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Simulation of Coastal Processes affecting pH with Impacts on Carbon and Nutrient Biogeochemistry

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Abstract

Naturally occurring microbial decomposition of organic matter (OM) in coastal marine environments cause increased acidity in deeper layers similar or even exceeding the future predictions for global ocean acidification (OA). Experimental studies in coastal areas characterized by increased inputs of OM and nutrients, coping with intermittent hypoxic/anoxic conditions, provide better understanding of the mechanisms affecting nutrients and carbon biogeochemistry under the emerging effects of coastal pH decrease. Laboratory CO₂-manipulated microcosm experiments were conducted using seawater and surface sediment collected from the deepest part of Elefsis Bay (Saronikos Gulf, Eastern Mediterranean) focusing to study the co-evolution of processes affected by the decline of dissolved oxygen and pH induced by (a) OM remineralization and (b) the future anthropogenic increase of atmospheric CO₂. Under more acidified conditions, a significant increase of total alkalinity was observed partially attributed to the sedimentary carbonate dissolution and the reactive nitrogen species shift towards ammonium. Nitrate and nitrite decline, in parallel with ammonium increase, demonstrated a deceleration of ammonium oxidation processes along with decrease in nitrate production. The decreased DIN:DIP ratio, the prevalence of organic nutrient species against the inorganic ones, the observations of constrained DON degradation and nitrate production decline and the higher DOC concentrations revealed the possible inhibition of OM decomposition under lower pH values. Finally, our results highlight the need for detailed studies of the carbonate system in coastal areas dominated by hypoxic/anoxic conditions, accompanied by other biogeochemical parameters and properly designed experiments to elucidate the processes sequence or alterations due to pH reduction.

Keywords: carbonate chemistry; carbon; nitrogen; phosphorus; microcosm experiment; Ocean acidification; coastal ocean; sediment.

Introduction

World Oceans currently absorb about one fourth of the anthropogenic carbon dioxide (CO₂) emissions into the atmosphere (Le Quéré *et al.*, 2015). The uptake of anthropogenic CO₂ from the oceans leads to pH decline and reduction of the carbonate ion concentration, a process usually referred as ocean acidification (e.g. Caldeira & Wickett, 2005). Since the beginning of the Industrial Revolution the average pH of ocean surface waters has fallen by about 0.1 units and is expected to decrease by 0.2 to 0.4 units by the end of the century (Ciais *et al.*, 2013).

In the Mediterranean Sea, recent studies report that anthropogenic carbon has penetrated throughout the water column, with higher concentrations than the global

average (Schneider *et al.*, 2010; Hassoun *et al.*, 2015). A net flux of anthropogenic carbon from the Atlantic towards the Mediterranean basin has been identified being responsible for 25% of the basin-wide CO₂ uptake over the last 200 years (Flecha *et al.*, 2015 and references therein). From the pre-industrial period until 2013 the pH in Mediterranean Sea has decreased by 0.055 - 0.156 pH units (Hassoun *et al.*, 2015) with an annual decreasing trend of 0.0044 ± 0.00006 (Flecha *et al.*, 2015). Model simulations predict a further decrease in the pH of Mediterranean surface waters of 0.3-0.4 units by the year 2100 (Howes *et al.*, 2015). In shallow nearshore Mediterranean areas, although seawater pH is influenced by various natural and anthropogenic processes other than CO₂ uptake (Borges & Gypens, 2010; Duarte *et al.*, 2013; Hagens *et al.*, 2015), long term data-based estimations at coastal

monitoring sites also indicate a shift towards more acidic conditions (e.g. Luchetta *et al.*, 2010; Howes *et al.*, 2015; Kapsenberg *et al.*, 2017).

The changes associated with the uptake of the excess CO_2 by the ocean cause an additional abiotic stressor for marine ecosystems (Andersson *et al.*, 2007). The oceans' response to acidification will not be uniform; different regions and different depths already exhibit different trends, and the activities of organisms may accelerate or modulate these trends (Beman *et al.*, 2011). In an acidifying ocean, microbial community responses to reduced seawater pH and elevated seawater partial pressure of CO_2 ($p\text{CO}_2$) may act as positive or negative feedbacks to carbon and nitrogen critical biogeochemical processes. In coastal areas, even in the presence of oxygen, the flow of the organic load is high and the aerobic degradation of organic matter leads to a higher CO_2 production, causing dissolution of existing sedimentary carbonates (Andersson *et al.*, 2007 and references therein). Furthermore, ocean acidification can interact with other natural and anthropogenic environmental processes in coastal areas, to accelerate local declines in pH and carbonate mineral saturation states (Feely *et al.*, 2010).

While acidification in the open ocean is mainly driven by the atmospheric CO_2 , in coastal zones this process may be minor and the signal of CO_2 -induced acidification may not be actually distinct, particularly at eutrophic marine areas where excessive nutrient loading and organic matter production have been associated with hypoxic events (Wallace *et al.*, 2014, Hagens *et al.*, 2015; Ingrosso *et al.*, 2016a).

In subsurface waters the same respiratory processes that lower dissolved oxygen concentrations, add CO_2 to solution, reduce pH, and, over longer time scales, can exacerbate the ocean acidification process (Cai *et al.*, 2011). There are some good examples available worldwide indicating the inverse relationship between pH and dissolved oxygen in stratified hypoxic coastal regions (Hagens *et al.*, 2015 and references therein). Elefsis Bay is a small (68 km^2) and shallow (with a mean and maximum depth of 20 m and 35 m, respectively), almost enclosed embayment with limited water exchange through narrow and shallow connections with the adjacent Saronikos Gulf (Aegean Sea, Eastern Mediterranean Sea; Fig. 1). During the period from May to late October, the development and establishment of a strong temperature-driven pycnocline results in the isolation of the deeper part of the water column, leading to insufficient oxygen supply. In parallel the organic matter degradation reduces oxygen levels promoting the development of hypoxic/anoxic conditions and the accumulation of high amounts of silicate, phosphate and ammonium at the near-bottom layers (Scoullou & Riley, 1978; Pavlidou *et al.*, 2013). Studies conducted in Elefsis Bay in late '70s during the stratification period, have reported surface pH values of 8.4 - 8.5 while for the bottom water the respective values were much lower 7.9-7.8 (Scoullou, 1979; Friligos, 1982) unequivocally linked to the CO_2 release during the microbial remineralization of organic matter in the subthermocline layer. It is thus evident that during summer stratified conditions the close to the bottom waters of Elefsis Bay would be an ideal system to study the co-evolution of various biogeochemical

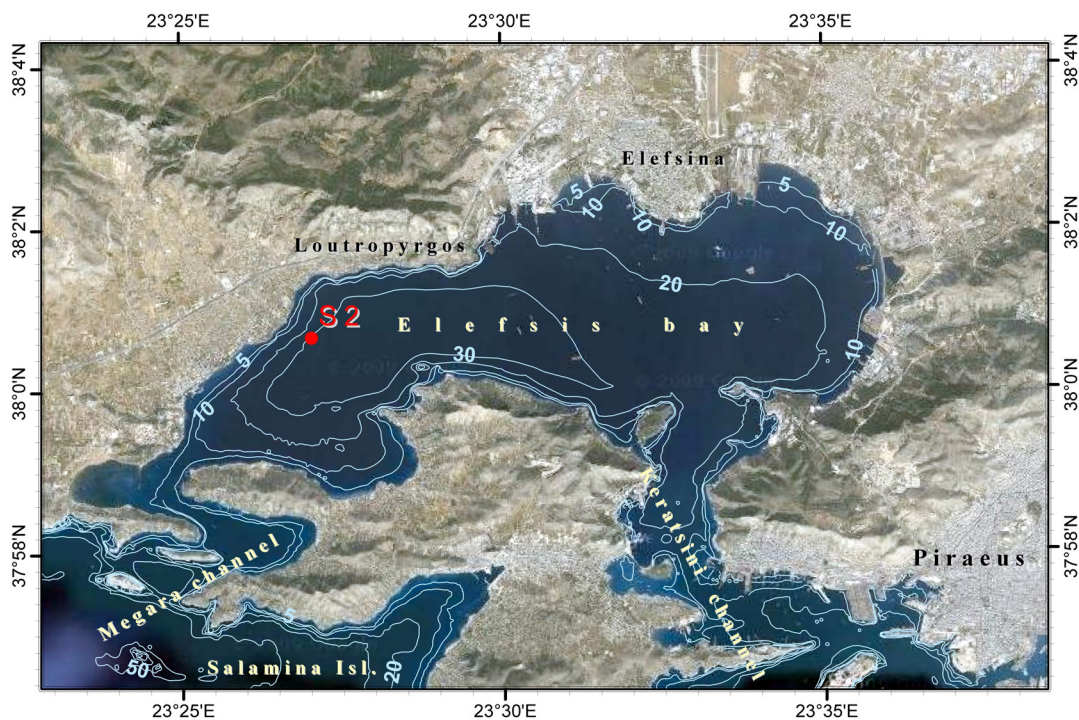


Fig. 1: Bathymetric map of Elefsis Bay (Saronikos Gulf, Eastern Mediterranean Sea). The location of the sampling site is also shown.

processes affected by the decline of both pH and dissolved oxygen.

The aim of this research is to investigate, in parallel, the response of carbon and nutrient biogeochemistry to the effect of pH decrease induced by (a) the organic matter remineralization and (b) the future anthropogenic increase of atmospheric CO₂. For this purpose, seawater and sediment from the deepest part of Elefsis Bay, an intermittently anoxic coastal system, were tested in a 20-days microcosm-scale lab experiment performed under controlled pH and temperature conditions. This experimental attempt intends to enhance our understanding of how pH changes could impact the basic but complex biogeochemical processes which are taking place in coastal areas coping with hypoxic/anoxic conditions.

Materials and Methods

Experimental Setup and Analyses

Field sampling was performed at the deepest western part of Elefsis Bay (Longitude: 23° 25' 48"E; Latitude: 38° 03' N; Depth: 33m; Fig. 1) in June 2014 with the *R/V Aegaeo* of the Hellenic Centre for Marine Research. Hydrographic parameters (salinity, temperature, depth) were recorded with a Sea Bird Electronics CTD instrument (SBE-9) associated with a General Oceanic rosette sampler, equipped with twelve 10 L Niskin bottles. Sub-samples of seawater from the deepest part of the water column (33 m) were collected and measured immediately for pH and redox potential with laboratory pHmeter and redox potential probe (Jenway 3310). In addition, seawater (unfiltered) was collected in polycarbonate bottles. Surface sediment sample (0-10 cm, untreated) was also collected using a 0.1m² box corer.

Seawater and sediment were carefully transferred to the laboratory into four 25 L polycarbonate bottles, at a proportion 80% and 20% v/v respectively, and kept in the dark in a temperature-controlled room that was set at the *in situ* temperature (15.6 °C). The seawater-sediment systems were left to settle, untreated, for a week with CO₂-free air supply through a pump; the CO₂-free air was introduced over the seawater surface in each tank in order to maintain the *in situ* oxygen saturation for the duration of the experiment. For the 20 days experimental period controlled CO₂ aeration was applied with a continuous flow system (IKS Aquastar, IKS Computer Systeme GmbH) which automatically adjusted CO₂ gas addition directly to the microcosms, in order to regulate and maintain stable pH value close to the target pH levels (Fig. 2). Each tank was monitored constantly (every 5 minutes) throughout the experiment by the IKS System using probes recording temperature (accuracy ±0.3°C), pH (accuracy ±0.05), and DO (accuracy ±12 μmol kg⁻¹). The pH probes were calibrated daily, using certified buffers to avoid drifting. The measured pH values by the IKS system were corrected with the parallel use of a Jenway 3310 laboratory pH meter calibrated in NBS scale (accuracy ±0.02); the pH

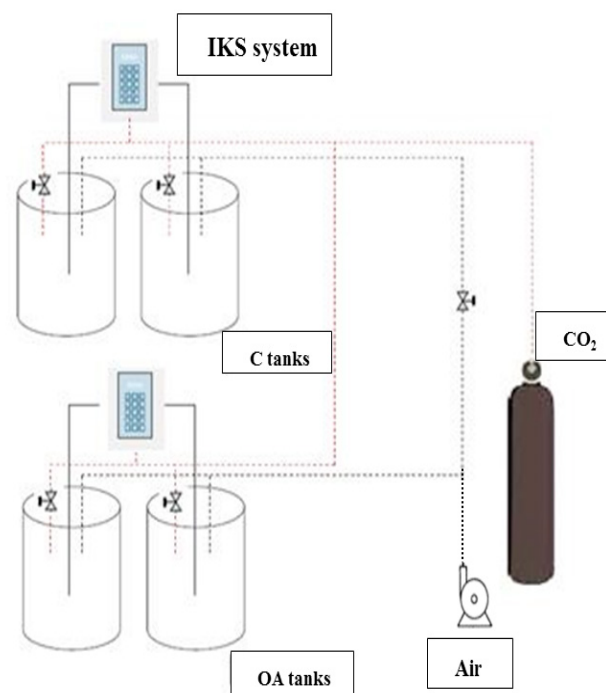


Fig. 2: Design of the experimental set-up demonstrating the two tanks for each pH treatment (OA and C), the IKS system monitoring the CO₂ gas supply and the air pump providing air to the systems.

values were then converted to the total scale (pH_T) (Dickson *et al.*, 2007).

The nominal pH values selected for the two treatments (experiment conditions) were: (i) for the control tanks (C) the pH value measured during sampling (7.85, NBS) and (ii) for the ocean acidification tanks (OA) the pH value predicted for the year 2300 (6.80 NBS), which corresponds to the highest total cumulative future CO₂ emission scenario, for latitudes corresponding to the Mediterranean Sea (Caldeira & Wickett, 2005). Each one of the two treatments was applied in two replicate tanks (Fig. 2). Thus all results regarding seawater samples for OA and C conditions refer to the mean value of two replicates (standard deviation is included in diagrams of each individual parameter). The temperature of the thermostated room and the pH selected for the C condition tanks were the recorded *in situ* values during sampling, in order to simulate as close as possible the natural system. Any change observed in the C condition tanks is considered to be a change naturally occurring, in order to investigate and fully comprehend the effects of acidification on the selected parameters.

Seawater samples were taken from the microcosms every 3 days and analyzed immediately for the determination of DO according to Winkler method, modified by Carpenter (1965) and the potentiometric determination of total alkalinity (A_T) according to Perez & Fraga (1987a) and Perez *et al.* (2000). The precision of A_T analysis was

determined by titrating bottled seawater samples having the same temperature and salinity and was $8.1 \mu\text{mol kg}^{-1}$, calculated as 3 times the standard deviation of 10 measurements. The accuracy was assessed by titration of sodium carbonate standard with known alkalinity fortified to the ionic strength of seawater and was $8.4 \mu\text{mol kg}^{-1}$, calculated as 3 times the standard deviation of 12 measurements. Samples for dissolved organic carbon (DOC) and nutrients were collected and filtered immediately through $0.45 \mu\text{m}$ polycarbonated membranes and stored in -18°C until their analysis. The DOC analysis was performed with the High Temperature Catalytic Oxidation method, using a Shimadzu 5000 total organic carbon analyzer, according to instrument's instructions; the Limit of Quantification (LOQ) for DOC was $39.5 \pm 4.9 \mu\text{mol kg}^{-1}$. Nutrients were determined spectrophotometrically with a Varian Cary 1E UV-visible spectrophotometer, using a 1 cm cell for nitrate, ammonium and silicate while for nitrite and phosphate a 5 cm cell was used. The analytical procedures followed were the ones described by Grasshoff *et al.*, 1999; for ammonium (4500-NH₃-F a modification of the Solorzano method (1969); LOQ: $0.8 \pm 0.2 \mu\text{mol kg}^{-1}$), nitrite (4500-NO₂-B; LOQ: $0.14 \pm 0.02 \mu\text{mol kg}^{-1}$), nitrate (4500-NO₃-E; LOQ: $0.21 \pm 0.07 \mu\text{mol kg}^{-1}$), phosphate (4500-P-E; LOQ: $0.11 \pm 0.03 \mu\text{mol kg}^{-1}$), silicate (4500-SiO₂-E; LOQ: $0.6 \pm 0.2 \mu\text{mol kg}^{-1}$). Total dissolved nitrogen (TDN; LOQ: $0.13 \pm 0.04 \mu\text{mol kg}^{-1}$) and total dissolved phosphorus (TDP; LOQ: $0.21 \pm 0.07 \mu\text{mol kg}^{-1}$) were determined as described by Valderrama (1981). Dissolved organic nitrogen (DON) and phosphorus (DOP) were calculated by subtraction of dissolved inorganic nitrogen forms (DIN) from TDN and of phosphate from TDP. Based on pH_T and A_T values, the rest of the carbonate system parameters, specifically pCO_2 , CO_2 concentration, dissolved inorganic carbon (DIC), bicarbonate (HCO_3^-) and carbonate (CO_3^{2-}) ions, aragonite's and calcite's saturation states (Ω_{ar} and Ω_{calc} , respectively) were calculated through the 'Seacarb' software package (Gattuso *et al.*, 2014) including also the respective phosphate and silicate concentrations. The 'Seacarb' calculations were performed using the apparent dissociation

constants of carbonic acid (K_1 and K_2) of Lueker *et al.* (2000), the equilibrium constant of hydrogen fluoride of Perez and Fraga (1987b), the stability constant of hydrogen sulfate ion of Dickson (1990) and the boron to chlorinity ratio of Lee *et al.* (2010).

Top 0-2 cm of surface sediments were sampled from each tank at the end of the experiment on the 20th day for supplementary analyses. In any case, the system volume, both water and sediment, was not reduced to more than 15% of the initial one (Riebesell *et al.*, 2010), in order to be considered undisturbed. The organic carbon (OC) and the total carbon (TC) and nitrogen (TN) of the sediment were determined with a Thermo Scientific FLASH 2000 CHNS elemental analyzer according to the analytical procedure described by Karageorgis *et al.* (2009). Inorganic carbon was calculated by subtracting organic carbon from the total carbon, and subsequently carbonate content was calculated. Total phosphorus and sulfur content was determined through X-ray fluorescence (XRF) analysis. Furthermore, X-ray powder diffraction (XRD) analyses were conducted to evaluate the main minerals prevailing in the sediment phases.

Data Analysis

A one-way analysis of variance (ANOVA) was performed to test the statistical significance of variation ($p < 0.05$) between the two experimental conditions for both water and sediment samples (Riebesell *et al.*, 2010; results are shown in Table 2). Data were first checked to ensure they conformed to the assumptions of ANOVA (normality: Kolmogorov-Smirnov test and homogeneity of variance test). Principal component analysis (PCA) was performed to evaluate the effects of the selected experimental treatments (pH) on nutrient species and carbon-carbonate parameters of the water-sediment laboratory systems. A Promax with Kaiser Normalization was used for rotation method, converged in 3 iterations (results are shown in Fig. 7). For PCA analysis, Ω -values were not used for data reduction since they are linearly correlated with CO_3^{2-} ; also, only pCO_2 values were used

Table 2. One-way ANOVA results (F, p) for all experiment parameters regarding seawater analyses (p values < 0.05 are marked bold, which indicate statistically significant difference between the two treatments).

Parameter	F	p	Parameter	F	p
DO	0.504	0.494	NO ₃ ⁻	9.982	0.010
A _T	7.688	0.020	NO ₂ ⁻	3.698	0.083
HCO ₃ ⁻	11.797	0.006	NH ₄ ⁺	6.474	0.029
CO ₃ ²⁻	54.706	0.000	DON	9.982	0.010
DIC	20.758	0.001	PO ₄ ³⁻	0.065	0.804
Ω_{ar}	56.934	0.000	DOP	0.191	0.672
Ω_{calc}	57.061	0.000	SiO ₄	1.993	0.188
DOC	2.712	0.131	DIN:DIP	5.620	0.039

while CO₂ concentrations were excluded as they are also inextricably related. Both ANOVA and PCA analyses were performed using the SPSS software package.

Results

During sampling, the difference in temperature between surface and bottom was 10°C; dissolved oxygen was found 225.77 µmol kg⁻¹ in the surface, whereas the respective bottom value was relatively low (99.45 µmol kg⁻¹). pH and Redox potential in Elefsis bottom seawater were 7.85 and 194.3 mV, respectively while A_T attained rather high concentration (2769 µmol kg⁻¹). Relatively elevated nitrate concentrations (4.35 µmol kg⁻¹) were measured, followed by nitrite (1.30 µmol kg⁻¹) and phosphate (0.08 µmol kg⁻¹) along with high silicate (13.83 µmol kg⁻¹) values. The pCO₂ calculated in the bottom seawater of Elefsis Bay was 797 µatm, almost double the current atmospheric pCO₂ value (<https://www.esrl.noaa.gov/gmd/ccgg/trends/>); however, both Ω_{ar} and Ω_{calc} indicated well saturated conditions.

During the experiment, temperature was kept quite stable at 15.1±0.3°C inside the incubator room. pH_T fluctuated slightly in both treatments (6.63±0.18 pH units for OA and 7.82±0.15 pH units for C treatment, Table 1). DO varied between 130±6 µmol kg⁻¹ and 138±12 µmol kg⁻¹ for OA and C conditions respectively (Fig. 6D) showing no statistical difference (F=0.504, p=0.494). Salinity was constant throughout the experiment at 38.7.

Carbonate System Parameters

Total alkalinity (A_T, Fig. 3A) in OA condition increased dramatically, with the significant increase occurring between the 4th and the 10th day of the experiment, while in C condition little variations were observed. Total dissolved inorganic carbon (DIC, Fig. 3B) also increased in both conditions, with elevated values for OA especially during the last days of the experiment (F=7.688, p=0.020). During the course of the experiment, bicarbonate ions (Fig. 3C) were found to increase in both C and OA conditions reaching considerably higher levels in OA (Table 1; F=11.797, p=0.006). Carbonate ions (Fig. 3D) decreased substantially in OA (F=54.706, p=0.000) in favor of HCO₃⁻ and DIC, probably reflecting the shift of carbonate system equilibria to compensate the CO₂ increase; CO₃²⁻ for C followed the pH slight variations. In C condition, saturation states for the two carbonate minerals (Ω_{ar}, Ω_{calc}, Table 1) were above 1, whereas in OA condition, both Ω_{ar} and Ω_{calc} were lower than 1.

Nutrient Species and Carbon

During the experiment, nitrate in C condition presented a sharp increase (from 2.7 µmol kg⁻¹ to 21.2 µmol kg⁻¹) until the 7th day, followed by a declining trend to 15.1 µmol kg⁻¹ (Fig. 4A). In OA condition, nitrate showed similar pattern until the 7th day (from 2.5 to 12.5 µmol

kg⁻¹), with final values remarkably lower than the ones determined in C condition (F=9.982, p=0.010). Nitrite (Fig. 4B) in C condition showed a similar increase (from 0.2 µmol kg⁻¹ to 1.6 µmol kg⁻¹), followed by a decreasing trend to values corresponding to the initial ones (0.3 µmol kg⁻¹). In OA condition, nitrite slightly increased from 0.2 to 0.5 µmol kg⁻¹, followed by a declining trend to values below the LOQ of the method (F=3.698, p=0.083). Ammonium (Fig. 4C) in C condition showed a decreasing trend from 1.9 to values below LOQ. On the contrary, in OA condition after the decrease observed until the 4th day (from 1.4 µmol kg⁻¹ to 0.8 µmol kg⁻¹), an increase was found until the end of the experiment (2.2 µmol kg⁻¹; F=6.474, p=0.029).

Phosphate (Fig.5A) showed a decreasing trend in C condition until the 10th day (from 0.6 µmol kg⁻¹ to 0.3 µmol kg⁻¹) followed by a slight increase (0.6 µmol kg⁻¹) until the end of the experiment; in OA condition, phosphate appeared constant until the 10th day (0.5 µmol kg⁻¹) and then followed the same trend with C condition to final values of 0.5 µmol kg⁻¹ (F=0.065, p=0.804). Silicate concentrations presented negligible variations between the two conditions (F=1.193, p=0.188).

Dissolved organic carbon (DOC; Fig.6B) concentrations showed the same trend in both conditions until the 10th day, while increased values were recorded for OA condition towards the end of the experiment (F=2.712, p=0.131).

Dissolved organic nitrogen (DON) followed the same decreasing pattern with ammonium in C condition till the end of the experiment (Fig. 4D); in OA condition DON varied between 21.9 µmol kg⁻¹ and 37.6 µmol kg⁻¹ reaching a final value of 27.3 µmol kg⁻¹ (F=9.982, p=0.010). Dissolved organic phosphorus (DOP) (Fig. 5B) showed similar increasing trend for both conditions until the 14th day of the experiment, and a decrease at the end of the experiment (F=0.191, p=0.672).

Dissolved inorganic nitrogen (DIN; stands for nitrate+nitrite+ammonium) to phosphate ratio (DIN:DIP from now on; Fig. 6C) in C condition increased from 8.0 to 79.4 until the 7th day followed by a decrease to 32.1. In OA condition, this increase was less significant (from 8.0 to 31.5 on the 10th day) and then the ratio remained constant at 21 (F=5.620, p=0.039).

Sediment composition

Sediment granulometry showed that Elefsis Bay is characterized by fine surface sediments of <63µm in a percentage between 95-100%. The main sediment minerals were calcite (CaCO₃) and quartz (SiO₂), followed by aragonite (CaCO₃) and clinocllore (Mg₅Al)(AlSi₃)O₁₀(OH)₈. This indicates sediment phases rich in carbonates and silica along with aluminium and magnesium. The carbonate content of the Elefsis Bay sediment was 46.8±0.95%; the respective values for OA and C were 47.6±2.6% and 47.0±1.3 respectively (Table 3). The OC analyses in sediment revealed high organic content

Table 1. Seawater carbonate system parameters (mean values, standard deviations, minimum and maximum values) for field and experiment microcosms (OA: Ocean Acidification condition, C: Control condition).

		pH_T	A_T	pCO_2	CO_2	HCO_3^-	CO_3^{2-}	DIC	$\Omega_{ar.}$	$\Omega_{cal.}$
			$\mu mol\ kg^{-1}$	μatm	$\mu mol\ kg^{-1}$	$\mu mol\ kg^{-1}$	$\mu mol\ kg^{-1}$	$\mu mol\ kg^{-1}$		
OA	field	7.72	2769.3	797	27.82	2413.8	146.25	2587.8	2.19	3.38
	mean	6.63	4017.3	2340	1153.3	3986.6	13.09	5152.9	0.20	0.30
	max	6.97	5341.0	2833	2458.6	5311.8	20.39	6825.3	0.31	0.47
	min	6.47	2772.7	1909	247.5	2720.0	2.23	2987.9	0.03	0.05
	st.dev.	0.18	569.8	822	768.1	1117.8	6.08	1379.8	0.09	0.14
C	mean	7.85	2749.1	974	31.0	2404.4	140.43	2575.9	2.10	3.26
	max	8.06	2858.5	1184	44.3	2606.2	202.77	2754.9	3.04	4.71
	min	7.69	2677.4	805	16.1	2181.5	97.99	2400.3	1.47	2.28
	st.dev.	0.15	329.6	364	11.9	153.6	40.85	126.1	0.61	0.95

The partial pressure of CO_2 (pCO_2), the concentration of CO_2 , HCO_3^- , CO_3^{2-} , dissolved inorganic carbon (DIC), and the saturation state of aragonite ($\Omega_{ar.}$) and calcite ($\Omega_{cal.}$) were calculated with 'Seacarb' package from pH_T , total alkalinity, salinity, temperature, phosphate and silicate data.

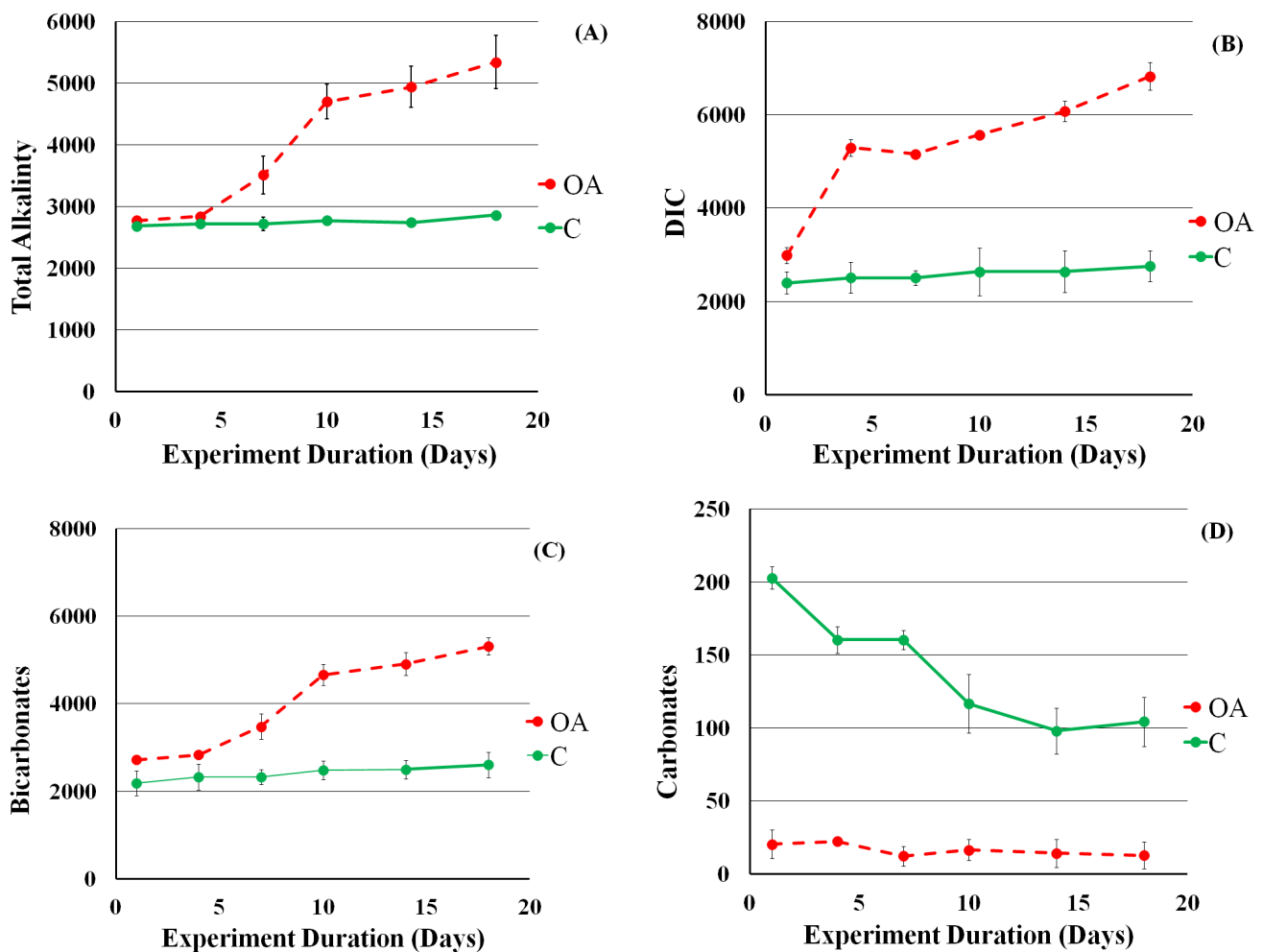


Fig. 3: Alkalinity, DIC, Bicarbonates (HCO_3^-) and Carbonates (CO_3^{2-}) concentrations (in $\mu mol\ kg^{-1}$) for the duration of the experiment; mean values and standard deviations for the two replicates of each treatment (OA: Ocean Acidification condition, C: Control condition).

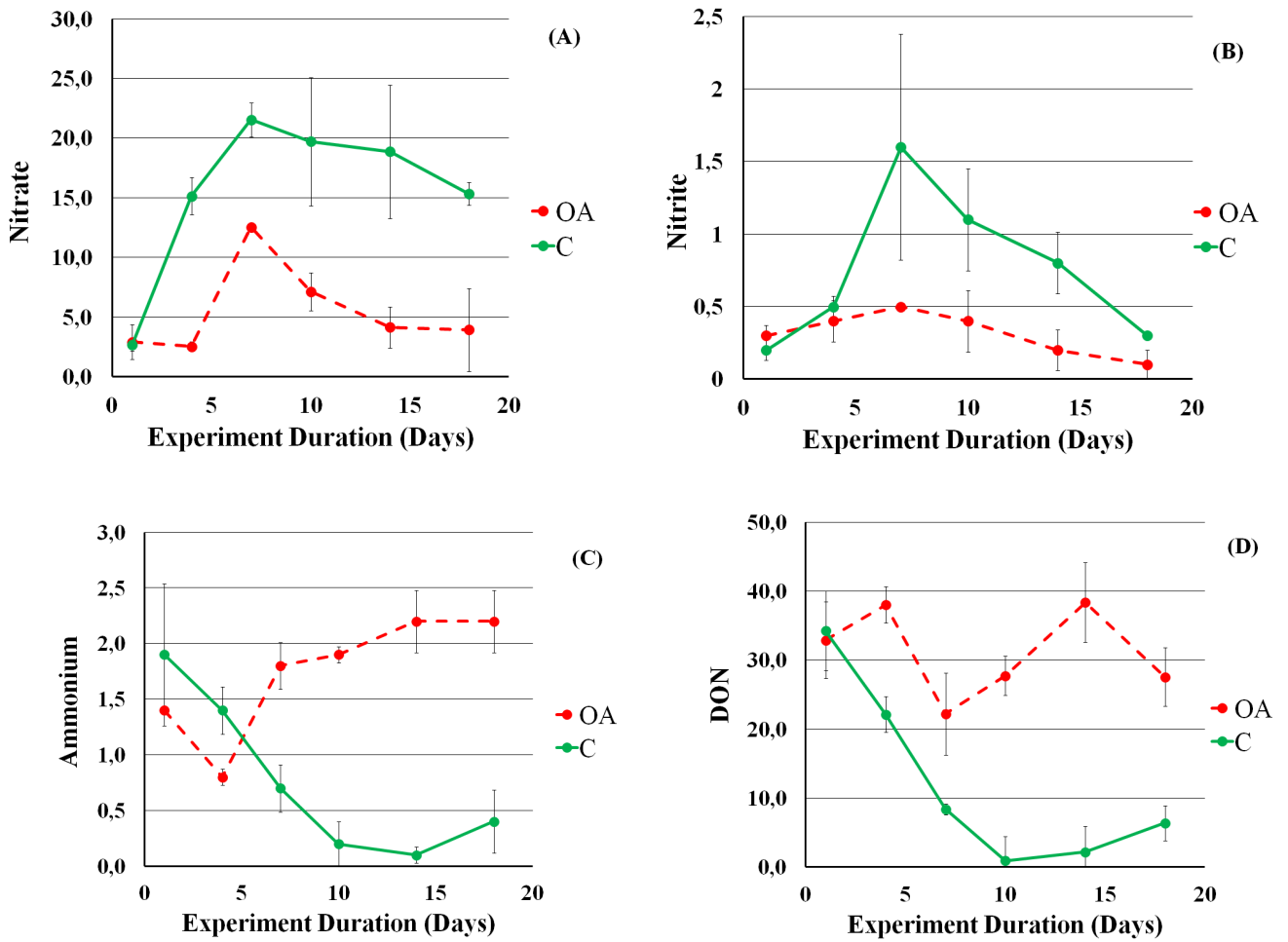


Fig. 4: Nitrate, Nitrite, Ammonium and DON concentrations (in $\mu\text{mol kg}^{-1}$) for the duration of the experiment; mean values and standard deviations for the two replicates of each treatment (OA: Ocean Acidification condition, C: Control condition).

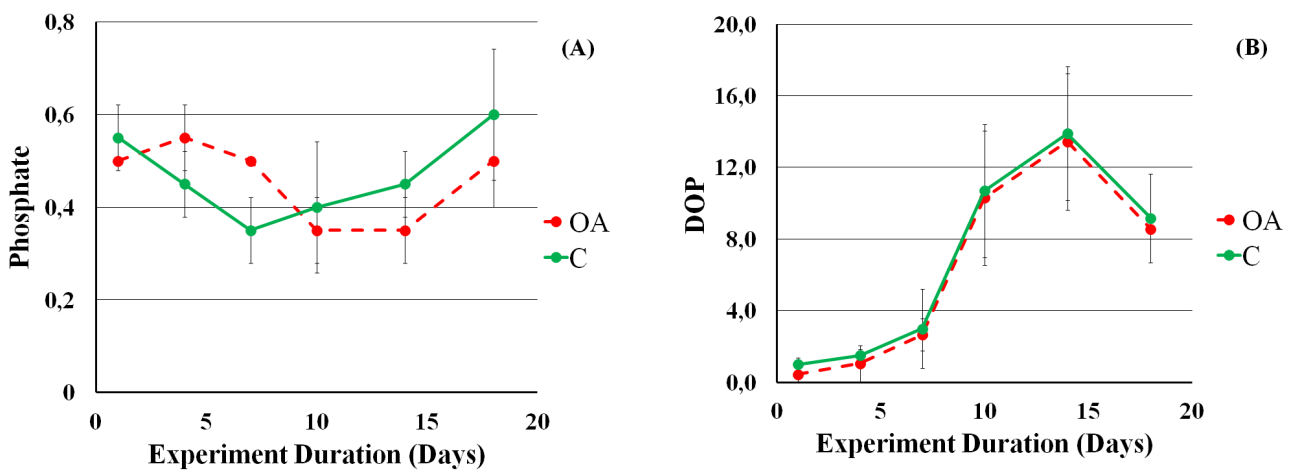


Fig. 5: Phosphate and DOP concentrations (in $\mu\text{mol kg}^{-1}$) for the duration of the experiment; mean values and standard deviations for the two replicates of each treatment (OA: Ocean Acidification condition, C: Control condition).

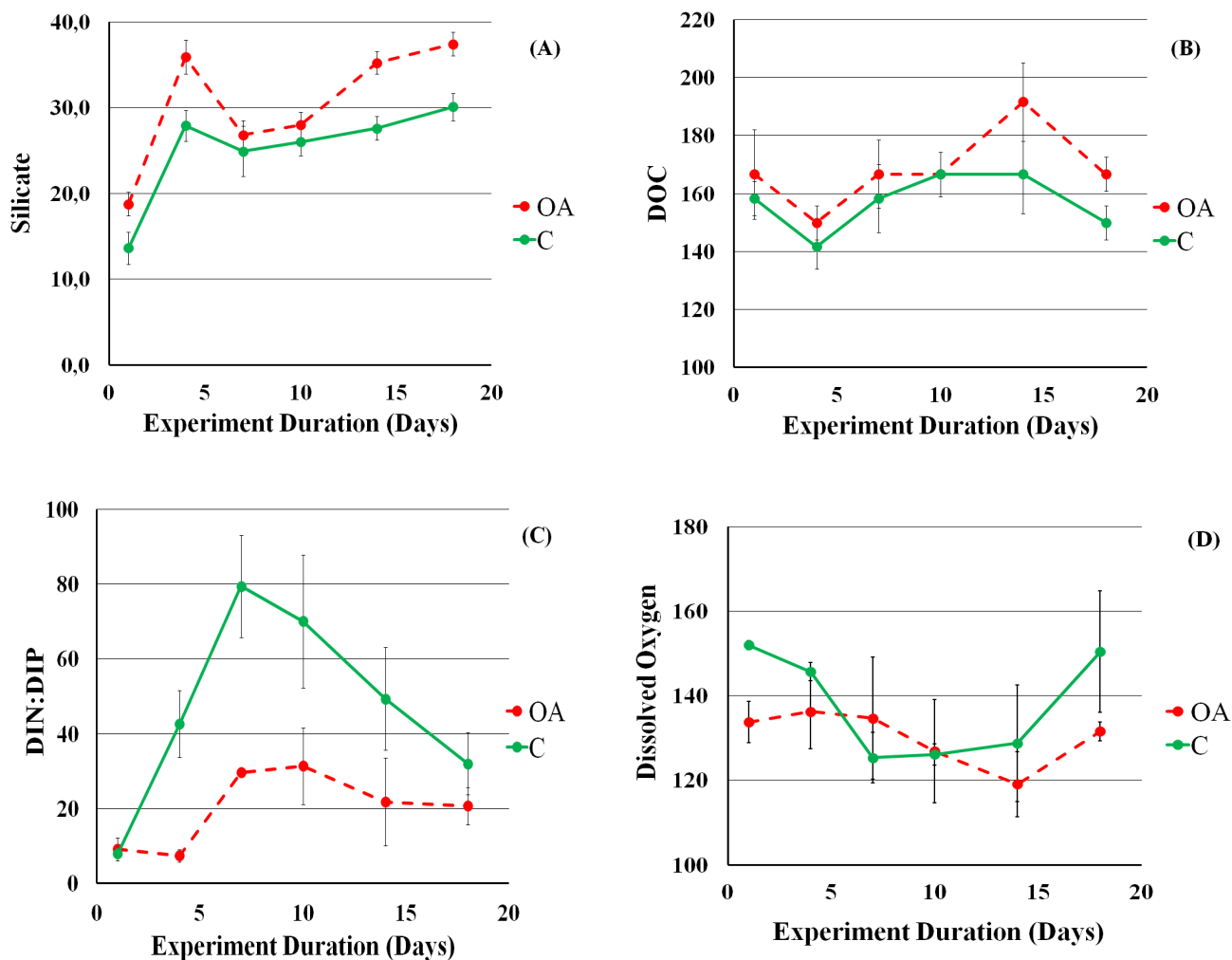


Fig. 6: Silicate, DOC and DO concentrations (in $\mu\text{mol kg}^{-1}$), and DIN:DIP molar ratio for the duration of the experiment ; mean values and standard deviations for the two replicates of each treatment (OA: Ocean Acidification condition, C: Control condition).

(2.35%) and showed a decrease throughout the experiment for both conditions ($1.77 \pm 0.14\%$, 1.67 ± 0.25 for OA and C respectively). The sulfur content was found $0.80 \pm 0.01\%$ with no variations during the experiment. The sediment nitrogen content (TN) for field samples was found $0.27 \pm 0.02\%$; for OA treatment TN varied between $0.20 \pm 0.01\%$ and for C treatment $0.19 \pm 0.02\%$. The phosphorus sediment content (TP) for field samples was found $0.07 \pm 0.02\%$ with negligible variations for both treatments (Table 3). The one-way ANOVA results, showed no statistically significant difference of the sediment characteristics (OC, CaCO_3 , TN, TP and S) between the two treatments.

Principal Components Analysis (PCA)

PCA for the C treatment explained 86.9% of variation in the first two principal components (Fig.7A). The first axis (PC1) explained 61.6% of total variance and was positively related to A_T (0.94), DOP (0.89), $p\text{CO}_2$ (1.00), HCO_3^- (1.00) and DIC (1.00) and was negatively related

to pH (-0.98), NH_4^+ (-0.81) and CO_3^{2-} (-0.96). The second principal component (PC2) explained 25.3% of total variation and was positively correlated with NO_2^- (0.92), DIN:DIP (0.98), DIN (0.87), DO (0.86), and NO_3^- (0.81) and was negatively correlated with PO_4^{3-} (-0.98).

PCA for OA treatment explained 70.1% of variation in the first two principal components (Fig.7B). The first component (PC1) explained 40.9% of total variance and was positively related with A_T (0.99), HCO_3^- (0.99), DOP (0.88), NH_4^+ (0.86), DIC (0.85), and was negatively related to PO_4^{3-} (-0.96). The second component (PC2) explained 29.3% of total variance and was only negatively correlated with $p\text{CO}_2$ (-0.90).

Discussion

This study, which was based on a microcosm lab simulation, was a first overall assessment of basic biogeochemical interactions/alterations of nutrients and carbon that are taking place in a restricted marine system under the emerging effects of coastal pH decrease. Previous

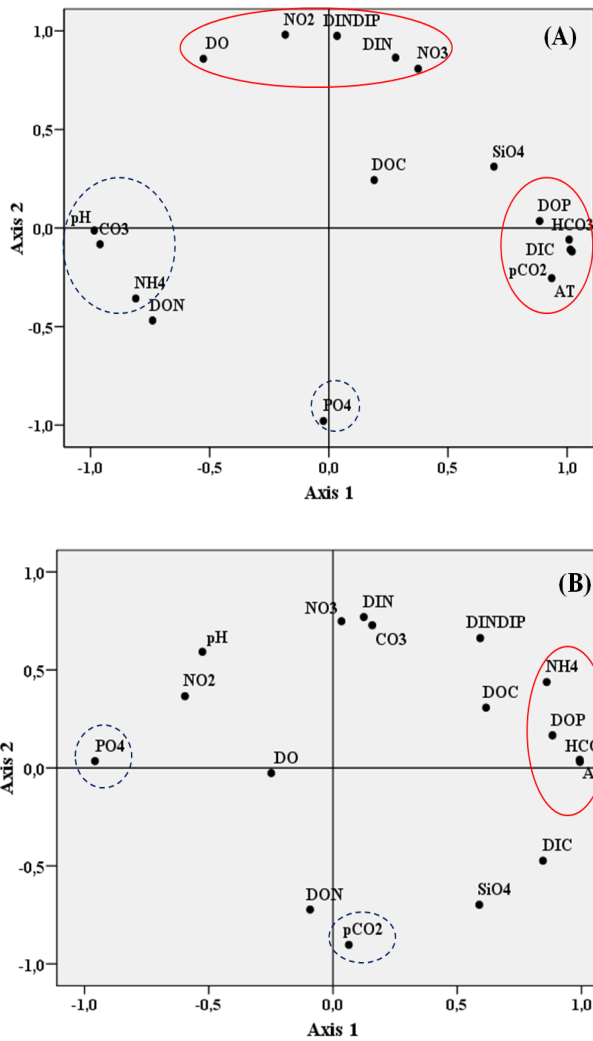


Fig. 7: Principal Component Analyses (PCA) graphs for (A) Control (C) and (B) Ocean Acidification (OA) treatments. Red circles indicate positive significant correlations while blue circles indicate negative significant correlations.

Table 3. Sediment analyses (OC, CaCO₃, TN, TP and S in % on dry weight) for OA (Ocean Acidification) and C (Control) experimental conditions; mean values and standard deviations for the two replicates of each treatment.

		OC	TN	TP	CaCO ₃	S
		%	%	%	%	%
	field	2.35	0.28	0.07	46.8	0.80
OA	mean	1.67	0.19	0.05	49.7	0.81
	stdev	0.14	0.01	0.03	3.00	0.08
C	mean	1.84	0.20	0.04	45.8	0.79
	stdev	0.25	0.02	0.02	1.8	0.07

studies were focused either in predicting the effects of future pH reduction or investigating the biogeochemical outcomes of coastal pH decline on certain organisms' response/survival or merely on the processes ultimately determining CO₂ fate and impact in aquatic systems (e.g. Andersson *et al.*, 2007; Kitidis *et al.*, 2011; Widdicombe *et al.*, 2009; Laverock *et al.*, 2014). Marine systems however, constitute of specific features, more complex than simple chemical or thermodynamic equilibria which are necessary, nonetheless, for any calculation or prediction model. So this experimental study tries to fill this gap by examining the co-evolution of basic geochemical processes.

Carbonate system processes - Evidence of alkalinity generation

Following the *in situ* DO measurements (99.45 μmol kg⁻¹), the experiment for both C and OA treatments was conducted under low DO conditions, characterized as mild hypoxic based on the threshold of 107 μmolO₂ kg⁻¹ cited by Hofmann *et al.* (2011).

In C condition, carbonate ions decrease throughout the experiment with a 50% decline in final values; carbonate mineral saturation states also present the same trend. Total alkalinity, DIC and bicarbonate ions slightly increase in this case. Although CO₂ was periodically injected in C condition tanks in order to maintain a stable pH value, CO₂ concentration and pCO₂ exhibit considerable increase from the beginning till the end of the experiment. The naturally occurring respiration and organic matter degradation as well as the modification of the equilibria between the different forms of dissolved inorganic carbon with an increase of the proportion of both CO₂ and bicarbonate ions at the expense of carbonate ions are the most likely processes to explain the observed trends. The carbonate precipitation would also be suggested as possible explanation of the carbonate ions decrease however, is not depicted on the sediment carbonate content, neither is supported by the declining trend of carbonate mineral saturation states.

In OA condition, an immediate decrease in carbonate ions in relation to C condition is found at the beginning with stable concentrations till the end of the experiment. These variations lead to Ω_{ar} and Ω_{cal} values below 1, while in sediments no differentiations were observed. The system response to CO₂ addition was immediate for DIC which was augmented probably as the combined result of decaying phytoplankton cells and zooplankton carcasses present in the experimental tanks in addition to system response to CO₂ addition. Total alkalinity and bicarbonate ions amplification followed as expected.

A significant increase of 90% was observed for A_T with a corresponding DIC increase of 120%, making clear that other mechanisms besides the carbonate system equilibria affect alkalinity. The enhanced A_T in more acidified conditions is generally attributed to carbonate minerals dissolution from sediment (Andersson *et al.*, 2007; Kru-

mins *et al.*, 2013). However, no noticeable decrease of sedimentary carbonates was found here. This is not surprising if the high CaCO_3 content of the sediment, as well as the relatively short duration of the experiment, are considered. Assuming that the observed A_T increase ($\Delta A_T = \sim 2500 \mu\text{mol kg}^{-1}$) in the overlying water was generated by the dissolution of sedimentary CaCO_3 , then the corresponding change in inorganic carbon percentage of the sediment would be about 0.002% w/w that is much lower than the methodological error of the particulate inorganic carbon determination and thus cannot be safely detected. However it seems very likely that the pH decline in the overlying water has led to an analogous shift of pH in sediment porewater that induced the development of under-saturated conditions for calcium carbonate minerals in pore waters leading to dissolution of CaCO_3 and DIC and A_T generation. Unfortunately, as no pH, A_T and/or DIC data were acquired for the sediment porewater during this study, it is impossible to assess the response of the sediment to pH changes in porewater and evaluate the contribution of this process to the observed ΔA_T . A previous study in Saronikos Gulf has shown that under dark conditions DIC efflux from the sediment towards the water column takes place that is negatively related with DO suggesting mineralization processes (Apostolaki *et al.*, 2010).

It has to be noted that variations in the overlying water A_T are not only due to carbonate dissolution; several other biogeochemical processes could contribute to the A_T increase that was recorded in the current study. Important alkalinity generation (benthic alkalinity) in shallow sediments due to anaerobic degradation of disposed organic matter has been well reported (Thomas *et al.*, 2009; Krumins *et al.*, 2013; Ingrosso *et al.*, 2016a). Remineralization of particulate organic matter and subsequent nutrient release directly affect A_T , depending on the reactive nitrogen species produced (e.g. 1 mole of ammonium or nitrate release leads to an increase or decrease, respectively, of A_T by 1 mole; Hernández-Ayón *et al.*, 2007). During more acidified conditions, in this study, the steady increase of ammonium over time along with the respective decrease in nitrate and nitrite concentrations could contribute positively to A_T levels. Moreover, recent estimates of alkalinity fluxes from coastal marine sediments underline the significant contribution of net sulfate reduction to alkalinity generation (up to 70-82%; Krumins *et al.*, 2013), especially in sediments in highly productive and/or oxygen-depleted coastal waters (e.g. in the Black Sea; Barker Jorgensen & Kasten, 2006). It is definite that organic matter degradation and nutrient related processes (e.g. denitrification) could not be solely responsible for the significant A_T increase reported here. During organic matter oxidation, when the available oxygen diminishes, respiration processes retreat while sulfate and Fe reduction take over; sulfate reduction producing hydrogen sulfide followed by Mn and Fe reduction result in bicarbonate generation which in turn increase total alkalinity (Krumins *et al.*, 2013; Hu & Cai, 2011; Hulth *et al.*, 1999).

PCA performed on the C treatment results revealed the effect of pH change on carbonate system parameters primarily, along with ammonium and DOP (PC1), as well as nitrogen and phosphorus species transformations through organic matter degradation and denitrification, as a result of oxygen availability (PC2). The same analysis performed on OA treatment results showed the strong impact of OM degradation, ammonium, organic phosphorus and bicarbonates on A_T , whereas $p\text{CO}_2$ even in excess was a factor that had minor effect on the system. The fact that under more acidified and low oxygen conditions, only OM degradation and bicarbonate ions are correlated in the first component could also be an indication of sulfate reduction processes releasing considerable amounts of bicarbonates. Through net denitrification and net sulfate reduction, nitrate and sulfate transfer their negative charge to HCO_3^- by oxidizing organic carbon producing the anion which contributes to DIC and alkalinity (Hulth *et al.*, 1999; Hu & Cai, 2011; Krumins *et al.*, 2013). High values of hydrogen sulfide measured *in situ* at the near bottom water layer support the sulfate reduction scenario (Pavlidou & Hatzianestis unpublished data). Bicarbonate, sulfide, ammonium and phosphate concentrations utterly contribute to the final A_T concentration (Dickson *et al.*, 2007). As it appears here, these chemical species affect A_T to a larger extent under lower pH values; from the PCA analysis for OA treatment, ammonium, DOP, HCO_3^- , and DIC were found to contribute strongly to A_T , while phosphate seem to have a negative feedback on it. Normally, phosphate contribute positively to A_T by definition, which indicates that in more acidified conditions with lower available oxygen, a strong complex of phosphate is formed with other substances (e.g. metals or carbonates) which in turn seem to be a reducing factor in the final A_T budget (Ge *et al.*, 2016). Furthermore, it has been shown that in areas with restricted mixing, and/or characterized by appreciable autotrophic DOM production and/or significant inputs of DOM from land, organic bases contribute significantly to A_T (Hernández-Ayón *et al.*, 2007; Kulinski *et al.*, 2014). Accumulation of DOM in the near-bottom layer of Elefsis Bay, which is enriched in phosphorus relatively to carbon and nitrogen, has been reported during conditions of stratification (Pavlidou *et al.*, 2013) which could be a further contribution to the system alkalinity.

Processes affecting organic carbon and nutrients

Considering that the basic biogeochemical processes were investigated, the normal mechanisms of the specific study area could be summarized as those pointed out for C condition. In C condition the DON appeared to convert to ammonium which in turn was consumed and rapidly oxidized to $\text{NO}_2^-/\text{NO}_3^-$; this process reached a plateau in the middle of the experiment after which $\text{NO}_2^-/\text{NO}_3^-$ were no longer produced (Fig. 4). Nitrite and nitrate, were favoured against ammonium in this case throughout the experiment, indicating nitrification to be the main prevailing

process. In addition, sedimentary TN decreased indicating dissolution into soluble forms; ammonium and nitrite efflux from the sediment to the water column have been reported for the specific area (Rousselaki *et al.*, 2014).

In OA condition, after the 5th day of the experiment, DON degradation was constrained, in relation to C condition (Fig. 4D). In parallel, the originally existing ammonium was no longer oxidized and as the experiment proceeded, ammonium accumulated while nitrate production declined, implying reduced nitrification processes. It has been reported that in Elefsis Bay sediment acts as a source of ammonium to the overlying water, under specific conditions (e.g. hypoxia; Rousselaki *et al.*, 2014). Additionally, OA was previously found to cause ammonium efflux from fine-grained sediment towards water (Widdicombe *et al.*, 2009); OA can also reduce nitrification rates, reducing oxidized nitrogen supply in the upper water layers (Beman *et al.*, 2011). The increased N-content, under lower pH with higher dissolved organic N forms pointed out here, indicates inhibited organic N mineralization processes and a possible N-buildup. The suppression in ammonium oxidation mechanism followed by decrease of nitrate production and subsequently ammonium accumulation, is in consistency with already published studies showing a deceleration of the ammonium oxidation mechanism as pH decreases (Beman *et al.*, 2011 and references therein). Efflux of nitrate and nitrite has been previously reported under lower pH values (Widdicombe *et al.*, 2009) depending on sediment granulometry. The signal of TN release from the sediment is not depicted in the concentrations of nitrite and nitrate, possibly indicating a denitrification mechanism, producing N₂ gas (and small amounts of N₂O) which likely escaped from the system. Phosphate presents similar trend with C condition (Fig. 5A), with negligible variation up to the 10th day. No significant acidification impact was observed in phosphate and silicate, as has been reported in previous studies (Widdicombe *et al.*, 2009; Tanaka *et al.*, 2008).

The concentration of the sedimentary organic carbon drops by 20% in C condition and by 30% in OA condition at the end of the experiment, as a result of the first step of its degradation, fueling the DOC pool of the overlying water that exhibits a 20% increase in DOC concentration in both cases. DOC was found relatively high throughout the experiment in both treatments (Fig. 6B). The final higher DOC concentrations observed for OA condition, although not statistically different from those in C condition, could implicate inhibition of dissolved organic matter decomposition under lower pH values. The limitation of DOC decay could be attributable to the reactivity of the compounds produced in the first step of organic matter degradation during which particulate organic carbon is converted to dissolved forms. The produced compounds may be resistant to further degradation, due to their inherent stability or as a result of abiotic reactions that protect the molecule from enzymatic attack (Hee *et al.*, 2001). Despite any indications revealed here, no similar published results were found; earlier experiments, con-

ducted under CO₂-perturbed conditions that also included the sediment phase, have not examined the features and fate of DOC released by the sedimentary organic carbon degradation. However, recent results from mesocosm experiments in the Mediterranean that examined the effect of increased *p*CO₂ and/or nutrient concentrations on dissolved organic matter dynamics 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 *p*CO₂ conditions (Aparicio *et al.*, 2016) influencing the organic matter lability. Furthermore, experimental observations demonstrated that at lower pH, dissolved organic molecules are becoming more condensed being thus less susceptible or even refractory to biodegradation (Chin *et al.*, 1998).

The DIN:DIP ratio was found decreased in more acidified conditions (Fig. 6C); since no prominent variation was observed in phosphate concentrations, the DIN reduction is considered to be the main factor affecting the DIN:DIP ratio. The decreased DIN:DIP ratio, the prevalence of organic nutrient species against the inorganic ones, the observations of constrained DON degradation and nitrate production decline and the higher DOC concentrations under lower pH values can also support inhibition of organic matter decomposition.

Implications for enclosed embayment in future CO₂ conditions

The results of this study showed that the near bottom waters of Elefsis Bay during June were characterized by mild hypoxic conditions (Hofmann *et al.*, 2011) and presented increased acidity (7.85 in NBS scale) in relation to typical surface values (pH≈8.1). In some of the oldest studies regarding Elefsis Bay (June 1975; May-June, 1977), surface pH values of 8.4-8.5 have been reported along with seasonal near bottom pH decrease reaching values of 7.9-7.8 (all in NBS scale; Scoullou, 1979; Friligos, 1982); this pH difference between surface and near bottom waters has been solely pointed out through individual studies and unfortunately systematic high-frequency pH measurements were never performed. The bottom pH nowadays (7.85, June 2014) was found to be at the same level with values reported about 40 years ago for corresponding time periods, implying that the area has been well affected by the seasonal pH decline manifoldly and in quite unpredicted pathways. Elefsis Bay is a restricted coastal system with intensive urban and industrial activities, receiving increased N and C inputs, which enhance biological primary productivity and promote subsequent organic matter decomposition. The dramatic DO decrease during stratification along with enhanced CO₂ production and more elevated *p*CO₂ values caused by OM degradation, induce non-equilibrium between bottom and surface layers.

Total alkalinity in Elefsis bottom water (2769 μmol kg⁻¹) was found markedly higher to the values reported recently for open Eastern Mediterranean Sea waters

(2600 $\mu\text{mol kg}^{-1}$; Hassoun *et al.*, 2015). This value is similar to those reported for the deep layer of the Gulf of Trieste in the northern Adriatic Sea that is a coastal system with shallow depths and seasonal stratification (e.g. Cantoni *et al.*, 2012; Ingrosso *et al.*, 2016a). This enhanced alkalinity first of all reflects the alkalinity input in Mediterranean coastal areas by the weathering of the land limestones; additionally, this study revealed the significant contribution of nutrient species and possibly of sulfate reduction in total alkalinity (Section 4.2). The penetration of anthropogenic CO_2 into the Mediterranean has led to lower saturation degree of calcium carbonate in relation to the preindustrial era (Hassoun *et al.*, 2015). During the period 1967–2003, the estimated Ω_{ar} in a coastal site in the western Mediterranean displayed a decreasing trend and fluctuated between the highest value of 4.3 observed in 1968 and a minimum of 3.1 in 2003 (Howes *et al.*, 2015); the calculated Ω_{ar} in the bottom waters of Elefsis Bay was found 2.19. Similar low Ω_{ar} values have been recorded in the deep layer of the Gulf of Trieste, under the occurrence of strong remineralization processes during summer (Cantoni *et al.*, 2012). Considering that the anoxic layer in Elefsis Bay is an intermittent feature that is developed annually from depths >15 m (Scoullou & Riley, 1978; Scoullou, 1983; Pavlidou *et al.*, 2013) and that the same processes promoting hypoxia also acidify the water column (Wallace *et al.*, 2014) during stratification periods, it would be expected to detect more increased DIC and CO_2 concentrations, higher $p\text{CO}_2$ (probably above 1000 μatm) and eventually even lower pH values. Concomitantly, the water column would be more corrosive in respect to aragonite and calcite, an effect relevant to what is expected in the future due to OA, but with undefined impacts on the ecosystem. Relevant findings were reported for the Gulf of Trieste (Cantoni *et al.*, 2012) where the intense remineralization of organic carbon in its deeper waters releases CO_2 , leading to $p\text{CO}_2$ values up to 1043 μatm during hypoxic periods. Water column mixing, drives the penetration of these increased amounts of CO_2 to the surface waters, resulting in their supersaturation, and eventually emitting carbon dioxide to the atmosphere (Ingrosso *et al.*, 2016b).

When hypoxic conditions characterize the marine ecosystem, both nitrification and denitrification processes can occur (Cai *et al.*, 2011) and since nitrite and nitrate both serve as substrates for denitrifying bacteria, it is possible that an inhibition of nitrification could result in a decrease of denitrification process as well (Huesemann *et al.*, 2002). Denitrification and nitrate reduction to nitrite are typical processes taking place during June in Elefsis bottom waters that are becoming evident from the decrease and disappearance of nitrate and the low values of oxygen (Friligos & Barbetseas, 1990; Pavlidou *et al.*, 2013). Moreover, the ocean acidification would decrease ammonium conversion to nitrate through nitrification processes and eventually would deplete nitrate supply in surface waters. In this way, smaller phytoplankton or-

ganisms, such as dinoflagellates will be favored, against diatoms which show a preference for growth on nitrate (Dortch, 1990), possibly triggering the development of HABs (Harmful Algal Blooms; Huesemann *et al.*, 2002; Beman *et al.*, 2011). In Elefsis Bay, the occurrence of HABs is sporadic in time and recurrence of the causative species (Ignatiades & Gotsis-Skretas, 2010). Eventually, more acidified conditions could impact the processes determining the final N-species available for biological consumption, a shift which is controlled by a series of biological and physical interactions including global climatic changes (Moncheva *et al.*, 2001).

Conclusions

This experimental study was a quite integrated simulation that examined the response, alterations and interactions of nutrients and carbon biogeochemistry under the emerging effects of coastal pH decrease. More acidified conditions have been previously reported in coastal areas characterized by temperature-induced stratification and increased inputs of OM and nutrients through anthropogenic activities and which have similar features with the Elefsis Bay. It is a fact that the ‘hot, sour, and breathless’ conditions predicted for the future open ocean (Gruber *et al.*, 2011) can already be found in today’s coastal zones during summer, and especially within the close-to-the-bottom layer where pH and DO levels are generally lower than the upper water column (Wallace *et al.*, 2014). Normally anthropogenic CO_2 is the major driver of OA in open ocean; in shallow coastal areas, this driver may have a minor role as multiple human activities operating at various spatial scales affect the carbonate system equilibria. The increase of anthropogenic nutrient and organic matter inputs by rivers, groundwaters, and atmosphere (eutrophication) is considered a significant driver of enhanced coastal acidification, especially in hypoxic bottom waters (Borges & Gypens, 2010; Strong *et al.*, 2014). The main differentiation between the evolution of acidification in coastal zone and in open ocean is that in the former human actions influence the coastal ocean carbonate system through an indirect mechanism which in addition has a strong bottom to surface orientation. Despite this significant discordance, both mechanisms are expected to present similar final outcomes in coastal systems’ biogeochemistry.

It has been demonstrated that under more acidified conditions, significant alkalinity release occurred, whereas the water became undersaturated in both carbonate minerals, namely aragonite and calcite. This alkalinity increase was partially associated with sedimentary carbonate dissolution and the reactive nitrogen species shift towards ammonium. In addition there is evidence that organic compounds containing basic functional groups originating from the DOM pool and sulfate reduction processes would contribute to the alkalinity production during the experiment.

The ammonium production by the organic matter degradation and the subsequent ammonium oxidation normally occurring under hypoxic conditions, are intercepted in more acidified conditions. The decline of nitrate and nitrite in parallel with ammonium increase, indicate a deceleration of the ammonium oxidation processes along with decrease in nitrate production. These could lead in a decline of nitrification mechanisms that could eventually affect the phytoplanktonic community composition and the relative species abundances. Phosphate and silicate were not affected by the further pH decline. Organic forms of N and P along with DIN:DIP ratio during more acidified conditions imply possible N-limitation and inhibition of organic matter decomposition.

Although Elefsis Bay is one of the most studied areas in Greece, there is a sparseness of recent pH measurements. The pH decline reported in this study, however, has been documented since the 1970's; the weak water mass renewal, in combination with organic load and high biological production, result in the entrapment and recycling of a large amount of organic matter (Pavlidou *et al.*, 2013). The microbial decomposition of this organic matter has caused a local bottom increase in CO₂ concentrations causing carbonate system alterations similar to the future predictions for global ocean acidification. This CO₂ release by respiratory processes, along with the development of hypoxia and anoxia has altered decidedly the biogeochemical cycling of carbon and nutrients in the subpycnocline waters of Elefsis Bay; upward spreading of these waters would have potentially important consequences in the biogeochemistry of the upper pycnocline layer.

Currently, OA is not the main factor affecting the Elefsis Bay but long-term trends in pH, resulting from increased prevalence of bottom-water hypoxia is known to be substantial compared to the pH trend resulting from anthropogenic CO₂ acidification, in coastal waters (Hagens *et al.*, 2015). It has also been suggested that the eutrophication-induced hypoxia could amplify the susceptibility of coastal waters to ocean acidification (Cai *et al.*, 2011), thus making the coastal realm most vulnerable to ecological and biogeochemical perturbations.

The key findings of this study may contribute to future research efforts regarding the carbon and nutrient cycling in relation with increasing atmospheric CO₂ in intermittently hypoxic/anoxic coastal systems of the Mediterranean Sea. However, in such highly variable coastal environments high-frequency monitoring of the marine carbonate system is essential in order to document and interpret the long-term trends in inorganic carbon dynamics and regional ocean acidification. In parallel, detailed studies of other biogeochemical parameters accompanied by properly designed experiments are needed to improve our understanding of the factors that regulate the carbonate system and elucidate the possible impact of the increasing CO₂ on the complex biogeochemical processes taking place in coastal areas.

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