INTRODUCTION: Is the ocean net autotrophic or heterotrophic? Is the biosphere losing nitrogen to nitrification and denitrification or is it in balance with nitrogen fixation and anthropogenic inputs? These questions cannot be resolved without accurate measurements of the physiological processes involved and yet our methodology lags far behind our curiosity and the demand to monitor our rapidly changing planet. This situation cries out for methods development. For this reason, as in Gomez et al. (1996), we continue to explore the use of the respiratory electron transport system (ETS) as a measure of potential respiration (R) and a proxy for respiration (R). Here we demonstrate: (1) how kinetic ETS assays document reaction-rate time-courses, cuts reaction-time in half, and eliminates turbidity blanks; (2) why substrate blanks do not measure ETS activity and why a Michalis-Menten Vmax should be measured instead; and (3) the biochemical meaning of R/phys (integrated) and CTC.

METHODS ETS assays reported here are based on the Packard and Williams (1981) method with the simplifications: (1) that turbidity and substrate blanks have been replaced by a reagent blank (an ETS assay with 1/1000 part of the sample’s biological material); (2) that succinate as a reactant (Savenkov et al., 1995) and cyanide as an inhibitor of cytochrome oxidase, have been eliminated. All measurements were made kinetically according to F. D. King (personal communication) with a computer-controlled spectrophotometer using automatic sample changing and temperature control. Homogenates were kept at 0-4°C and were measured within 20 min after being removed from either liquid nitrogen or -80°C storage.

RESULTS Fig. 1A-1C show that kinetic analysis offers the advantages of: (1) detecting errors during the ETS reaction; (2) halving the time of the analysis; (3) increased sensitivity and resolution through better slope definition and higher data acquisition rate. Figs. 2A-2C shows that ETS activity cannot be measured in zooplankton, phytoplankton, or seawater (microplankton) without adding substrates (NADH and NADPH). Fig. 3 illustrates why an irreproducible result is obtained if substrate is not added to an enzyme assay based on disrupted cells. Fig. 4 compares the currently used reagent blank with an ETS assay done on a seawater sample.

To reconcile known variability in the ratio of respiration (R) to potential respiration (φ) in bacteria (Fig. 5A and 5B) with the use of a constant R/phys in oceanographic fieldwork (Fig. 6A), we can begin to consider a kinetic formulation of respiration (Box 1). Upon doing this, it becomes apparent that R/phys in the field (Arístegui and Montero, 1995) can be an oceanographic average of the expression, [NADPH][NADH]/Kcφ,[NADPH][NADH], as explained in Box 1. This is an unproven, but testable hypothesis. ETS activity, as currently measured, is based on a specific absorbance (Amax) of 15.9 absorbance units m⁻¹ in a 1 cm cuvette of the INT formazan at a wavelength of 490 nm. Here we show that the peak at 490 nm in phosphate buffer is incorrect (Fig. 6A), but that Amax may be 17.8 (Fig. 6B) rather than 15.9. We also tested another tetrazolium salt (CYT) to see if it could replace INT as an electron acceptor. We found its absorption maximum at 455 nm (Fig. 6C) and its Amax to be 22.9 absorbance units m⁻¹ (Fig. 6D).

Box 1 ETS reaction (R) in seawater, phytoplankton, bacterioplankton, and zooplankton can be represented by the equation:
\[ R = \Phi[NADPH][NADH]/Kc[NADPH][NADH] \]
where \( \Phi \) = potential respiration, NADH and NADPH are the principal reactants (substrates) for the ETS, and
\[ Kc = (KcA[H][H]) + (KcNADPH)[NADH] + KcA[H][NADH] \]
where \( Kc \) is the dissociation constant for the ETS-NADPH complex. \( KcA[H][H] \) is the affinity of the ETS for NADH, and \( KcNADPH [NADH] \) is the affinity of the ETS for NADPH.

Then for seawater, recombination between Figs. 5A, 5B, and 5C is found when [NADPH][NADH] = 3.5 [NADPH][NADH] for seawater equals the slope of the calibration plot between seawater R and seawater φ. In other words for a seawater average (Fig. 5C) according to Arístegui and Montero (1995):
\[ [NADPH][NADH]/[KcNADPH][NADH] = 0.59 \]

CONCLUSIONS AND SUMMARY
1. Kinetic analysis provides more sensitivity, reliability, information, and a higher data acquisition rate than endpoint analysis (Figs. 1A-1C).
2. An ETS assay measured without adding substrates (substrate blank) yields practically no activity and therefore cannot serve as a measure of φ. Furthermore, it is not a measure of invivo ETS activity because the reactants, during homogenization, are driven far lower than their occurrence in the living organism (Figs. 2-4).
3. Fig. 5 and Box 1 argue that a measurement of R/phys in the ocean is a measure of [NADPH][NADH]/[KcNADPH][NADH] in the plankton.
4. Fig. 6 supports the use of INT as an efficient electron acceptor for the ETS.
5. Fig. 7 illustrates the facility of using yeast as a biological standard for ETS measurements.

REFERENCES
Gómez et al. (1996) Modification of the electron transport system (ETS) method for routine measurements of respiratory rates of zooplankton. S. 47, P. 335-345