Eutrophication and acidification: do they induce changes in the dissolved organic matter dynamics in the coastal Mediterranean Sea?

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Abstract

Two mesocosms experiments were conducted in winter 2010 and summer 2011 to examine how increased pCO_2 and/or nutrient concentrations potentially perturbate dissolved organic matter dynamics in natural microbial assemblages. The fluorescence signals of protein- and humic-like compounds were used as a proxy for labile and non-labile material, respectively, while the evolution of bacterial populations, chlorophyll a (Chl *a)* and dissolved organic carbon (DOC) concentrations were used as a proxy for biological activity. For both seasons, the presence of elevated pCO_2 did not cause any significant change in the DOC dynamics (p-value < 0.05). The conditions that showed the greatest changes in prokaryote abundances and Chl *a* content were those amended with nutrients, regardless of the change in pH. The temporal evolution of fluorophores and optical indices revealed that the degree of humification of the organic molecules and their molecular weight changed significantly in the nutrient-amended treatment. The generation of protein-like compounds was paired to increases in the prokaryote abundance, being higher in the nutrient-amended tanks than in the control. Different patterns in the magnitude and direction of the generation of humic-like molecules suggested that these changes depended on initial microbial populations and the availability of extra nutrient inputs. Based on our results, it is expected that in the future projected coastal scenarios the eutrophication processes will favor the transformations of labile and recalcitrant carbon regardless of changes in pCO_2.

Keywords: Acidification; eutrophication; mesocosms, DOM, Mediterranean Sea, optical indices

1. – Introduction

As a result of human activities, atmospheric CO_2 levels have increased from approximately 280 ppm in pre-industrial times to 395 ppm in 2013 (Le Quéré et al., 2015 and references therein). A large portion of the atmospheric CO_2 is dissolved in the ocean and, thanks to the 'solubility pump', it is transported from the ocean's surface to its interior in form of dissolved inorganic carbon (Volk and Hoffert, 1985). In addition to this passive diffusion of CO_2 into the ocean, marine biota plays an active role in the uptake of carbon dioxide from the atmosphere in what is known as the 'biological pump' which refers to the processes that involve the biologically-mediated uptake and transport of carbon from the upper to the deep ocean (Volk and Hoffert, 1985; Passow and Carlson, 2012). Thus, marine ecosystems play an important role in regulating atmospheric CO_2 concentrations and, in this way, in moderating climate change. However, the physical, chemical, and biological mechanisms governing the fluxes between the different carbon compartments in the marine system are still poorly understood.

The diffusion of CO_2 into the ocean is determined by temperature and salinity that provide a dependent solubility coefficient (Henry's law, Henry, 1803). When a CO_2 molecule is finally taken up by the ocean, two main paths may follow: i) it may remain in a dissolved inorganic form, altering the marine carbonate chemistry equilibrium and leading to ocean acidification (Hönisch et al., 2012; Zeebe, 2012), or ii) may be captured by a photosynthetic marine organism, fixing it in the form of organic carbon. The pathways that this new biologically generated organic molecule may follow within the trophic chain are very diverse and vary from being incorporated into a larger organism (reaching higher trophic levels) to being excreted or respired as part of a variety of metabolic processes. The size of the excreted compounds varies widely, contributing to both the particulate organic matter (POM) and the dissolved organic matter (DOM) fractions. Regarding the DOM, this pool is mainly produced by phytoplankton exudation (Hopkinson et al., 2002; Romera-Castillo et al., 2011b; Sarmento et al., 2013), viral lysis (Brussaard, 2004; Motegi et al., 2009), the sloppy feeding carried out by protists and metazoans grazers and the POM solubilization by bacterial and archaeal hydrolases (Nagata et al., 2000; Sala and Güde, 2004).

These mechanisms determine the quantity and the complexity of the molecules contained in the DOM, as well as their fate along the biogeochemical cycles.

The estimations of oceanic CO_2 assimilation by phytoplankton to generate cellular structures or its subsequent release of C as exudates (particulate and dissolved primary production, respectively) range between 3 and 4 Pmol C $year^{-1}$ (Berger, 1989; Antoine and Morel, 1996; Behrenfeld and Falkowski, 1997; Chavez et al., 2011). Research undertaken in the context of the US Joint Global Ocean Flux Study (Schlitzer et al., 2003) concluded that a fraction of this carbon is rapidly removed from surface waters and exported to the ocean's interior. In addition, Jiao et al. (2010) emphasized the role of oceanic in transforming POM and DOM into recalcitrant DOM, material susceptible of staying sequestered in the ocean for long periods of time. The processes that transform labile organic matter into refractory compounds are termed 'microbial carbon pump' (MCP, Jiao et al., 2010).

The chromophoric dissolved organic fraction (CDOM; Coble, 1996) of the DOM pool absorbs light at both ultraviolet (UV) and visible wavelengths. A sub-fraction of this CDOM, the fluorescent DOM (FDOM; (Coble, 2007, 1996), fluoresces when irradiated with UV light. Since 1990, (Coble et al., 1990) the characterization of marine DOM has been performed by applying fluorescence excitation-emission matrices (EEM). Although this technique does not permit the quantification of specific molecules, it has been extensively used to track the origin and transformations of DOM (Coble et al., 1990; Cory and McKnight, 2005; Nieto-Cid et al., 2005; Romera-Castillo et al., 2011a, Catalá et al, 2015) because it is relatively inexpensive, low-time consuming and provides valuable information about the quality of the DOM.

As it has been shown over the last years, ocean acidification affects marine organisms and ecosystems in several ways (Gattuso et al., 2015 and references therein). In addition, nitrogen (N) and phosphorous (P) pollution has increased over the past decades, primarily due to the utilization of active N and P for fertilizer use (Galloway et al., 2004). This utilization has enhanced the nutrient loads from land to coastal zones and may favor an increase of

eutrophication episodes in the near future (Howarth & Marino, 2006). Since the beginning of the 20th century, eutrophication has been a persistent problem and a subject of different studies. Bio-assay experiments in lake and coastal systems were done to test the effect of eutrophication on phytoplankton dynamics in the seventies and eighties (Pomeroy et al., 1972; Carpenter & Capone, 1983). Since then, numerous studies have been addressed this issue in different aquatic systems (Statham, 2012 and references therein).

A convenient procedure to gain insight on the possible changes that ocean acidification and eutrophication may induce on marine systems is the deployment of mesocosms experiments (Kim et al., 2011; Teeling et al., 2012; Riebesell et al., 2007, 2013; Bunse et al., 2016). Three recent mesocosms studies (Yamada et al., 2013; Riebesell et al., 2013; Zark et al., 2015) have examined the effects of ocean acidification on DOM transformation processes. Yamada et al. (2013) did not find a significant effect of increased CO_2 concentration on the short-term decomposition of labile DOM in Sagami Bay (Japan), yet the study did not look at the possible changes in organic matter quality. The study conducted by Riebesell and collaborators (2013) in Svalbard (Norway) shed light on the pathways that the organic matter followed when the system was amended with nutrients and increased in pCO_2. They found that the combination of these two stressors triggered a synergistic effect inducing an increase in the dissolved organic carbon fraction. The study of Zark et al. (2015) tracked the transformations suffered by DOM molecules in a mesocosms study using Fourier transform ion cyclotron resonance mass spectrometry (FT-ICR-MS) and they concluded that ocean acidification alone did not induce changes in the composition of the DOM pool in the Gullmar Fjord (Sweden).

We investigated the effects of increasing pCO_2 and its synergy with increasing nutrient availability on the dynamics of organic matter in a Mediterranean coastal area. We have particularly examined the optically active fractions of the DOM as they have been used as indicators of recalcitrant material and can provide useful information about DOM transformations. In addition, the study of these fractions is of remarkable interest in the Mediterranean waters where CDOM to chlorophyll ratio is higher than the global average (Morel & Gentili,

2009; Claustre et al., 2002). We enclosed coastal water in mesocosms and performed two experimental studies in which we manipulated pCO_2 and nutrient concentrations. In order to assess the importance of the initial conditions in regulating the responses to reducing pH and increasing nutrients, one mesocosm experiment was performed in winter and the other in summer, displaying contrasting initial oceanographic and biological characteristics.

2. - Materials and methods

2.1. - Experimental setup and initial conditions at the sampling site

Two experiments were conducted in winter 2010 and summer 2011 (W and S, respectively) to examine the dynamics of microbial communities and organic matter under different pH conditions and nutrient levels. Natural seawater from the Blanes Bay Microbial Observatory, NW Mediterranean (BBMO; 41º40'0" N, 21º48'0" E; Gasol et al., 2012), was enclosed in eight 200 L tanks and maintained in a temperature-controlled chamber, with a 12:12 h light:dark cycle. Gro-lux and cool-white lamps where positioned in the walls of the chamber surrounding the tanks. Light intensity inside the containers was 121.3 ± 3.5 µmol m^{-2} s^{-1} during the winter experiment and 230 ± 25 µmol m^{-2} s^{-1} during the summer experiment, measured using a spherical radiometer (Biospherical Instruments Inc., Model QSL 2100, San Diego, CA).

Four experimental conditions were randomly assigned to duplicated containers: K1 and K2 (controls), KA1 and KA2 (reduced pH) N1 and N2 (nutrient amended), and NA1 and NA2 (nutrient amended and reduced pH). The pH in the KA and NA treatments was manually adjusted by bubbling CO_2 every morning in a controlled way, to lower their pH in approximately 0.2 units respect to the controls, so as to simulate future conditions in a medium-level mitigation scenario such as the Representative Concentration Pathway (RCP) 4.5 (Taylor et al., 2015). For reproducibility, the control tanks were also bubbled with compressed air at current atmospheric CO_2 concentrations.

The seasonal cycle in Blanes Bay is characterized by a late winter phytoplankton bloom dominated mostly by diatoms (Guadayol et al., 2009). In contrast, during summer, when nutrient concentrations are lower,

picophytoplankton is the most representative group (Alonso-Sáez et al., 2008). Moreover, DOC accumulates during summer, while annual minimum concentrations are found in winter (Vila-Reixach et al., 2012; Romera-Castillo et al., 2013). Due to this seasonality, the initial seawater of the winter experiment was relatively rich in inorganic nutrients and poor in DOC. On the contrary, in summer the water was depleted of inorganic nutrients and enriched in DOC, generated via metabolic pathways during the bloom phase within the previous spring season (Romera-Castillo et al., 2013). The summer nutrient depletion limits the bacterial activity, reducing the microbial degradation of DOM and leading to a DOC accumulation in this season (Thingstad et al, 1997) Thus, the starting point conditions of the experiments differed in the original concentrations of organic matter and inorganic nutrients.

2.2. – Measured variables

Measurements of the following variables were taken every day during 9 days. Duplicate containers for each of the four treatments were simultaneously and independently sampled. Temperature was monitored daily using a digital thermometer *VWR 8202-156* (VWR International, LLC). This variable was set to 14 ± 1 ºC and to 22 ± 1 ºC for W and S experiments, respectively. The pH in the mesocosms was determined every morning by spectrophotometry in the laboratory, following standard procedures (Clayton and Byrne, 1993). In addition, pH was continuously recorded using glass electrodes (Ecotrode Plus, Metrohm) connected to a D130 data logger (Consort, Belgium) that were calibrated on a daily basis with a Tris buffer following standard procedures (SOP6a of Dickson et al, 2007). Chlorophyll a (Chl *a*) was measured according to Yentsch and Menzel (1963): seawater (50 mL) was filtered through Whatman GF/F glass fiber filters, which were subsequently placed in 90% acetone at 4 ºC for 24 h and the fluorescence of the extract measured using a fluorometer (Turner Designs, Sunnyvale, CA).

Dissolved inorganic nutrient concentrations, nitrate (NO_3^-), phosphate (PO_4^{3-}) and silicate (SiO_2), were determined by standard segmented flow analyses with colorimetric detection (Hansen & Grasshoff 1983) using a CFA Bran + Luebbe autoanalyser. Precisions were ±0.01 µmol kg^{-1} NO_3^-, ±0.02 µmol kg^{-1} PO_4^{3-}, and

±0.01 µmol kg^{-1} SiO_2. Inorganic nutrients were added to N and NA treatments to reach a final P:N:Si molar concentration of 1:16:30 and 0.25:4:8 in the winter and summer experiments, respectively. Initial and post-addition nutrient concentrations are summarized in Table 1. In both cases, the nitrogen enrichment was increased at least eight times from the seasonal average concentration measured in the BBMO during the last 10 years. Nitrogen and phosphorus were added at a Redfield ratio, whereas silicate was added in excess, so diatom growth was not affected by lack of elemental compounds.

Table 1. Concentrations of the main inorganic nutrients measured before and after the additions. Values are expressed in µM. The standard deviations were calculated using duplicated containers of the same experimental condition.

	Winter		Summer	
	Before addition	After addition	Before addition	After addition
NO_3^-	2.55 ± 0.00	17.38 ± 0.45	0.10 ± 0.01	4.70 ± 0.06
PO_4^{3-}	0.11 ± 0.00	1.14 ± 0.07	0.03 ± 0.00	0.24 ± 0.03
SiO_2	2.33 ± 0.00	31.41 ± 0.79	0.37 ± 0.03	6.51 ± 0.13

Samples for dissolved organic carbon (DOC), FDOM and CDOM were prefiltered under reduced pressure through precombusted (450 ºC, 4h) Whatman glass fiber filters (GF/F). DOC samples were collected in 10 mL precombusted (450 ºC, 24 h) glass ampoules, acidified with 50 µL 25% H_3PO_4 to pH <2 and heat-sealed and stored in the dark at 4 ºC until analysis. A Shimadzu TOC-CSV organic carbon analyzer was used to carry out analysis. Three to five injections of 150 µL per sample were performed, and DOC concentrations were calculated by subtracting a Milli-Q blank and dividing by the slope of a daily standard curve of potassium hydrogen phthalate. The precision of these measurements was ±0.7 µM. All samples were checked against deep Sargasso Sea reference water (2600 m).

CDOM absorption spectra were determined from 250 to 600 nm using a Varian Cary 100 Bio spectrophotometer equipped with 10 cm quartz-cells. Milli-Q water

was used as a blank. Absorbance was converted into napierian absorption coefficient (a_λ, Green and Blough, 1994) using the equation:

$$a_\lambda = \frac{2.303 \cdot Abs_\lambda}{l} \quad (1)$$

where Abs_λ is the absorbance at a given wavelength, the factor 2.303 converts from decadic to natural logarithms, and l is the cell path-length in meters. In addition, the UV absorption at 254 nm was also normalized to the dissolved organic carbon (DOC) concentration to obtain the specific UV absorbance coefficient (SUVA, in m^{-1} mg^{-1} L) following Weishaar et al. (2003). SUVA values are correlated with DOM aromaticity and provide information on the complexity of molecules (Helms et al., 2008; Weishaar et al., 2003). Furthermore, the dimensionless slope ratio of a short wavelength region (275-295 nm) to that of a longer wavelength region (350-400 nm) was determined (S_R; Helms et al., 2013). S_R is inversely correlated to SUVA and is related to the molecular weight of the DOM (Helms et al., 2013).

A Perkin Elmer LS55 luminescence spectrometer was used to measure FDOM. This instrument was equipped with a xenon discharge lamp equivalent to 20 kW for an 8-µs duration. Both, single point measurements and emission excitation matrices of the samples were acquired. The scan speed was set at 250 nm min^{-1} and slit widths for the excitation and emission wavelengths were fixed at 10 nm. Measurements were performed in a 1 cm quartz fluorescence cell. Following Coble 1996, the Ex/Em wavelengths used for the single point measurements were: Ex/Em 280 nm/350 nm (peak-T) indicative of the presence of protein-like compounds, Ex/Em 320 nm/410 nm (peak-M) as indicator of marine humic-like substances, Ex/Em 340 nm/440 nm (peak-C) to trace terrestrial humic-like substances and Ex/Em 250 nm/435 nm (peak-A) to track humic materials in general. Additionally, EEMs were obtained by concatenating 21 excitation/emission spectra of the sample. The fluorescence intensities were reported as quinine sulfate units (QSU) by calibrating the instrument at Ex/Em: 350 nm/450 nm against a quinine sulfate dehydrate (QS) standard made up in 0.05 mol L^{-1} sulfuric acid. Optical analyses of tryptophan

(Try) dissolved in seawater at different levels of pH were performed to test the pH influence in the fluorescence properties of the protein-like substances.

The humification index (HIX) describes the diagenetic state of the DOM and it was calculated by dividing the peak area under the emission spectra at 435-480 nm by the peak area under the emission spectra at 300-345 nm, at an excitation of 254 nm. The aromatic humic acids are known to have high HIX values (Zsolnay, 2003; Giering et al., 2014).

Heterotrophic prokaryotes were enumerated with a FACSCalibur (Becton Dickinson) flow cytometer equipped with a 15 mW argon-ion laser (488 nm emission) as described by Gasol and del Giorgio (2000). Samples (1.8 mL) were immediately fixed with 1% paraformaldehyde plus 0.05% glutaraldehyde (final concentrations), incubated for 10 min at room temperature, frozen in liquid nitrogen and stored at -80 ºC. Before analysis, samples were unfrozen, stained with SYBRGreen I (Molecular Probes) at a final concentration of 10 µM and left in the dark for about 15 min. Each sample was then run at low speed (~12 µL min^{-1}) for 2 minutes with Milli-Q water as a sheath fluid. We added 10 µL per sample of a solution of yellow-green 0.92 µm Polysciences latex beads (10^6 beads mL^{-1}) as an internal standard. Bacteria were detected by their signature in a plot of side scatter versus FL1 (green fluorescence). Data analysis was performed with the Paint-A-Gate software (Becton Dickinson).

2.3. – Statistical analyses

The software SigmaPlot v11.0 (Systat Software Inc.) was used to perform the two-way ANOVA and the t-tests. Two-way ANOVA was carried out to test if differences between conditions and experiments were significant and t-tests to discriminate if the temporal evolution of the different variables measured in an experiment (winter or summer) could be considered significant. The software XLSTAT 2016 (Addinsoft ©) was used to perform Mantel tests. The Pearson correlation implemented in the Mantel tests was performed to discriminate if changes in the intensity of the fluorophores were significant between conditions. p-values were set to $p < 0.05$ for all types of test.

3.- Results

3.1. – Plankton dynamics

Figure 1 shows the differences between prokaryotic abundances and Chl *a* levels for the different treatments during winter and summer experiments. Prokaryotic abundances started at 5.0 ± 0.1·10^5 cells mL^{-1} at t_0 in the winter experiment. Between t_3 and t_4, the abundances in K and KA conditions reached the highest numbers 4.0 ± 0.2 and 3.7 ± 0.4·10^6 cells mL^{-1}, respectively. Within a short time lag, slightly higher values were reached in N and NA tanks (4.4 ± 0.3 and 4.0 ± 0.3·10^6 cells mL^{-1}, respectively). Between t_5 and t_7, prokaryotic abundances decreased markedly and, by the end of the experiment the abundances increased again in all conditions (Figure 1a). Prokaryotic abundances at t_0 ranged from 7.3 ± 0.2 to 8.4 ± 0.2·10^5 cells mL^{-1} in the summer experiment (Figure 1b). An initial drop was observed in all conditions, reaching the lowest values at t_3. After this time point, the prokaryotic populations started to increase in all treatments. Prokaryotic numbers in K and KA treatments were lower than those in treatments N and NA.

Chl *a* concentrations varied in a similar way in both the winter and summer cases: Chl *a* under N and NA experimental conditions (Figure 1e, f) reached higher concentrations than under K and KA treatments (Figure 1c, d). The Chl *a* values were about three to eight times higher in the winter experiment, which relates to the higher nutrient enrichment induced in that experiment.

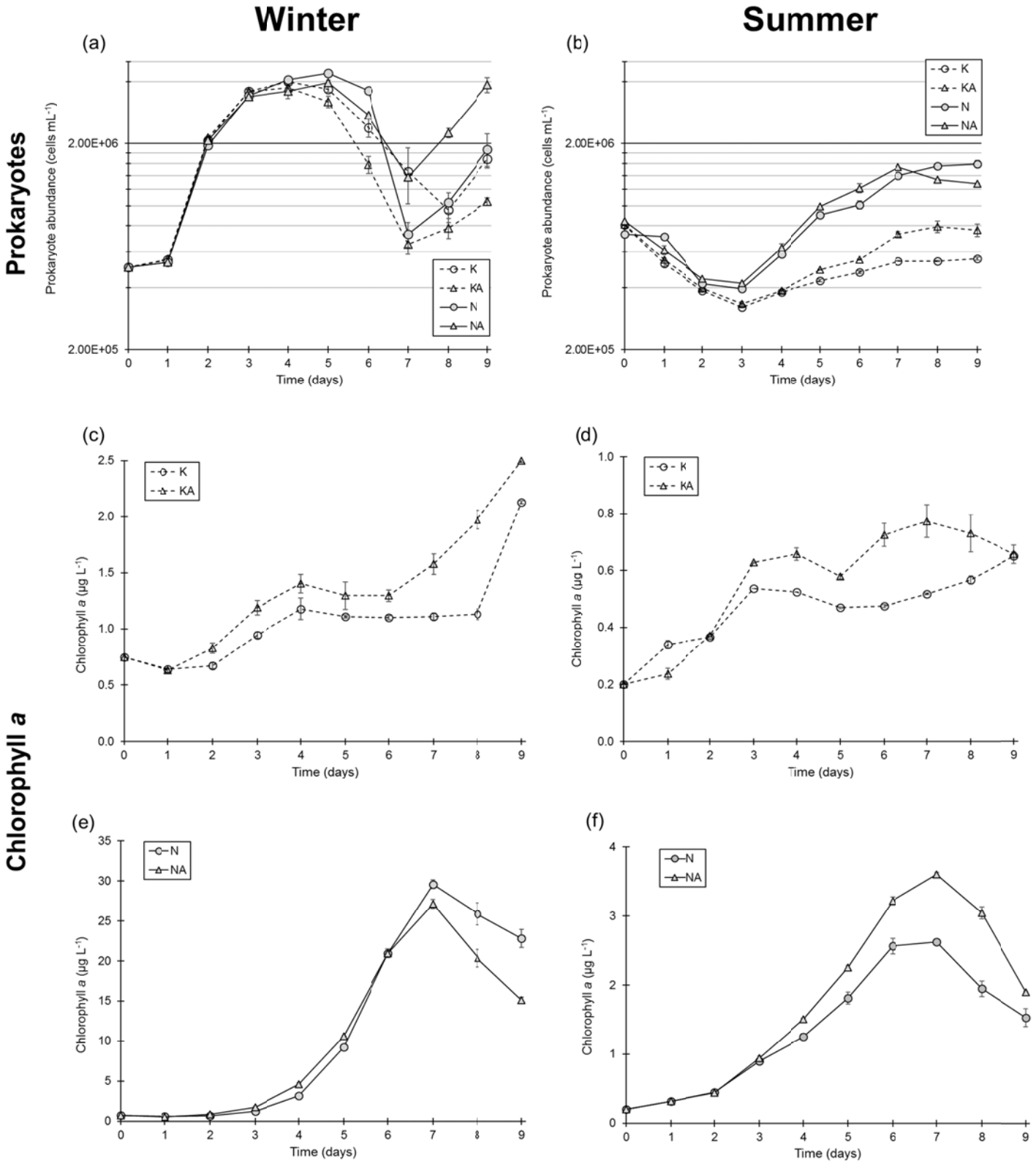

Figure 1. Temporal dynamics of prokaryote abundances (cells mL^{-1}) in (a) winter and in (b) summer; chlorophyll *a* pigment concentration (µg L^{-1}) for K and KA treatments in (c) winter and (d) summer; chlorophyll *a* pigment concentration (µg L^{-1}) for N and NA treatments in (e) winter and (f) summer. Note that, in (c), (d), (e) and (f) panels, the scales of the vertical axes are different. Error bars indicate the standard error of 2 replicates.

3.2. – DOC

DOC concentration in the winter experiment increased in the four treatments (Figure 2a) reaching maximum values at t_7 in treatments K and KA, and t_9 in N and NA. From t_7 to t_9, DOC decreased in both K and KA treatments, while DOC concentration kept increasing in N and NA conditions, coinciding with the decay of phytoplankton bloom. During the summer experiment, small variations in DOC concentration were observed (Figure 2b). In general, a positive trend to higher concentrations was identified during the entire incubation. The starting point conditions were about 80-85 µmol L^{-1} and the final concentrations increased to 90-95 µmol L^{-1}. Regarding nitrate concentrations, they generally decreased from the beginning of the experiment in the N and NA tanks. Similar patterns were found for phosphate concentrations in these enriched conditions, while small variations occurred during the whole experimental period in the control treatments.

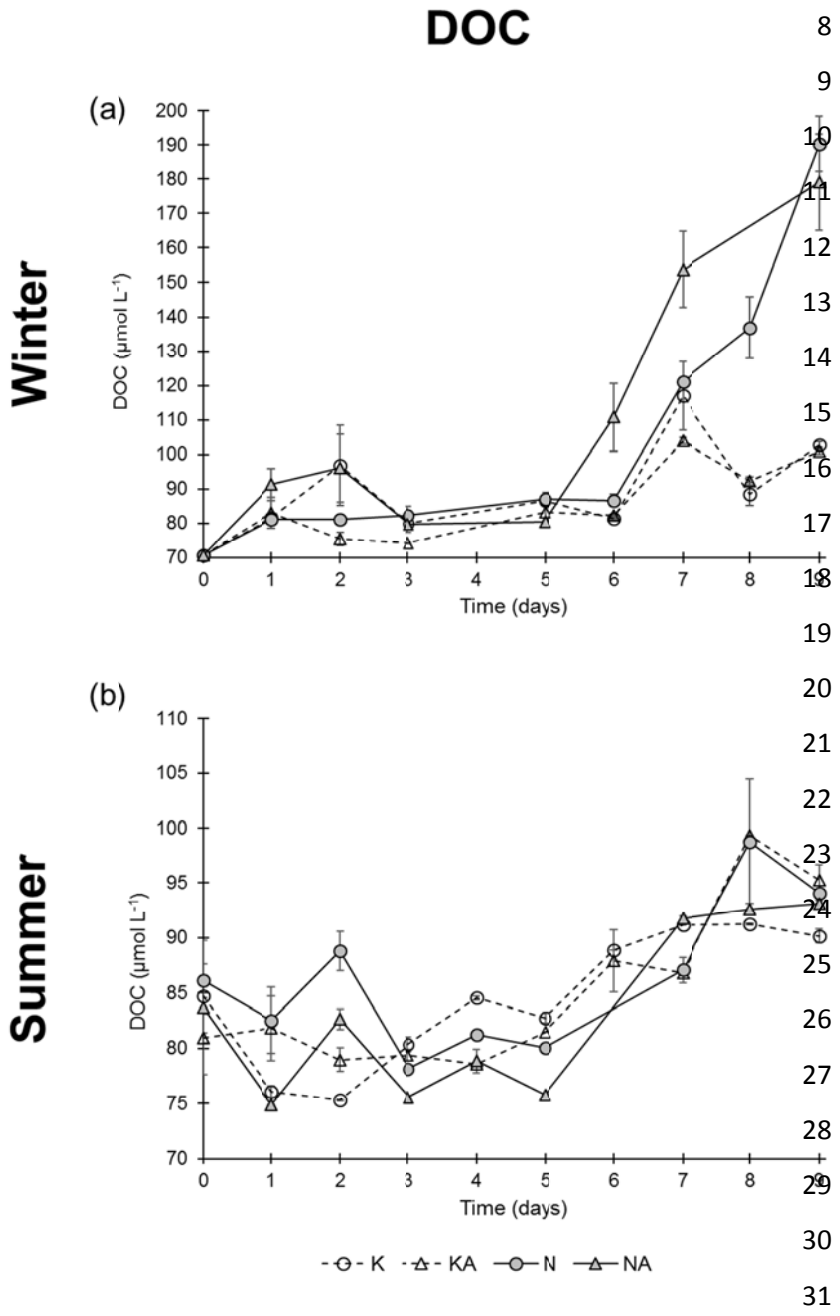

Figure 2. Changes in DOC concentrations (µmol L^{-1}) in the (a) winter and in the (b) summer experiments. Open symbols represent control (K) conditions and filled colored symbols nutrient-amended (N) conditions. Note the change of scale in vertical axes. Error bars indicate the error of 2 replicates.

3.3. – Optical analyses of the DOM

Fluorescence intensities during the experiments were measured to track changes in the quality of organic matter. We visualized the differences by subtracting the EEMs at t_0 from those at t_9 (Figure 3). In the winter experiment, the most remarkable feature was the increase in the fluorescence signal of the protein-like material (peak-T) in all treatments, including the control, which reached 2.3 QSU (Figure 3a). However, in the acidified scenario (Figure 3b), the increase was slightly smaller in the non-enriched treatments (~1.6 QSU). Regarding the enriched mesocosms (N and NA, Figure 3c, d), we also observed slighter increases of the fluorescence signal of the humic-like compounds (A, C and M regions), in addition to those of the peak-T.

The patterns identified in summer and winter were similar: the main changes were found around the protein-like fluorescence region, which increased in all four conditions (~0.5 QSU in K and KA to ~1.4 in N and ~1.0 QSU in NA). Again, slight increases of humic-like fluorescence were detected during the experiments, mainly in the nutrient-enriched treatments (Figure 3g, h).

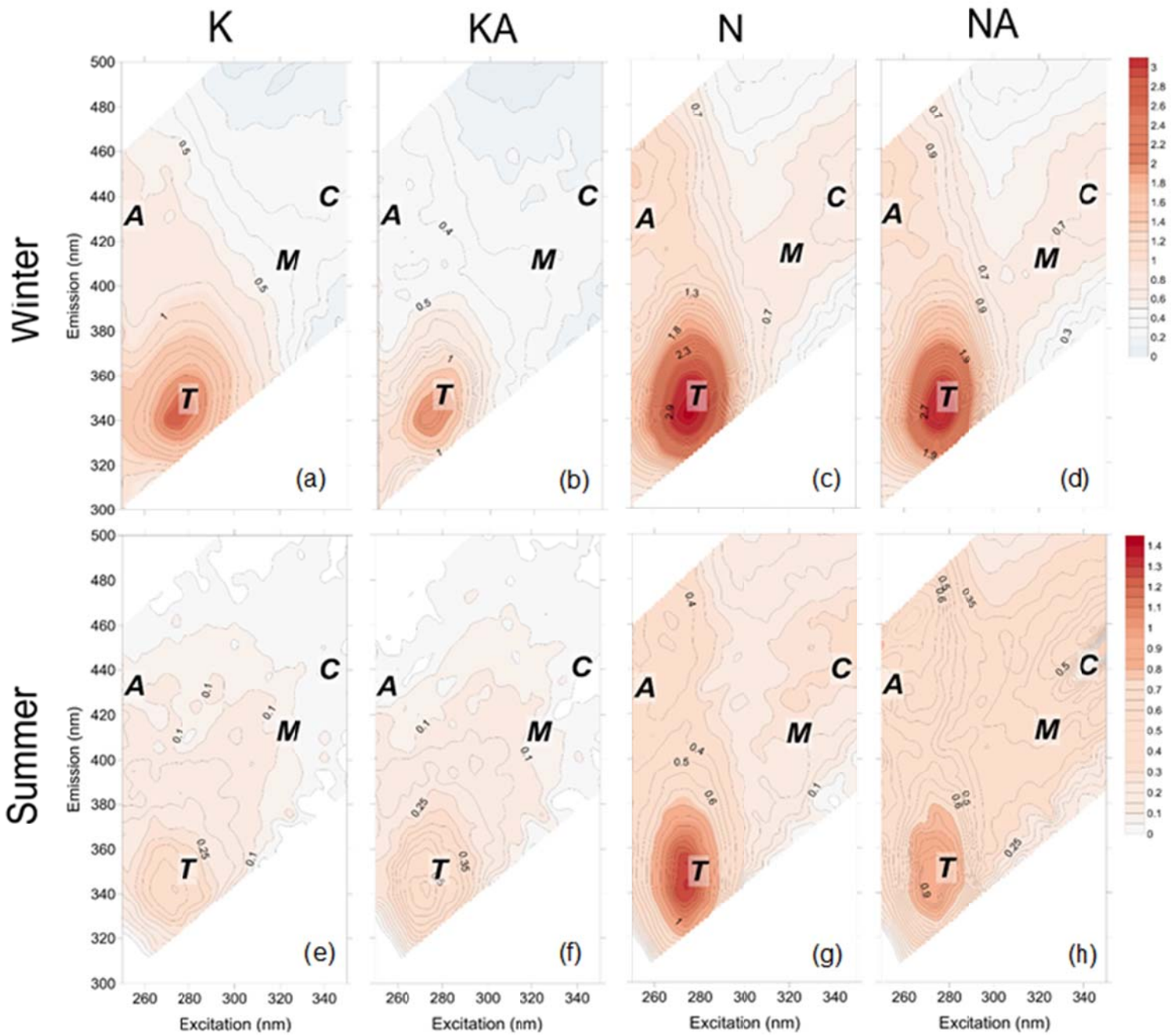

Figure 3. EEMs showing increases/decreases in fluorescence intensity over the 9 days of the mesocosm experiments ($\Delta EEM = EEMt_9 - EEMt_0$) for the different fluorophores in winter (a-d) and summer (e-h). Values reported as quinine sulfate units (QSU). Humic-like fluorophores indicated as A, C and M; protein-like fluorophore indicated as T. Note the change in scale between the W and S experiments.

The temporal evolution of peak-C to peak-M ratio (peak-C:peak-M) helped us to explore, in more detail, the changes experienced by the humic-like substances (Figure 4a, b). In general, the ratios were lower in winter than in summer, but both experiments showed the highest ratios in the N and NA tanks at the end of the experiment. In K and KA conditions, no clear trends were identified in winter, while the evolution of the non-nutrient-enriched and nutrient-enriched tanks was relatively parallel in summer. After a decrease observed from t_0 to t_1, the values tended to increase until the end of the experiment reaching higher values in the enriched conditions. The SUVA evolution (Figure 4c, d) did not show a clear temporal pattern in the winter experiment, although by t_9 NA and N conditions presented the highest increase in relation to the initial values. In summer, after t_3, the enriched- and non-enriched treatments diverged, reaching significantly higher values in the N-conditions (p-value < 0.05) and, again, the highest increase at the end of the experiment occurred in the NA treatment (0.040 ± 0.004 m^{-1} mg^{-1} L) followed by the N treatment (0.030 ± 0.005 m^{-1} mg^{-1} L). In winter the K treatment displayed higher increases in SUVA than the acidified control condition (KA) but this fact was not observed in summer. The temporal evolution of HIX differed during the first days of the experiment between the winter and summer scenarios, this index decreased until t_5 and then increased until t_9 in winter, whereas it increased during the whole experiment in summer. The HIX values reached at the end of the incubation were always higher in the nutrient enriched conditions than in the non-enriched ones. Furthermore, the values of the NA treatments were higher than the N ones in both experiments.

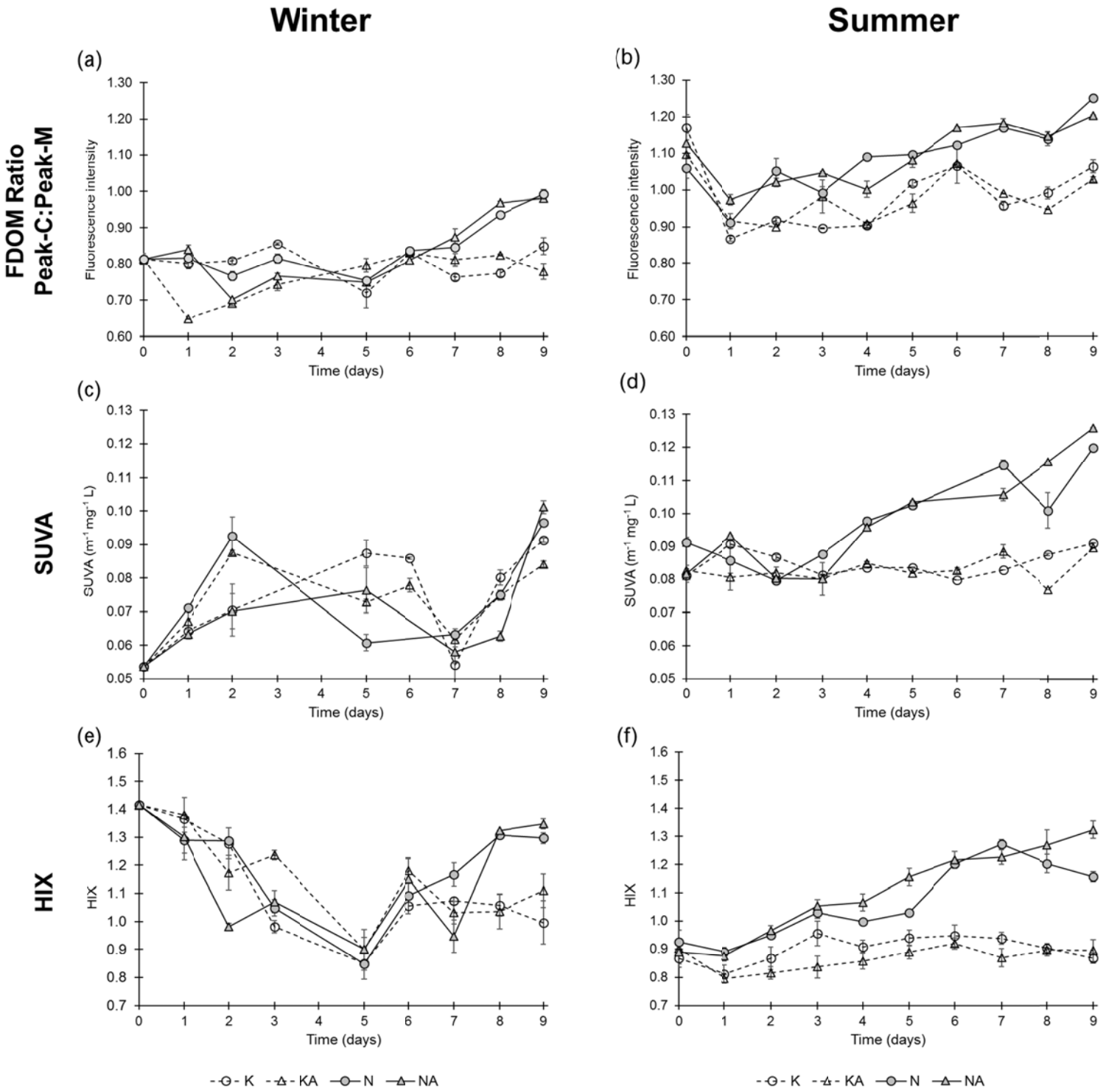

Figure 4. Time evolution of the quotient between peak-C and peak-M in (a) winter and (b) summer; specific UV absorbance at 254 nm (SUVA) in (c) winter and (d) summer; and humification index (HIX) in (e) winter and (f) summer. Peak-C:peak-M ratio and HIX are dimensionless variables. Error bars indicate the standard error of 2 replicates.

4. – Discussion

4.1. - DOC dynamics

DOC net accumulation occurred always after the end of the exponential-growth phase (coinciding with the phytoplankton post-bloom phase) either with high or low pCO_2 levels (Figures 1, 2). Thus, the production of DOC, without distinction of the seasonality or the addition of nutrients, was not significantly different between the non-acidified and acidified tanks (K and N with respect to KA and NA, p-value < 0.05). In the same way, MacGilchrist et al. (2014) found no significant effect of pCO_2 on the DOC dynamics in five shipboard bioassay experiments in the northwest European shelf seas. These results are also in agreement with the mesocosm study by Maugendre et al. (2014) in the Bay of Villefranche (France, NW Mediterranean Sea), where no significant effects of elevated temperature and/or CO_2 were found on most biological parameters and processes, including the generation of DOM. On the other hand, Yoshimura et al. (2010) conducted incubation experiments with sea surface water (depleted in nutrients) from the Sea of Okhotsk and detected a decrease in the generation of DOC when pCO_2 levels were >480 µatm.

The evolution of DOC and nutrient dynamics in previous mesocosm experiments, can be contradictory. In 2007, Riebesell and collaborators found that although the CO_2 uptake was higher in conditions with elevated pCO_2, no differences in the phytoplankton POC flux were observed. Thus, they suggested that the extra CO_2 incorporated was lost as DOC or respiration. More recently, in 2013, a mesocosm experiment was conducted in Svalbard to examine the influence of high pCO_2 and nutrient availability on microbial activities (Riebesell et al., 2013). In that experiment, pico-phytoplankton growth and DOC exudation increased at elevated CO_2 concentrations after inorganic nutrients were supplied. Another mesocosm experiment conducted in waters off the Baltic Sea during 4 weeks in the summer season (Paul et al., 2015) revealed that under high pCO_2 an important percentage of the organic matter production was in dissolved form. In our study, the abundance of small phytoplankton (pico- and nanoeukaryotes) was stimulated in the enriched conditions of the summer experiment (Sala et al., 2016). However, this stimulation was not accompanied by a net increase of DOM. In the nutrient-enriched conditions of the winter

scenario, we found an increase of DOC due to the phytoplankton bloom (dominated by diatoms) reaching discrete higher values (although not significant) during the bloom phase when high pCO_2 were induced. Moreover, the experiments performed by Kim et al. (2011) with mesocosm enclosures in Korean coastal waters, showed that when the pCO_2 and temperature increased, the production of DOC was enhanced. A different study conducted by Yoshimura et al. (2013) in the sub-Arctic Pacific obtained higher concentrations of DOC in the lowest pCO_2 treatment (300 μatm) over the first 10 days of incubation. Thus, in discordance with Kim et al. (2011), Riebesell et al. (2013), Yoshimura et al. (2013) and Paul et al. (2015), no significant differences were observed in DOC dynamics between acidified and non-acidified conditions.

Looking in further detail to the relationship between DOC and phytoplankton biomass (DOC:Chl *a* ratio*,* Figure 5), the highest values of this ratio were found under non-enriched conditions in both experiments. This fact could be due to a nutrient limitation of prokaryote growth as it has been described in open and coastal Mediterranean waters during low nutrient concentration episodes (Thingstad et al., 1997; Sala et al., 2002). Besides, for the non-enriched conditions, we found higher values of this ratio in the acidified conditions in winter, while the opposite pattern was observed in summer. The high values found for this quotient in summer have been previously discussed in different studies conducted in Mediterranean waters (Morel and Gentili, 2009; Organelli et al., 2014). As described in the work of Romera-Castillo et al. (2013), at the Blanes Bay sampling site, the DOC accumulates during summer when the degradation of organic matter by heterotrophic prokaryotes is reduced due to the depletion of inorganic nutrients. In that scenario, DOC accumulates and the ratio DOC/Chl *a*, increases with respect to winter.

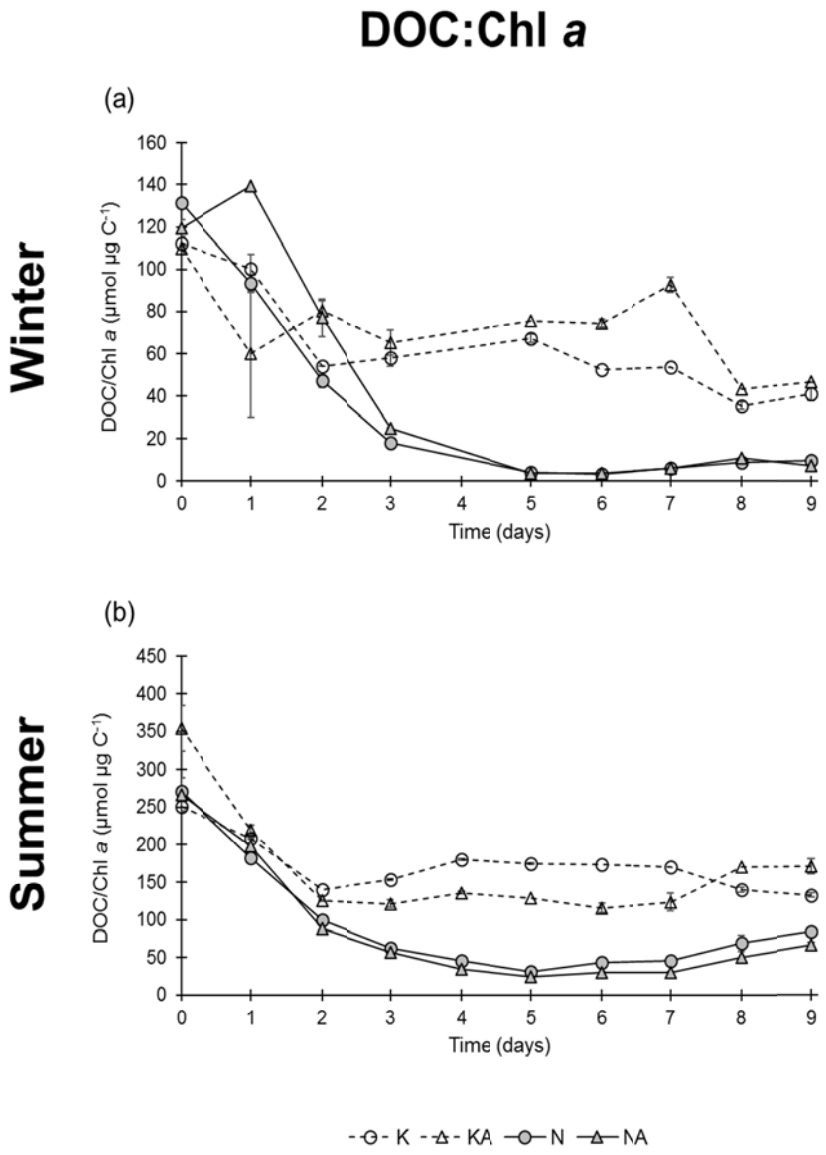

Figure 5. Time evolution of the quotient between DOC and Chl *a* in (a) winter and (b) summer. Units are expressed in µmol µg C^{-1}. Error bars indicate the standard error of 2 replicates.

4.3. – FDOM dynamics

Since fluorescence excitation-emission matrices are spectral signatures of the FDOM, they are useful to track the changes of different DOM constituents over time. As explained above, previous studies have hypothesized that the increasing concentration of CO_2 in seawater could channel the extra organic carbon fixed by photosynthesis into the dissolved fraction. Despite not finding accumulation of DOC in the treatments amended with high pCO_2, we

consistently observed changes in the quality of DOM (Figure 3). The temporal evolution of the four main fluorophores revealed an increase with time, so no net consumption of FDOM was detected in any of the treatments. Data depicted in Figure 3, confirms that the increase in the humic-like fluorescent signal was greater in the tanks where nutrients were added than in the non-enriched tanks. This would agree with the increase of CDOM compounds after enrichment with nitrate found in the experiments performed by Lekunberri et al. (2012) and by Yuan et al. (2016) in the Mediterranean Sea and the South China Sea, respectively.

Acidification conditions could potentially alter the optical properties of the protein- and humic-like portions of FDOM. Previous studies indicated that only pH levels above and below specific values (i.e., a pH <3 or >8) have potential to significantly change the structure of the DOM and thus induce a reduction of the fluorescence efficiency of the humic-like molecules (Laane, 1982; Dryer et al., 2008; Yan et al., 2013). Equally, it has been reported that the fluorescence signal of protein-like compounds as tyrosine and tryptophan can be only altered above and below specific pH values (i.e., a pH < 3 or >9; White, 1959). Consequently, given that the pH values achieved in KA and NA conditions were ~7.81 and ~7.76 for winter and summer, respectively, and that the original seawater pH values were 7.99 in winter and 8.02 in summer, we can discard that the changes in fluorescence were due to the alterations caused directly by the pH levels reached. In addition, we also tested the possible effects of pH on FDOM measurements (see Material & Methods section). And, as expected, we found no differences of FDOM intensities within the range of pH observed in our experiments. Therefore, we can assume that the changes in FDOM intensity were induced only by biological activity (i.e. FDOM intensities were not affected by pH).

The increment in peak-T fluorescence (Figure 3) indicated the generation of protein-like compounds in all the experimental conditions. It is common to find this type of fluorescence increases when studying microbial assemblages because it is associated to high productivity periods (Coble, 1996, 2007). Also, since microbial cells have protein-like fluorescence themselves (Determann et

al., 1998), several authors have found a positive direct relationship between fluorescence intensities and microbial biomass, in estuaries (Boyd and Osburn, 2004; Chen et al., 2004; Nieto-Cid et al., 2006; Huguet et al., 2009), coastal waters (Para et al., 2010; Romera-Castillo et al., 2011a, 2010), open ocean (Yamashita and Tanoue, 2003; Aparicio et al., 2015) or lakes (Yao et al., 2011; Catalán et al., 2013). The increase in peak-T intensity was lower in acidified conditions (Figure 3) except for the non-enriched summer conditions. However, the Pearson correlation performed in the Mantel tests revealed that differences between acidified and non-acidified treatments were not significant (p-value < 0.05). We found a significant correlation between prokaryote abundance and peak-T in summer experiment but not in winter, probably due to a larger influence of other variables not measured here, such as grazing on bacteria.

Although the production of protein-like material was the most relevant fluorescence feature in both experiments, the generation of humic-like substances (peak-A, -C and -M) was also notable. Nutrient enriched mesocosms (N and NA) presented an important increase in humic signals regardless of the experiment. This is consistent with these humic signals being by-products of the microbial respiration processes (Nieto-Cid et al., 2005; Coble, 2007; Romera-Castillo et al., 2011b, 2013; Jørgensen et al., 2011, 2014; Catalá et al., 2015). In order to elucidate whether the presence of higher fluorescent signals in the humic-like substances area was accompanied by a change in the quality of the FDOM, we made use of three different fluorescent indices that are related to the quality of the DOM. The temporal trend of the peak-C:peak-M ratio (Figure 4a, b) indicated that nutrient additions affected the quality of the fluorescent organic matter, however changes in pCO_2 did not induce significant changes in the quantity nor in the quality of the fluorescent organic material (p-value < 0.05). It has been previously demonstrated that the fluorescence in the peak-C region is associated with prokaryote respiration (Lønborg et al., 2010) and exudation of prokaryote by-products (Romera-Castillo et al., 2011b). Therefore the high peak-C:peak-M ratios found at the end of the nutrient enriched experiments, compared to the non-nutrient amended, could be linked to an increase of prokaryote respiration which would be induced by the elevated nutrient availability.

The high pCO_2 did not seem to influence the SUVA index. However, the nutrient addition generated an increase of the SUVA values with respect to the non-amended mesocosms, but only in the summer season. This contrasting response to nutrient additions in summer could have resulted from differences in the initial quality of the DOM (note the initial values of the three indices tested, Figure 4). The generation of high molecular weight aromatic humic acids in the enriched mesocosms was distinguishable by the increase in HIX values at the end of the experiment. The HIX starting values in the two experiments differed between seasons and, most likely due to the intense solar radiation, we found lower values of HIX in the summer experiment. It has been demonstrated that photobleaching of humic-like materials results a loss of aromaticity and a decrease in the molecular weight of irradiated organic matter (Moran and Zepp, 1997; Osburn et al., 2001; Rochelle-Newall and Fisher, 2002; Helms et al., 2008; Para et al., 2010; Catalán et al., 2013). Nevertheless, because our mesocosms were not exposed to UV radiation (only photosynthetically active radiation -PAR- was provided) changes in HIX values were most parsimoniously ascribed to differences in biological activity.

It is clear that the evolution of the optical indices differed between seasons (Figure 4). In winter, nutrient and control conditions showed differences, but only in the last days of the experiment. In summer these differences were observed already at the beginning of the incubations, although the differences were statistically significant (p-value < 0.05) from t_4 (for SUVA and HIX indices) and from t_6 (peak-C:peak-M ratio). In this way, the normalization of the FDOM to DOC (peak-C/DOC and peak-M/DOC; Figure 6) also revealed that the quality of the DOM was highly influenced by the initial conditions. Although the starting values were similar for all the conditions, the different initial microbial populations could have conditioned the evolution of the DOC and FDOM dynamics. Diatoms clearly predominated over other phytoplanktonic group during winter, in the four tanks, whereas, during the summer experiment, this group was only present at small proportions in N and NA conditions and almost inexistent in the control conditions (Sala et al., 2016). Thus, a synergistic effect can be extracted from our results regarding the composition of the initial

microbial population and the nutrient availability. These results agreed with those obtained in the work of Zark and collaborators (2015), indicating that the ocean acidification *per se* does not influence the accumulation of DOM in coastal environments.

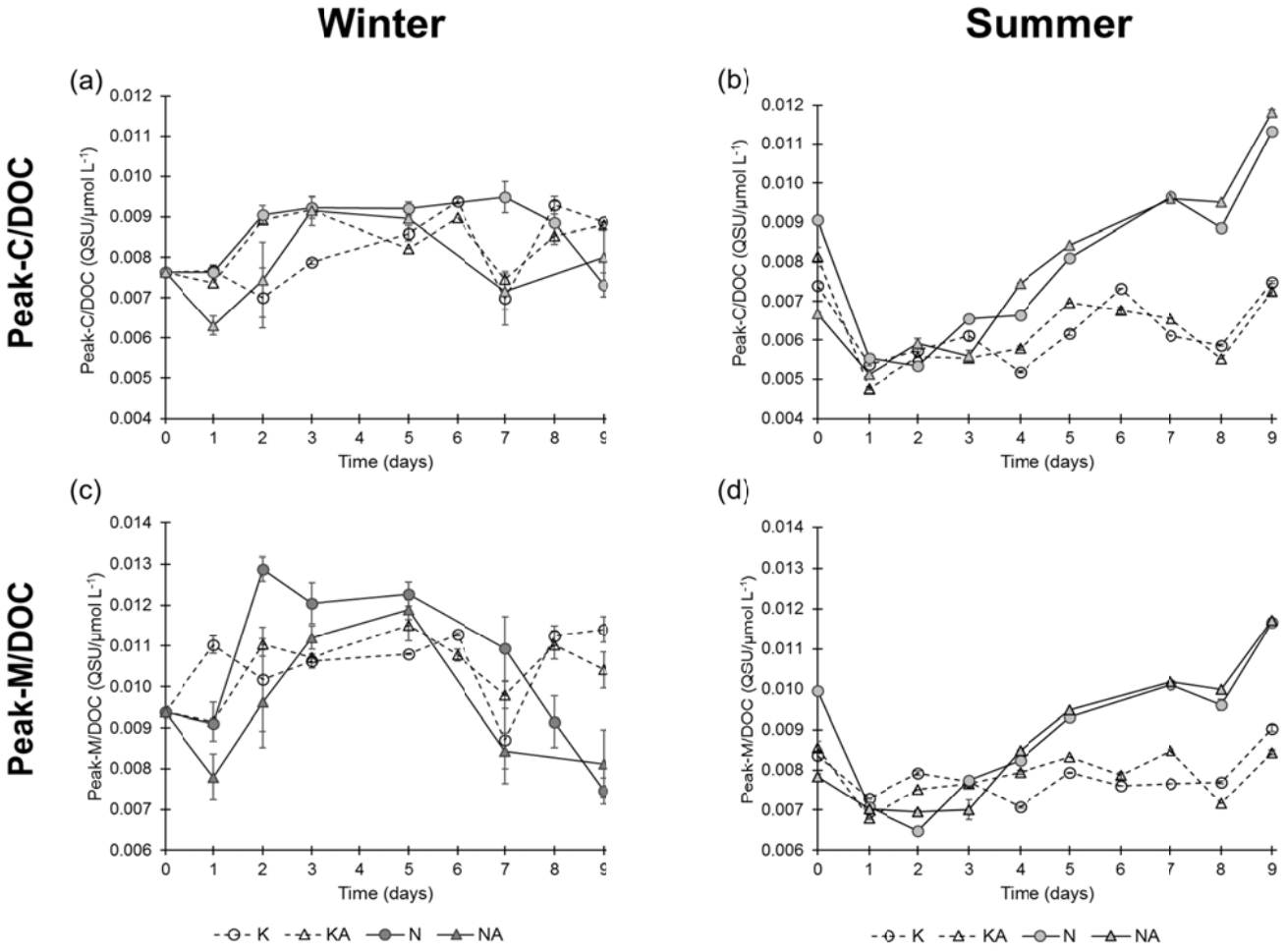

Figure 6. Time evolution of the quotient between peak-C and DOC in (a) winter and (b) summer; quotient between peak-M and DOC in (c) winter and (d) summer. Units are expressed in QSU/µmol L^{-1}. Error bars indicate the standard error of 2 replicates.

5. – Conclusions

The results extracted from this study highlight the value of mesocosm experiments as a way to assess possible responses of DOM dynamics to future environmental changes. To our knowledge, this is the first study that quantifies the influence of high levels of pCO_2 on the fluorescent properties of DOM in the Mediterranean Sea.

Although we found, in general, higher phytoplankton biomass under high-pCO_2 conditions, we did not observe differences in the DOC dynamics between acidified and non-acidified treatments.

Transformations of DOM composition tracked by changes in its optical properties (absorbance and fluorescence) indicated that eutrophication modified the structure of the organic matter into more complex material, while a weak aromatization of the DOM was observed under higher pCO_2 conditions.

The effects of eutrophication, in terms of quantity and quality of organic matter, varied depending on the initial conditions, which highlights the importance of conducting experiments under different seasons/regimes to account for temporal variability in the response of the ecosystem to the studied variables.

Acknowledgements

The authors would like to acknowledge S. Arnautovic, V. Balague, C. Cardelús, L. Cros, E. Vázquez-Domínguez, J. Movilla and À. López-Sanz for technical assistance during the experiments; the Experimental Aquarium Zone (ZAE) of the ICM-CSIC for providing the installations and the analyses provided by the laboratory of Analytical Chemistry at the ICM-CSIC. We would like to extend thanks to the three anonymous reviewers, whose comments have improved this work. The experiments have been possible thanks to the funding of projects: STORM (CTM2009-09352), DOREMI (CTM2012-342949) and ANIMA (CTM2015-65720) from the Spanish Ministerio de Economía y Competitividad (MINECO) and the Group de Recerca Consolidat financed by the Agència de Gestió d'Ajuts Universitaris i de Recerca (AGAUR) from the Generalitat de Catalunya. F.L.A. benefited of a JAE-pre pre-doctoral fellowship from the

Consejo Superior de Investigaciones Científicas (CSIC). M.N.-C. was funded by the CSIC Program 'Junta para la Ampliación de Estudios' co-financed by the ESF. E.B. benefited of a FPI pre-doctoral fellowship from the Spanish Ministerio de Economía y Competitividad.

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