Plankton Community Respiration and ETS Activity Under Variable CO_2 and Nutrient Fertilization During a Mesocosm Study in the Subtropical North Atlantic

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The enzymatic electron transport system (ETS) assay is frequently used as a proxy of respiratory activity in planktonic communities. It is thought to estimate the maximum overall activity of the enzymes associated with the respiratory ETS systems in both eukaryotic and prokaryotic organisms. Thus, in order to derive actual respiration rates (R) from ETS it is necessary to determine empirical R/ETS conversion algorithms. In this study we explore the temporal development of R and ETS activity in natural plankton communities (from bacteria to large phytoplankton) enclosed in mesocosms, treated with different CO_2 concentrations. The experiment lasted 30 days, during which abrupt changes in community structure and biomass occurred through a sharp transition from oligotrophy (phase I) to highly eutrophic conditions (phase II) after nutrient-induced fertilization (day 18). R and ETS did not show any response to CO_2 under oligotrophic conditions, but R increased significantly more in the two high CO_2 mesocosms after fertilization, coinciding with a sharp rise in large phytoplankton (mostly diatoms). R and ETS were significantly correlated only during the eutrophic phase. The R/ETS ranged more than threefold in magnitude during the experiment, with phase-averaged values significantly higher under oligotrophic conditions (0.7–1.1) than after nutrient fertilization (0.5–0.7). We did not find any significant relationship between R/ETS and community structure or biomass, although R correlated significantly with total biomass after fertilization in the four mesocosms. Multiple stepwise regression models show that large phytoplankton explains most of the variance in R during phases I (86%) and II (53%) and of ETS (86%) during phase II, while picophytoplankton contributes up to 73% to explain the variance in the ETS model during phase I. Our results suggest that R/ETS may be too variable in the ocean as to apply constant values to different communities living under contrasting environmental conditions. Controlled experiments with natural communities, like the present one, would help to constrain the range of variability of the R/ETS ratio, and to understand the factors driving it.

Keywords: plankton respiration, ETS activity, R/ETS, ocean acidification, nutrient fertilization, mesocosm, subtropical North Atlantic

INTRODUCTION

Respiration is a key factor in organic carbon utilization and energy flow in oceanic ecosystems. It is regarded to be an ubiquitous process, occurring at all depths and in all regions, so it can be used as an indicator of community metabolism for aquatic ecology studies (del Giorgio and Williams, 2005). The rates of community respiration are, however, generally low and consequently difficult to measure in the ocean, particularly in the deep cold waters, where long term (>24 h) *in vitro* incubations are necessary to measure significant changes in oxygen concentration. Several studies have reported changes in bacterial abundance, phylogenetic composition (e.g., Massana et al., 2001) and metabolic rates (Jürgens et al., 2000; Massana et al., 2001; Gattuso et al., 2002) during ≥24 h incubations, caused by a number of factors, like bacterivory pressure (Hopkinson et al., 1989) or changes in inorganic and organic nutrients inside the incubation bottles (Gasol and Morán, 1999). Other studies, however, have shown that *in vitro* incubations may not drastically affect the respiratory rates of bacterial populations, in spite of shifts in the bacterial assemblage composition during the incubation (e.g., Baltar et al., 2012).

In order to overcome the potential difficulties in measuring community respiration by oxygen consumption during long incubation periods, other alternative approaches have been used (see review in Robinson and Williams, 2005). Among these, Packard (1971) introduced the electron transport system (ETS) assay as an index of potential respiration for planktonic organisms. The determination of ETS activity in plankton is thought to estimate, under saturation of substrates, the maximum overall capacity of the enzymes associated with the respiratory ETS (i.e., the potential respiration) in both eukaryotic and prokaryotic organisms (Packard, 1985). Since the introduction of the ETS method, this approach has been widely used to estimate the respiratory activity of specific components of marine plankton (e.g., Kenner and Ahmed, 1975; King and Packard, 1975; Christensen et al., 1980; Bidigare et al., 1982; Finlay et al., 1983; Packard et al., 1983; Packard, 1985; Hernández-León, 1988; Schalk, 1988; Martinez, 1991). However, like other enzymatic methods, the ETS represents a proxy of activity, which needs to be transformed to actual respiration rates (R) by means of a R/ETS ratio or relationship. Paradoxically, few studies have investigated the variability of R/ETS, with the aim to elucidate whether ETS could be universally used to derive actual respiration in marine ecosystems (Packard and Williams, 1981; Vosjan et al., 1987, 1990; Mimura et al., 1988; Arístegui and Montero, 1995; Arístegui et al., 2005; Reinthaler et al., 2006). Published empirical R/ETS ratios for bacteria, phytoplankton and zooplankton show a wide range of values (0.2 to >1), with large standard deviations (**Table 1**), suggesting that it may vary with community structure and specific metabolism of the different members of the community. Unfortunately, the meager database on R/ETS is insufficient to conclude on the causes of its variability. In spite of this, R has been frequently inferred from ETS using constant R/ETS ratios, sometimes without any reasoning for the selection of the applied conversion factor (e.g., Bangqin et al., 2005; Ramírez et al., 2006).

Here we aim to contribute to the understanding of the causes and magnitude of variability of R, ETS, and their ratio. This is the first study where R and ETS are simultaneously measured under controlled conditions, inside mesocosms, during a relatively long period of time (30 days), where the ecosystem undergoes sharp changes in nutrients and CO_2 concentrations. The objective of our work was twofold. First, to investigate how the variability in the structure and biomass of the autotrophic and heterotrophic communities, under different trophic conditions (oligotrophic versus eutrophic environments), may affect community respiration, ETS activity and the R/ETS index. We wanted to test whether pulses in nutrient fertilization (e.g., due to local upwelling events) could modify the community structure, and how this is reflected in the respiratory metabolism of the whole community, estimated both as oxygen consumption and respiratory enzymatic activity. Second, to examine the effects of acidification on plankton community respiration, with the aim of predicting how different CO_2 scenarios due to anthropogenic activities might affect plankton respiration in a future ocean. In case there is a response to high CO_2 concentrations, we wanted to investigate whether it is due to a direct effect on the specific metabolism of the organisms, or rather to an indirect effect caused by the changes in community composition and total planktonic biomass.

MATERIALS AND METHODS

Experimental Design and Sampling

The study (KOSMOS Gran Canaria 2016) was carried out at the pier of Taliarte, Gran Canaria (Canary Islands), from 2nd March to 5th April 2016. Eight 11 m^3 mesocosm bags were installed and supplied with seawater pumped directly from the adjacent offshore waters outside the harbor. Different pCO$_2$ levels were applied to each mesocosm in a random distribution: the gradient spanned from 400 μatm (the present-day level in the atmosphere and sea surface) up to 1450 μatm (simulating a future scenario of an acidified surface ocean). We chose the following four mesocosms for our study: M5, M6, M7, and M8 with target pCO$_2$ levels of 400, 1450, 700, and 1150 μatm, respectively, although actual values varied during the different phases of the experiment (see Hopwood et al., 2018).

Depth-integrated water samples were collected using 2.5 m long custom-made samplers with an internal volume of 10 L, constructed from polypropylene tubing with valves at both ends. Samples were collected every 2 days, from the beginning of the experiment (t1) until the depletion of nutrients (t17) (phase I: t1–t17). On t18, a single macronutrient addition was applied to reach the following concentrations: 3.1 μmol L^{-1} nitrate, 1.5 μmol L^{-1} silicate, and 0.2 μmol L^{-1} phosphate. Then, the sampling was changed to daily in order to closely follow the development of the phytoplankton bloom (t19–t25). Over the last days (t25–t29), the sampling shifted again to every 2nd day (phase II: t18–t29). A wide variety of measurements were carried out in the mesocosms to study the effects of acidification on both ecological and biogeochemical processes (see Hopwood et al., 2018, for

TABLE 1 | Published empirical actual respiration to ETS ratios (R/ETS) [means and standard deviations (SD)] for various groups of marine organisms and for freshwater and marine plankton communities.

Location	R/ETS	Organisms	Reference
	Average ± SD (n)		
Cultures	0.43 ± 0.09(98)	Senescent bacteria	Christensen et al., 1980
	5.02 ± 1.45(49)	Active bacteria	
Cultures	0.75 ± 0.16	Bacteria	Packard, 1985
Cultures	0.71 ± 0.23(11)	Phytoplankton	Martinez, 1992
Cultures	0.17 ± 0.01(106)	Phytoplankton	Kenner and Ahmed, 1975
Cultures	0.25 ± 0.02(6)	Protozoa	Finlay et al., 1983
Canadian lakes	0.22 ± 0.11(60)	Microplankton	del Giorgio, 1992
Med Sea (SML)	1.09 ± 0.71(22)	Microplankton	Mimura et al., 1988
Banda Sea	0.32 ± 0.71(10)	Microplankton	Vosjan and Nieuwland, 1987
North Sea	0.23 ± 0.71(9)	Microplankton	Vosjan et al., 1990
Gulf of Maine	0.25 ± 0.05(14)	Microplankton	Packard and Williams, 1981
Oceanic regions	0.89 ± 0.4(197)	Microplankton	Arístegui and Montero, 1995
Subtropical NE Atlantic	1.14 ± 0.22(4)	Microplankton	Hernández-León et al., 1999
Mesopelagic NE Atlantic	0.68 ± 0.11(28)	Bacterioplankton	Arístegui et al., 2005
Bathypelagic N Atlantic	5.3	Bacterioplankton	Reinthaler et al., 2006

n, number of observations; SML, surface micro layer.

additional information on the water samplers and set up of the experiment).

Community Respiration

Community respiration (R) was determined by oxygen consumption in borosilicate bottles. Water samples (5 L) were carefully siphoned using a silicone tube into four replicate "time-zero" and "dark" 125 mL-BOD bottles. Dark bottles were kept in a temperature-controlled chamber (21°C) at *in situ* temperature for ~24 h. R was estimated from the difference in oxygen concentration between the time-zero and dark bottles.

Dissolved oxygen was measured by the Winkler technique, following the recommendations of Carrit and Carpenter (1966), Bryan et al. (1976), and Grasshoff et al. (1983). The entire contents of the bottles were titrated during ~3 min by means of an automated, precise titration system, with colorimetric end-point detection (Williams and Jenkinson, 1982). The precision achieved in replicates was %CV < 0.07.

Enzymatic ETS Activity

ETS activity was measured by means of the tetrazolium reduction technique according to Arístegui and Montero (1995). Briefly, a tetrazolium salt [2-(4-iodophenyl)-3-(4-nitrophenyl)-5-phenyl tetrazolium chloride salt (INT)] is incorporated through the respiratory ETS and subsequently reduced by dehydrogenase enzymes to a non-fluorescent formazan crystal, INT-formazan. The amount of INT-formazan produced can be estimated from its absorption at 490 nm and be converted to the equivalent oxygen utilization. Seawater (about 3 L of the same carboy where water was collected for R) were poured into acid-cleaned plastic carboys, before being filtered through 47 mm Whatman GF/F filters, at a low vacuum pressure (<1/3 atm). The filters were immediately flash-frozen in liquid nitrogen and stored at −80°C until being assayed in the laboratory within a few weeks. ETS determinations were carried out according to the Kenner and Ahmed (1975) modification of the tetrazolium reduction technique as described in Arístegui and Montero (1995). An incubation time of 15 min at 18°C was used. ETS activities measured at 18°C were converted to activities at *in situ* temperature by using the Arrhenius equation. A mean activation energy of 16 kcal mole^{-1} was used (Arístegui and Montero, 1995).

Chlorophyll a

Samples (250–500 mL) for chlorophyll a (Chla) were filtered and collected on GF/F Whatman glass-fiber filters (nominal pore size of 0.7 µm). Chla was estimated fluorometrically by means of a Turner Designs bench fluorometer, previously calibrated with pure chlorophyll a (Sigma Co.), as described in Strickland and Parsons (1960). Pigments were extracted in cold acetone (4°C) for 24 h. For the final determination of Chla, the acetone extracts were acidified allowing Chla and phaeopigments to be independently estimated.

Prokaryotic and Eukaryotic Abundances and Biomasses

We used a FACScalibur (Becton and Dickinson) flow cytometer to look for heterotrophic bacteria (HB), small photosynthetic eukaryotic cells (picoeukaryotes), and *Prochlorococcus* and *Synechococcus* type cyanobacteria. Sea water samples (about 1 mL) were analyzed 30–60 min after sampling. *Prochlorococcus* was not found in the samples throughout the experiment. In other similar experiments we observed that *Prochlorococcus* vanished during the 1st day of enclosure in the mesocosms. Heterotrophic bacteria (HB) were fixed with a 2% final concentration of formaldehyde, after keeping them 30 min at 4°C, and then stored frozen in liquid nitrogen until analysis. Subsamples (400 µL) were stained with the fluorochrome SYBR

Green I, Molecular Probes (final concentration 1000× dilution of the commercial product) at room temperature before analyses. HB were identified by their signatures in a plot of side scatter (SSC) versus green fluorescence (FL1). Samples were run at low speed for HB (16 μL min^{-1}) and medium speed for the other photosynthetic cells (60 μL min^{-1}). A suspension of yellow–green 1 μm latex beads ($\sim$10^5–10^6 beads mL^{-1}) was added as an internal standard (Polysciences, Inc., Warrington, PA, United States). Pigmented nanoeukaryotes (2–20 μm; Nano) were counted on fresh samples with a Cytobuoy cytometer (Dubelaar and Gerritzen, 2000), provided with flow-image. Samples (about 3–5 mL) were analyzed *in vivo* for 7 min at a flow rate of 300 μL min^{-1}. Microphytoplankton (mostly diatoms and dinoflagellates; Micro) were fixed with alkaline Lugol's iodine (1% final concentration), sedimented in Utermöhl chambers and counted by means of an inverted microscope.

Biomass of picoplankton (Pico: *Synechococcus* and picoeukaryotes) and HB were estimated by multiplying their abundances by the average cell carbon content for each group, using the conversion factors obtained by MF Montero (unpublished) from samples collected in coastal waters of Gran Canaria: 18 fgC/cell (bacteria), 120 fgC/cell (*Synechococcus*), 420 fgC/cell (picoeukaryotes). Nanoeukaryotes' abundances were converted into biomass using an estimated average biovolume of 20 μm^{-3} for organisms between 2 and 6 μm and a biovolume of 125 μm^{-3} for organisms between 6 and 11 μm, applying the conversion factor of 220 fgC μm^3 proposed by Borsheim and Bratbak (1987). Measured average biovolumes for the different diatoms and dinoflagellates species were used to estimate their biomass using the biovolume-carbon conversion factors given by Menden-Deuer and Lessard (2000).

Total Organic Carbon (TOC) and Chromophoric Organic Carbon (CDOM)

TOC in seawater was measured using a Shimadzu TOC-V analyzer. Samples were collected in 10 mL high-density polyethylene bottles (Nalgene®) and stored at −20°C. Prior to that, bottles were acid-cleaned, flushed with MQ water and dried. Before analysis, samples were acidified with 50 μL of 50% phosphate, and sparged with CO_2-free air for 2 min to remove inorganic carbon. To ensure the precision and accuracy of the measurement, reference Sargasso Sea deep-water samples (44–45 μM C) and blank reference water, distributed by the Rosenstiel School of Marine and Atmospheric Science (University of Miami), were analyzed daily. TOC concentrations were determined from standard curves (30–200 μM C) of potassium hydrogen phthalate produced every day (Thomas et al., 1995).

CDOM absorption spectra were measured with a 100 cm, 250 μL capillary (LPC100CM) connected via an optical fiber to a light source (DH2000BAC) and a USB2000+UV-VIS ES detector (Ocean Optics). The system was controlled using Spectra-suite software (Ocean Optics). Samples were injected into the capillary with a peristaltic pump at a flow rate of 1 mL min−1. Relative molecular weight was estimated from CDOM absorption by deriving the slope ratio (SR), calculated as the ratio of the slope of the shorter wavelength region (275–295 nm) to that of the

longer wavelength region (350–400 nm) (Helms et al., 2008). The spectral slopes were calculated from the linear regression of the log-transformed absorption spectra.

Statistical Analysis

Model II (Reduced Major Axis) linear regressions were conducted to determine potential correlations between log-normalized data of R and ETS activity, for the whole study and for each of the phases. Statistical significance was accepted for p-values higher than 0.05 (95% of confidence level). In order to quantify the error of the correlation predictors and to compare between the different models, the Mean Absolute Percentage Error (MAPE) was calculated.
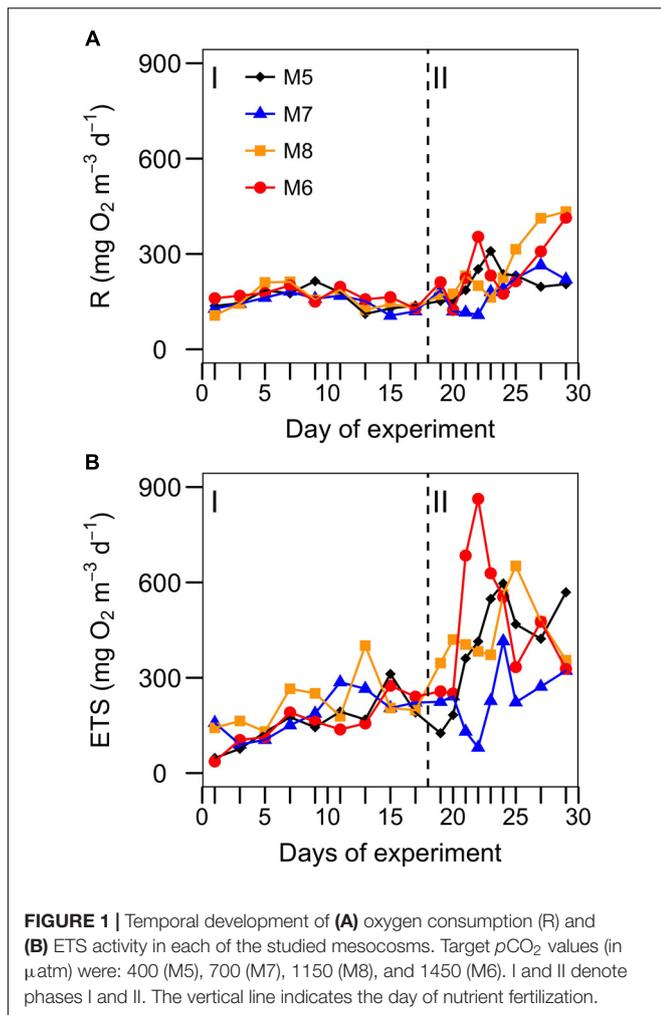
To investigate the variables (Micro, Nano, Pico and HB biomass, Chla and TOC) that influence R and ETS activity during the two phases, multivariate regression models were fitted via Stepwise Multiple Linear Regressions (SMLR). Models were built both by adding predictor variables to an initial model in which R and ETS would not be correlated with any predictor variable (Forward SMLR method), and by removing predictor variables from a full model in which R and ETS would be correlated with all predictor variables (Backward SMLR method). The selection of the best explanatory model was based on Akaike's Information Criterion (AIC). The model with lower AIC was considered as the best explanatory option for our data. All data were tested for multicollinearity using Variance Inflation Factors (VIF). Models were graphically tested for homoscedasticity. The contribution of every statistically significant predictor variable to the explained variance (R^2) was quantified calculating the Relative Importance (RI; %) using the LMG method (Lindeman et al., 1980) All statistical computations were performed using R software[1].

RESULTS

Community Respiration (R) and ETS Activity

The rates of R over the course of the experiment ranged from 106 to 433 mg O$_2$ m^{-3} d^{-1}, with the highest values at the end of the experiment in the mesocosms with highest CO_2 concentrations (**Figure 1A**). These rates are about 2–7 times higher than average values reported for open ocean waters of the Canary Islands (Arístegui and Montero, 1995). Despite the different concentrations of CO_2 in the mesocosms, a common general trend was observed during phase I (from t1 to t17). R increased from t1 to t5 to t10 and decreased thereafter until nutrient fertilization (t18). ETS activity showed a steadier increase from t1 to t17, also with no significant differences among mesocosms during phase I (**Figure 1B**). After nutrient fertilization (phase II), R in M5, M6, and M8 increased and peaked on days t21–t23, decreasing toward the end of the experiment. R in M6 and M8 – the two mesocosms with the highest CO_2 – started to increase after t23 reaching the highest rates at t29. R in M7 showed a delayed pattern with respect to other mesocosms, decreasing at t20–22 and peaking at t27. ETS

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[1]http://www.r-project.org

FIGURE 1 | Temporal development of **(A)** oxygen consumption (R) and **(B)** ETS activity in each of the studied mesocosms. Target pCO_2 values (in μatm) were: 400 (M5), 700 (M7), 1150 (M8), and 1450 (M6). I and II denote phases I and II. The vertical line indicates the day of nutrient fertilization.

during phase II showed similar trends as R, except at the end of the experiment, where ETS decreased in M6 and M8, while R increased.

Influence of Community Structure on R and ETS

The temporal development of the planktonic community, from HB to microplankton (Micro: diatoms and dinoflagellates), is represented in **Figure 2**. There were significant correlations between total biomass and R for all the mesocosms only during phase II, while ETS only correlated with total biomass in MK5 in phases I and II (**Table 2**). Some patterns of R and ETS can be attributed to changes in community structure. The relatively higher values of R from days t5 to t10 coincided with a transition period, with a decreasing pattern in the biomass of HB and Micro and increased biomass of nanoeukaryotes (Nano) (**Figure 2**). After nutrient fertilization (t18), the peaks of R and ETS observed on t21–24 in M5, M6, and M8 coincided both with peaks in Chla and in biomass of Nano. M7 behaved differently with respect to the other mesocosms in terms of the development of Chla and planktonic organisms. The

exceptionally high zooplankton concentration observed in this mesocosm after nutrient fertilization (data not shown), probably prevented the fast development of the phytoplankton bloom (as occurred in the other mesocosms), showing a delay of several days in the Nano burst and thus in the increase in Chla, R, and ETS. The further increase in R with maximum rates at the end of the experiment in the two highest CO_2 treatments matched the highest Chla and biomasses of HB and Micro (mostly large diatoms; data not shown) in M6 and M8, although HB decreased sharply the last day of the experiment in the two mesocosms. On the contrary, M5 and M7 presented lower Micro and HB biomasses with a relative dominance of Nano and picophytoplankton (Pico: *Synechococcus* + picoeukaryotes). Indeed, average planktonic biomasses at the different mesocosms show a transition in M5 and M7 from a diatom-dominated (phase I) to a Nano-dominated community, decreasing in total biomass, while in M6 and M8, the diatom-dominated community of phase I shifted to a co-dominance of HB and large diatoms (but see discussion below), with a clear increase in total biomass (**Figure 3**).

Relationship Between R and ETS

R and ETS show a significant, although weak, positive correlation ($r^2 = 0.31$, $P < 0.001$) for the whole experiment (**Table 3**). However, when splitting the data set into the two phases, the relationship of R and ETS before and after fertilization turns out to be very different (**Figure 4** and **Table 3**): Phase I shows no significant correlation ($r^2 = 0.02$, $P > 0.05$), while the two variables are significantly correlated during phase II ($r^2 = 0.38$, $P < 0.0001$), when the phytoplankton bloomed after nutrient fertilization.

The average R/ETS ratios for all the mesocosms were markedly different during the two phases, being close to 1 in phase I and about 0.5 (except in M7) in phase II (**Table 4**).

DISCUSSION

One of the goals of this study was to explore the effect of CO_2 concentration on the respiratory metabolism. Linear regression analysis detected no significant relationship (F-test, $p > 0.05$) between R and CO_2 in any of the mesocosms, agreeing with previous observations by other authors (Egge et al., 2007; Li and Gao, 2012; Motegi et al., 2013; Tanaka et al., 2013). However, when looking into the temporal development of R and ETS during the experiment (**Figures 1A,B**), we observed significant enhancements of R (but not ETS) at high CO_2 during the last 5 days of the experiment. These results contrast with the conclusions of Spilling et al. (2016) from another recent ocean acidification mesocosm experiment performed in the Baltic Sea. They observed that lower R and bacterial remineralization occurred at elevated CO_2 levels, resulting in high dissolved organic carbon accumulation. Although they discussed several mechanisms that might explain their results, they also acknowledge that the cause for reduced respiration at high CO_2 remains unresolved. In our study, the high R associated with high CO_2 treatments is probably not due to a direct effect
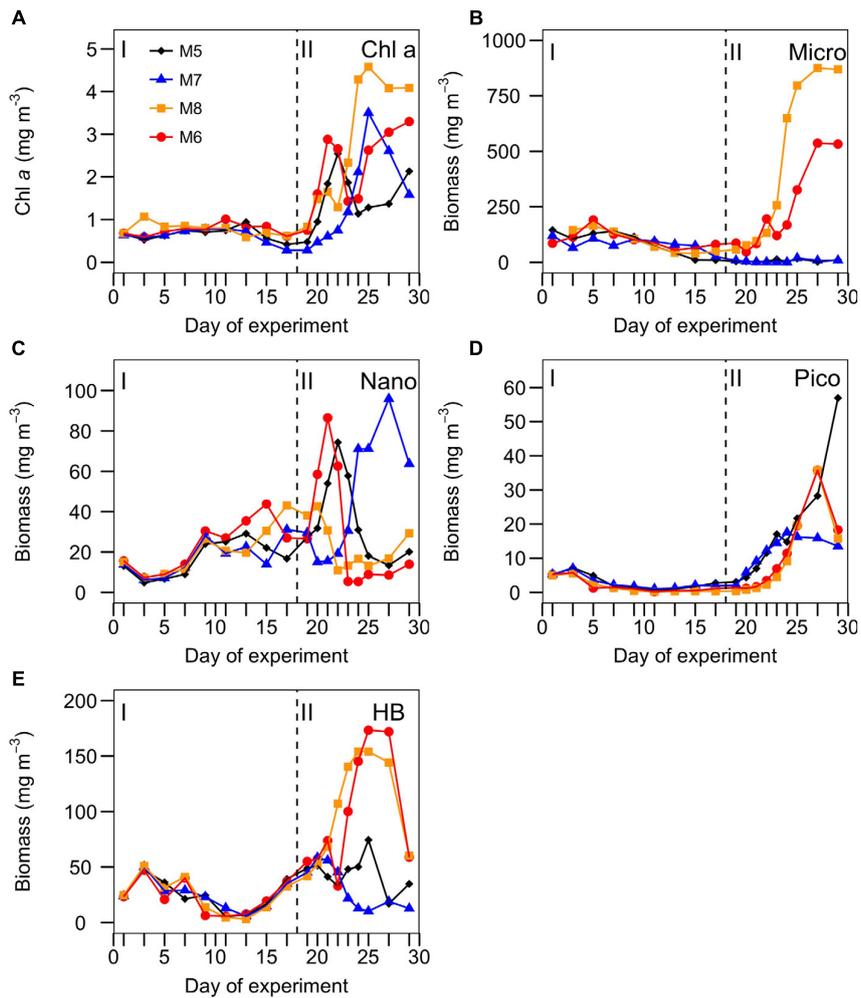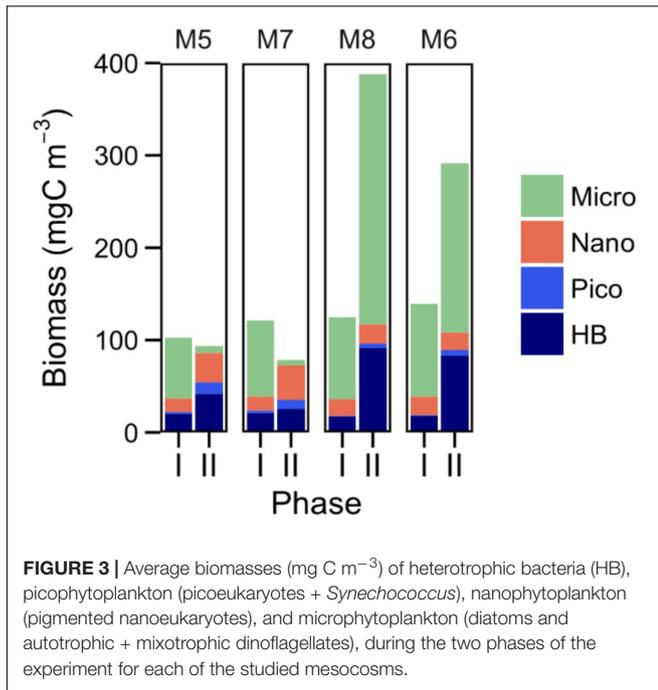
FIGURE 2 | Temporal development of **(A)** chlorophyll a, and biomasses (in mg C m^{-3}) of **(B)** microphytoplankton (diatoms + autotrophic and mixotrophic dinoflagellates), **(C)** nanophytoplankton (pigmented nanoeukaryotes), **(D)** picophytoplankton (picoeukaryotes + *Synechococcus*), and **(E)** heterotrophic bacteria (HB) in the studied mesocosms. Target pCO$_2$ values (in μatm) were: 400 (M5), 700 (M7), 1150 (M8), and 1450 (M6). I and II denote phases I and II. The vertical line indicates the day of nutrient fertilization.

TABLE 2 | Pearson pairwise correlation coefficients between community respiration (R) and ETS activity and the biomass of heterotrophic bacteria (HB), picophytoplankton (Pico), nanophytoplankton (Nano) and microphytoplankton (Micro), and the sum of all them (Total), during the two phases (I and II) of the experiment (target pCO$_2$ concentrations in brackets).

Phase	Variable	M5 (400)		M7 (700)		M8 (1150)		M6 (1450	
		R	ETS	**R**	ETS	**R**	ETS	**R**	ETS
I	HB	0.03	0.16	0.00	0.56*	0.08	0.28	0.00	0.00
	Pico	0.01	0.44*	0.04	0.78**	0.11	0.21	0.03	0.35
	Nano	0.03	0.27	0.02	0.70**	0.26	0.10	0.12	0.30
	Micro	0.31	0.50*	0.14	0.02	0.51*	0.21	0.18	0.05
	Total	0.33	0.61**	0.11	0.20	0.35	0.32	0.08	0.01
II	HB	0.00	0.02	0.65**	0.4	0.06	0.45*	0.04	0.00
	Pico	0.31	0.72**	0.08	0.02	0.65**	0.31	0.24	0.01
	Nano	0.13	0.00	0.87**	0.45*	0.01	0.18	0.01	0.00
	Micro	0.08	0.02	0.36	0.07	0.64**	0.32	0.58*	0.01
	Total	0.43*	0.44*	0.58*	0.23	0.63**	0.34	0.40*	0.01

All data were previously log transformed. *$p \leq 0.05$, **$p \leq 0.01$.

FIGURE 3 | Average biomasses (mg C m^{-3}) of heterotrophic bacteria (HB), picophytoplankton (picoeukaryotes + *Synechococcus*), nanophytoplankton (pigmented nanoeukaryotes), and microphytoplankton (diatoms and autotrophic + mixotrophic dinoflagellates), during the two phases of the experiment for each of the studied mesocosms.

FIGURE 4 | Relationship between community respiration (R) and ETS activity for the two phases of the experiment (see **Table 3** for the statistics of the regressions). Data are log transformed for the regression analysis.

on the specific metabolism of the organisms but to the increase in total planktonic biomass at the end of the experiment. HB increased in abundance in phase II right after the Nano decline (probably relieving the grazing pressure on HB) in parallel to the Micro rise, with both groups together reaching the highest biomasses (**Figures 2, 3**). Other studies carried out in mesocosms show a similar trend, with increased bacterial abundance and metabolism stimulated after the development of phytoplankton blooms (Grossart et al., 2006; Endres et al., 2014; Bergen et al., 2016).
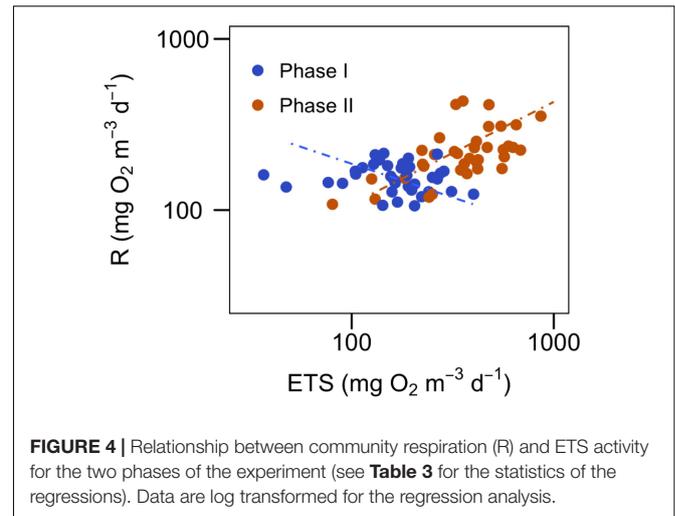
M6 holds the highest plankton biomass in phase I, as well as in phase II (together with M8). This is reflected in higher concentrations of total organic carbon (TOC) excreted by the phytoplankton and accumulated during the experiment in M6 during phase I, and M6 and M8 during phase II (**Figure 5A**). The molecular weight of the colored dissolved organic matter (CDOM) – represented by the slope ratio (SR) – increased during the course of the experiment in all the mesocosms (**Figure 5B**; decreasing SR), suggesting that bacterial production of high molecular weight CDOM exceeded the rate of photochemical bleaching, responsible of breaking high molecular weight into low molecular weight organic compounds. Indeed, the only change in the decreasing trend of SR during phase I occurred around t10, when bacterial biomass was at its minimum (**Figures 2E, 5B**). Moreover, in the two mesocosms with highest CO_2 there were significant correlations (Pearson
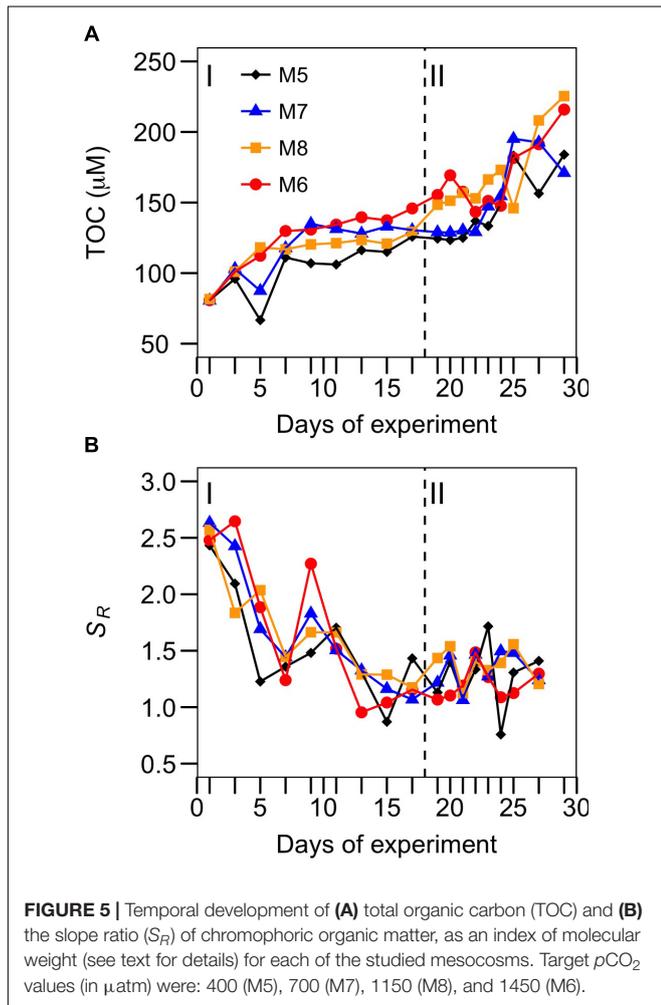
TABLE 3 | Regression statistics for the relationship between actual respiration (R) and ETS activity in our study (phases I and II), compared with the study of Arístegui and Montero (1995) (A&M95) (see their **Figures 2, 6**).

Study	n	Slope (± se)	y-intercept (± se)	r2	F	Significance (F-test)	CF	MAPE (%)
This study (Ph. I+II)	72	0.508* (0.058)	1.050* (0.138)	0.31	77.4	<0.001	1.04	21
This study (Phase I)	36	−0.393[NS] (0.090)	3.057* (0.260)	0.02	19.3	>0.1	1.02	15
This study (Phase II)	36	0.672* (0.058)	0.616* (0.259)	0.38	44.0	<0.001	1.04	20
A&M95[&]	197	0.750* (0.031)	0.357* (0.057)	0.75	578.2	<0.0001	1.08	34
A&M95 + Phases I+II	269	0.865* (0.024)	0.186* (0.046)	0.80	1272.1	<0.001	1.09	34
A&M95 + Phase I	233	0.896* (0.030)	0.111* (0.046)	0.75	882.8	<0.001	1.10	35
A&M95 + Phase II	233	0.844* (0.024)	0.286* (0.046)	0.83	1290.0	<0.001	1.07	33

*Values (in mg O_2 m^{-3} d^{-1}) were log transformed to compute regression statistics. The data are fitted to the model logR = a(± se) + b(± se) × logETS, where a is the slope and b the intercept. The correction factor (CF) is used to convert from log to arithmetic scales. CF = antilog (1.513 × RMS), RMS being the square of the mean residual value of each regression. n, number of data; se, standard error. *P < 0.001; NS, not significant; MAPE, Mean Absolute Percentage Error.*

TABLE 4 | Average ($\pm$ SD) ratios of R/ETS from the two phases of the experiment: before (Phase I: t1–t17) and after (Phase II: t19–t29) fertilization, in the four mesocosms studied (target pCO_2 concentrations in brackets).

	Phase I	Phase II
M5 (400)	0.98 ± 0.31	0.52 ± 0.43
M7 (700)	0.79 ± 0.37	0.75 ± 0.56
M8 (1150)	0.73 ± 0.46	0.59 ± 0.99
M6 (1450)	1.06 ± 0.31	0.52 ± 0.43

FIGURE 5 | Temporal development of **(A)** total organic carbon (TOC) and **(B)** the slope ratio (S_R) of chromophoric organic matter, as an index of molecular weight (see text for details) for each of the studied mesocosms. Target pCO_2 values (in μatm) were: 400 (M5), 700 (M7), 1150 (M8), and 1450 (M6).

pairwise coefficient) between TOC and HB biomass ($r = 0.55$, $p < 0.05$ for M6; $r = 0.64$, $p < 0.01$ for M8), suggesting a strong link between organic carbon dynamics and bacterial biomass.

The specific contribution of HB to R and ETS, however, is uncertain. Multiple stepwise regressions (**Table 5**) show that R and ETS, average over the mesocosms, can be mostly explained by the phytoplankton community. Large phytoplankton explains most of the variance in R during phases I (86%) and II (53%) and of ETS (86%) during phase II, while picophytoplankton contributes up to 73% to explain the variance in the ETS model during phase I.

Past studies on plankton respiration reported significant correlations between ETS and Chla, (e.g., Packard et al., 1983; Martinez, 1991; del Giorgio, 1992; Estrada et al., 1992; Packard and Christensen, 2004), suggesting that when seawater Chla is high, respiratory activity is mainly due to phytoplankton (Harrison, 1986; Martinez, 1991; Packard and Christensen, 2004). Our results, however, in spite of the large contribution of phytoplankton to biomass, show values ranging from 4 to 13 mg O_2 mg $Chla^{-1}$ d^{-1}, which are similar to those reported by Martinez et al. (1990) for the western Mediterranean Sea (2–10 mg O_2 mg $Chla^{-1}$ d^{-1}). In our study, in spite of the phytoplankton dominance during the two periods (**Figure 3**), ETS and R do not consistently correlate with Micro biomass, which largely contributes to total biomass in most of the mesocosms and phases. R, however, correlates with total biomass in the four mesocosms during phase II (**Table 2**), when diatoms' biomass increased. This lack of a consistent correlation could result from the presence of a complex and variable in time planktonic community (**Figures 2**, **3**), where HB do not seem to be a key player in the total community respiration. Indeed, recent studies from the North Atlantic indicate that the contribution of bacterial respiration to total community respiration may be highly variable, ranging from <5 to >75% (García-Martín et al., 2017, 2018).
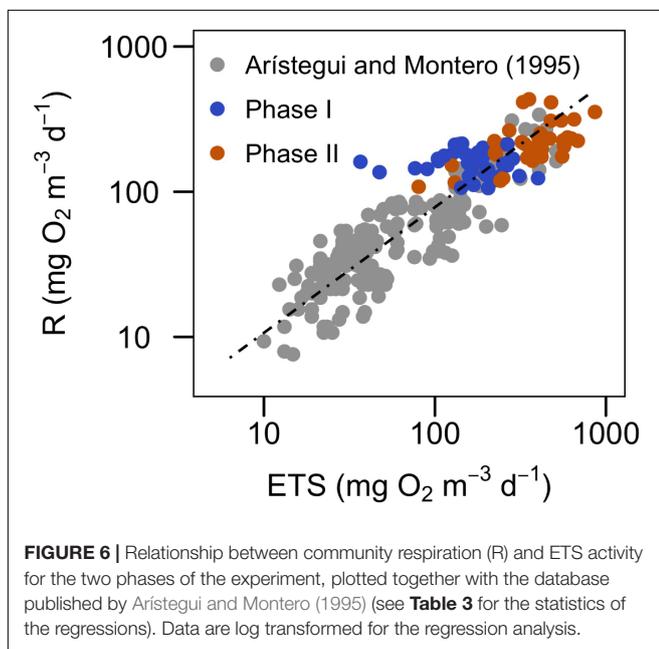
Earlier studies showed that the R/ETS ratio may vary depending on the planktonic community structure and seasonality (e.g., Hobbie et al., 1972), with a general perception that autotrophic organisms have lower R/ETS ratios than heterotrophs (Kenner and Ahmed, 1975; Span, 1988; del Giorgio, 1992). Photosynthesis, like respiration, involves an ETS. Therefore, when autotrophs dominate the plankton community, the ETS assay would measure the potential activity of both processes, yielding higher ETS estimates, and hence lower R/ETS, than measured taking into account only the respiratory process (Packard, 1985). Supporting this hypothesis, Martinez, 1991) reported the highest values of ETS at the chlorophyll maximum layer for all the sampled stations during a cruise in the Barents Sea. Conversely, R/ETS would be higher with increased bacteria contribution to overall respiration (Fuhrman and Azam, 1980; Williams, 1984; Harrison, 1986). In our experiment, the R/ETS ratios were lower during the second period, after nutrient fertilization, coinciding with the phytoplankton bloom and the rise of diatoms. Our phase-average R/ETS data show significant variability between mesocosms during the course of the experiment (ranging from 0.5 to >1). This is however not surprising when looking at the published R/ETS ratios that span several fold (0.2 to >1) in phytoplankton cultures and natural samples (**Table 1**).

The application of a regression equation has been found to be a more reliable approach than the use of a mean R/ETS ratio to transform ETS activity to actual respiratory rates (e.g., Packard and Williams, 1981), but unfortunately there are very few published studies from natural communities to compare with. The only study where community R/ETS from different marine biogeographical regions (spanning from polar to tropical waters) are compared was published by Arístegui and Montero (1995).

TABLE 5 | Stepwise multiple linear regression statistics between actual respiration (R) and ETS activity and plankton biomasses (Micro, Nano, Pico, HB), Chlorophyll (Chla) and Total Organic Carbon (TOC) during the two phases (I and II) of the experiment.

Phase	Y	X_i	R^2-adj	p-value	F-statistic	RI (%)
I	R	Micro	0.35	<0.001	9.99	85.64
		Pico				14.36
	ETS	Micro	0.43	<0.001	13.56	26.95
		Pico				73.05
II	R	Micro	0.57	<0.001	14.93	53.33
		Nano				15.50
		Pico				31.17
	ETS	Micro	0.18	0.023	4.302	86.45
		Nano				13.55

Y, Dependent variable; X_i, Statistically significant predictor variables; R^2-adj, Adjusted correlation coefficient; RI (%), Relative importance.

FIGURE 6 | Relationship between community respiration (R) and ETS activity for the two phases of the experiment, plotted together with the database published by Arístegui and Montero (1995) (see **Table 3** for the statistics of the regressions). Data are log transformed for the regression analysis.

Their study shows that in all except one of the regions, measured R and ETS were significantly positively correlated, although the slopes and y-intercept of the regression equations differ up to 50% among regions. The regression equation obtained, considering all their data together, yields a mean error of ± 34% in the prediction of R from ETS, similar to the errors obtained applying the equation at each area, but much lower than applying an average R/ETS ratio (Arístegui and Montero, 1995). The regression between R and ETS in our study (even if we use only the data from phase II) yields a lower slope, a higher y-intercept and a lower r^2 than the general equation for the global ocean (**Table 3**). The error in the prediction of R from ETS using only the data of phase II yields a mean error of ±20%, even lower than when including the data in the global equation. However, if we include the dataset of this study in the global equation of Arístegui and Montero (**Figure 6** and **Table 3**), the slope, y-intercept and r^2 do not change significantly, although the correlation is higher if we only include the data from phase II. All our data are placed

in the highest range of the regression, closely together with the data from the eutrophic ecosystems of the Gulf of California and Baltic Sea (**Figure 6**; Arístegui and Montero, 1995). Nevertheless, the variability in the slopes and intercepts of our data with respect to the global equation raises the question of whether we can universally use a single R/ETS relationship to derive R from ETS activity in different communities and environmental conditions.

CONCLUSION

This work represents one of the few studies where actual respiration rates (R) and ETS activity are measured simultaneously in natural marine communities. Moreover, it is the only study where R and ETS are measured under controlled conditions – inside mesocosms – and during a relatively long period of time (30 days), where the community undergoes drastic changes in nutrient and CO_2 concentrations, as well as community structure.

R and ETS do not show any significant correlation with CO_2 or community structure for the whole experiment, although R increased in the high CO_2 treatments at the end of the experiment, matching the rise in diatoms after nutrient fertilization (phase II). Only during this phase R correlates significantly with total biomass in all the mesocosms.

The temporal development of R and ETS can be mostly explained by changes in the phytoplankton community. Large phytoplankton explains most of the variance in R during phases I and II and of ETS during phase II, while picophytoplankton contributes to explain the variance in ETS during phase I.

The R/ETS ratio ranged more than threefold in magnitude during the experiment. The average R/ETS is higher under nutrient depleted conditions (0.7–1.1), compared to the bloom period after nutrient fertilization, where the ratios reached lower and more similar values among the mesocosms (0.5–0.7).

Our results (together with other published studies) indicate that R/ETS may be too variable as to apply constant ratios in different marine regions and trophic conditions. The application of a regression equation is a more reliable approach than the use of a mean R/ETS ratio to transform ETS activity to actual respiratory rates. Nevertheless, further research, like the one

carried out in this study, is necessary to constrain the variability of the R/ETS and to elucidate the universality of this equation.

AUTHOR CONTRIBUTIONS

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