representative signal. So our first step is to determine the best conditions for the use of this improvement in the technique.

METHODS

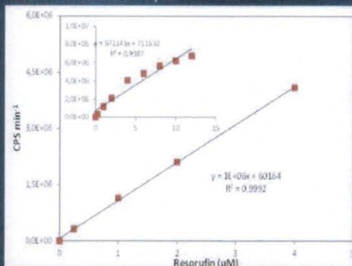
The definition of the method is due to a progressive series of experiments, where the determination of each optimum condition is applied to the following test. Fluorescence was measured using a Horiba-FluoroMax-4 Spectrofluorometer with a xenon lamp. All measurements where made with a 2 nm bandpass, 0.5 s integration time For the reagent optimum determinations, a 3 ml Helma® GS quartz cuvettes were used.

The first step was to know the emission and excitation peaks of the resorufin. It is important to note that we have two kinds of this dye. The firs is pure for the calibration curve, the second is the resorufin as the product of the reduction by the reaction mixture of Substrate buffer (NADH + NADPH +Succinate), Diaphorase solution, in a relationship 3:1:1 respectively (Kenner and Ahmed 1975; Owens and King, 1975). When the reaction achieves the plateau, it is assumed that all the resazurin is reduced to resorufin. Then one needs to determine the peaks of emission and excitation. Further more one needs to know if, at the same concentration of resorufin, both kinds give the same signal (CPS).

The key actors in a enzymatic reaction of respiration are the substrates (NADH, NADPH and succinate) the artificial electron acceptor (resazurin) and the enzymatic complex (Diaphorase from *Clostridium kluyvery*). So we determine individually the optimum concentration of each reactant. Substrate concentrations are from a range of values that lie between those reported by Owens and King (1975), Kenner and Ahmed (1975) where used . As we learned, succinate does not react with the diaphorase, but to know if succinate makes any affect on the activity when all substrates are together as is used for phytoplankton community (Kenner and Ahmed, 1975) two experiments were made to determine if succinate can make a synergic effect in the reaction.

A good calibration plot will assure a good interpretation of the resorufin production in biological samples. A broad range of resorufin concentrations was used, since this dye is highly fluorescent. Reproducibility, limit of detection (LOD) and quantification (LOQ) detection values were also determined.

Figure 6. Calibration plots of pure resorufin. In the box, we test whole range of resorufin concentration (0-12 µM). After 6 µM the fluorescence becomes saturated, so to obtain linearity (r^2 0.996) the maximum concentration used is 5 µM.



CONCLUSIONS

1. The pure resorufin and the one obtain from the reduction behave in the same way. They gave the same emission and excitations peaks, and also gave the same signal at equal concentrations. This means that the two resorufins are totally comparable.
2. The NADH and NADPH substrates at the concentration tested are in saturating levels and do not limit the reaction.
3. Succinate does not react nor contribute to the velocity of the reaction. So it will not be used.
4. The low LOD and LOQ (0,014 and 0,033 uM) will favor the application of this new dye and technique to very low concentrations of biomass. This will translate into a decrease in the time needed for plankton fishing or water filtration.

RESULTS

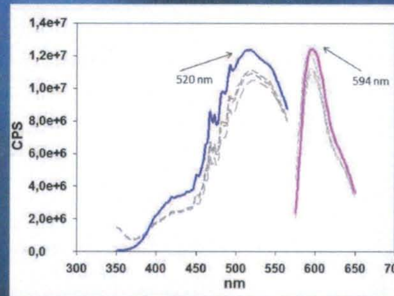


Figure 1. Excitation and Emission spectra of resofurin obtained from the reduction of resazurin (colored lines), compared with the emission and excitation spectra of pure resorufin (gray colors). Both types of resorufin coincide in the peaks of emission (520 nm) and excitation (594 nm) in both tests, meaning that they are the same.

Figure 2. The signal obtained (CPS) from the pure resorufin and the one from the reduction of resazurin. In the two lowest concentrations the signals are almost the same. The other ones are very closed, and this could be because the reaction was followed only for 10 minutes, not enough time to react all the resazurin. This is why the total concentration of resazurin was not reduced to resorufin.

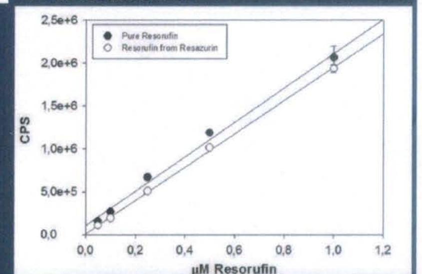


Figure 3. Fluorescence production in time (slope), at different NADH concentrations. There is no difference between the different concentrations. This indicates that at concentrations grater than 0,1µM, the reaction is not limited or affected by NADH. Since substrates are expensive, for further experiments 1mM concentration is selected. On the diagram is an explanation of the reaction.

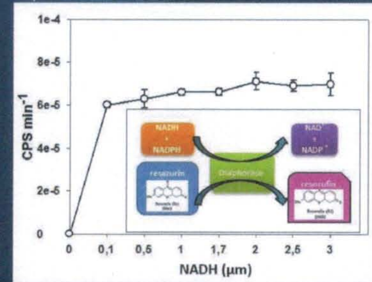


Figure 5. Fluorescence production in time (slope), at different diaphorase concentrations. In the range tested there is no limitation or interference of the reaction by the diaphorase.

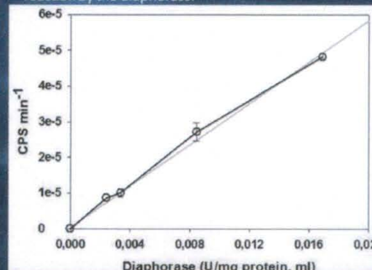
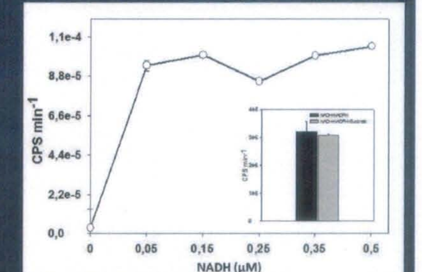


Figure 4. Fluorescence intensity production in time (slope), at different NADH concentrations. There is no difference between the different concentrations. This indicates that at a concentration greater than 0,05µM, the reaction is not limited or affected by NADPH. Since substrates are expensive, for further experiments 1µM concentration is selected. Succinate did not react (data not shown). In the box we demonstrate that succinate does not have any synergic effect on the velocity of the reaction



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