Growth impacts of Saharan dust, mineral nutrients, and CO_2 on a planktonic herbivore in southern Mediterranean lakes

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HIGHLIGHTS

- Impact of multiple global stressors on *Daphnia* growth rates was investigated in Mediterranean lakes.
- No single effect of rising CO_2 was detected unless supplemented with Saharan dust or inorganic nutrients.
- These factors affected the growth of Daphnia via alteration in the quantity of its food.
- CO_2 effects on herbivores are expected to intensify as the Mediterranean region becomes dustier.

GRAPHICAL ABSTRACT
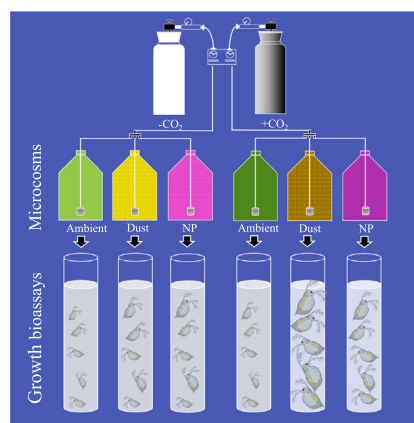
ABSTRACT

Rising levels of CO_2 can boost plant biomass but reduce its quality as a food source for herbivores. However, significant uncertainties remain as to the degree to which the effect is modulated by other environmental factors and the underlying processes causing these responses in nature. To address these questions, we carried out CO_2-manipulation experiments using natural seston from three lakes under nutrient-enriched conditions (mimicking eutrophication and atmospheric dust-input processes) as a food source for the planktonic *Daphnia pulicaria*. Contrary to expectations, there were no single effects of rising CO_2 on herbivorous growth. Instead, synergistic $CO_2 \times$ nutrient interactions indicated that CO_2 did not support higher zooplankton growth rates unless supplemented with dust or inorganic nutrients (nitrogen, N; phosphorus, P) in two of three studied lakes. The overall positive correlation between zooplankton growth and seston carbon (C), but not seston C:P, suggested that this was a food quantity-mediated response. In addition, we found that this correlation improved when the data were grouped according to the nutrient treatments, and that the response was largest for dust. The synergistic $CO_2 \times$ nutrient effects reported here imply that the effects of rising CO_2 levels on herbivorous growth may be strongly influenced by eutrophication processes and the increase in dust deposition predicted for the Mediterranean region.

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1. Introduction

Carbon dioxide (CO_2) increase in the atmosphere is perhaps the most notorious global-change anomaly, from around 230 ppm in preindustrial times to over 400 ppm today and, based on the model used for future projections, its concentration is expected to reach 750 to 1000 ppm in the next hundred years (IPCC, 2013). Studies on the influence of atmospheric CO_2 on aquatic ecosystems have shown that CO_2 addition promotes the growth (Kim et al., 2013) and production of phytoplankton (Jansson et al., 2012), as less light energy is required to operate the carbon-concentrating mechanisms (Hopkinson et al., 2011). However, various adverse effects has also been reported. These include the pH decline of surface ocean waters by >0.1 units since the beginning of Industrial Revolution (Orr et al., 2005). Increased pCO_2 levels in aquatic environments can shift the carbonate buffer system towards more acidic conditions, which can affect carbonate and bicarbonate chemistry and ultimately alter the structure of phytoplankton communities due to differences in the competitive ability of species (Shi et al., 2017) and their susceptibility to lower pH values (Hurd et al., 2011; Kroeker et al., 2013). Also higher pCO_2 levels can alter food quality for herbivores by rising the C:N:P ratios of autotrophs (Riebesell et al., 2007; Verschoor et al., 2013). Thus, some studies have reported a decrease in the growth rate of herbivores with monospecific diets in response to rising CO_2 levels (Urabe et al., 2003; Schoo et al., 2013), although these adverse effects were mitigated when herbivores were offered multialgal diets (Urabe and Waki, 2009). While these studies provide valuable perspectives on the food-quality effects of CO_2, it remains unassessed whether these findings can be translated to the outside world. This is because in experiments using natural seston mixtures (living and nonliving suspended matter in a water body), as opposed to controlled assays with single or multialgal diets, not all carbon (C) is equally edible for zooplankton. For example, *Daphnia* are filter feeders that ingest algae and detritus, but also bacteria which can be an important source of nutrients due to the flexibility in their P content with up to 3% of bacterial dry mass (Godwin and Cotner, 2015). In addition, most studies that investigate the effects of CO_2 have been conducted under *ad libitum* food concentrations and, therefore, do not separate effects of food quality from those of food quantity that are likely to prevail in low- to medium-trophic conditions. Lastly, the influence of CO_2 on herbivores is most often studied independently, whereas the interactive effect of CO_2 with other factors is noticeably lacking.

From this ecological background, it is becoming increasingly clear that realistic studies on the effects of increased CO_2 not only need to consider natural seston mixtures but also the effect of multiple interactive factors. For example, there is evidence showing that nutritional conditions of phytoplankton determine their response to rising CO_2 (Verspagen et al., 2014). Higher levels of nutrients might be a consequence of anthropogenic eutrophication processes induced by the discharge of limiting nutrients contained in detergents, sewage or fertilizers into the aquatic ecosystems. At the same time, human activities are also affecting the Earth's nutrient budgets by altering atmospheric concentrations of key gases and bulk dust aerosols (Sala et al., 2000; Hartmann et al., 2013). Mineral dust particles are produced mainly by the disintegration of aggregates following creeping and saltation of larger soil particles over deserts and other arid surfaces (Koch, 2001; Zhao et al., 2006). Dust aerosols not only exert a major radiative effect on the Earth's energy balance, but also constitute major vectors for nutrient transfer (e.g., iron [Fe], P or N) between distant worldwide regions, affecting the productivity of ecosystems (Herut et al., 2002). Thus, P deposition due to increased dust and biomass burning emissions has increased by 1.4 times the preindustrial rate (Brahney et al., 2015). The Sahara and the Sahel are by far the largest sources of dust particles (Prospero and Lamb, 2003), contributing ~56% of the Earth total dust emissions (Jickells and Moore, 2015), and particularly in the nearby Mediterranean basin where the occurrence and intensity of desert dust episodes are increasing (Bullejos et al., 2010; Gkikas et al., 2013).

However, the role of dust on ecosystem productivity is still controversial (Gallisai et al., 2014). Some experimental studies have shown that mineral-dust inputs can strongly stimulate the primary production of P-limited communities (González-Olalla et al., 2017), boosting the biological pump and reinforcing the role of oligotrophic areas as key CO_2 sinks (Cabrerizo et al., 2016). In contrast, other studies have reported negative effects of dust inputs on phytoplankton growth due to the presence of toxic microelements such as copper (Paytan et al., 2009; Jordi et al., 2012). In addition, the impact of dust deposition on microbial plankton can be quite heterogeneous as a result of the uniqueness in the intensity (Gallisai et al., 2016) and composition of aerosols (anthropogenic versus mineral; e.g. Marín et al., 2017).

There is increasing awareness among scientist that the effect of multiple factors are key to understand how species and biological interactions respond to all the stressors that are acting on an ecosystem at a given time (Villar-Argaiz et al., 2018a). So far, there is empirical evidence showing that CO_2 and nutrients interactively impact the growth and elemental content of primary producers (Verspagen et al., 2014), but the extent of their joint effect to the next trophic level of herbivores in nature is quite unexplored. Finally, the overall net effects of dust, specifically its interactive effect with CO_2 on zooplankton are largely unknown (Christou et al., 2017). To examine the single and interactive effects of CO_2, Saharan dust and mineral nutrients on herbivorous growth, we designed a series of microcosm experiments composed of two steps. In the first, we exposed natural seston from three southern Mediterranean lakes to the effects of CO_2 (ambient and elevated levels), dust, and inorganic nutrients. In the second, we tested the nutritional suitability of the raised seston on the growth of the cladoceran *Daphnia pulicaria* as a target species. The following specific hypothesis were tested: (1) the single effect of CO_2 enhances the growth rate of *D. pulicaria* and, (2) inorganic nutrients and dust synergistically increase the role of CO_2 on *D. pulicaria* growth rate. The experimental manipulation proposed here should provide with relevant information on how future scenarios of augmented eutrophication and dust might offset or enhance CO_2 effect on planktonic food webs.

2. Material and methods

2.1. Study sites

This study was conducted in three Mediterranean lakes in southern Spain. Two of these lakes (Dulce [37°3′10.96″ N, 4°50′6.78″ W] and Archidona [37°6′31.87″ N, 4°18′9.20″ W], province of Malaga) are hard-water lakes with high carbonate/bicarbonate content. However, while Lake Dulce is a temporary shallow lake that dries out during the summer period, Archidona is a permanent, deeper lake. The third lake (Cogollos [37°12′29.47″ N, 3°10′1.06″ W], province of Granada) is a low-alkalinity lake located on a siliceous bedrock. The three lakes covered a moderate trophic gradient according to their Total Phosphorus (TP) content from the oligotrophic Lake Cogollos to the mesotrophic Lake Dulce (TP: 15.2–39.8 μg P L^{-1}).

2.2. Field sampling

These lakes were sampled in 2014 (Lake Dulce in May 27; Lake Archidona in June 2) and 2015 (Lake Cogollos in March 1) at a station located at the maximum depth of each lake. Vertical profiles of radiation (UVR at 305, 320, and 380 nm and photosynthetically active radiation, PAR) were measured at noon using a submersible BIC compact 4-channel radiometer (Biospherical Instruments Inc., San Diego, CA, USA). Diffuse attenuation coefficients for downward irradiance (k_d) were determined from the slope of the linear regression of the natural logarithm of downwelling irradiance vs. depth for each wavelength range considered ($n > 200$ per profile). Vertical profiles of pH and temperature were measured using a multi-probe system (YSI MPS-556, YSI Incorporated, OH, USA). A representative water-column sample for each

lake was constructed from equal volumes of water collected with a 6-L horizontal Van Dorn sampler from three depths within the water column (0.5, 1.5 and 2.5 m in Lake Dulce; 0.5, 5, and 9 m in Lake Archidona; and 0.5, 1.5 and 2 m in Lake Cogollos). This composite lake sample was pre-filtered through a 45 μm mesh to remove zooplankton and transported in a 75-L covered container to the laboratory.

2.3. Microcosm experiments

Water from the 75-L container was transferred to six 8-L polycarbonate microcosms in the laboratory (Fig. 1). Microcosms were incubated at a similar temperature to that of the lake water (20 ± 1 °C in Dulce and Archidona, and 12 ± 1 °C in Cogollos), with photosynthetically active radiation (~115 μmol photons m^{-2} s^{-1}) in a 14 h light: 10 h dark cycle. Microcosms were manipulated to assess the single and interactive effects of CO_2, Saharan dust and nutrients using a 2 × 3 factorial experiment with: (1) two CO_2 levels, lake water pCO_2 (hereafter −CO_2), and elevated lake water pCO_2 (hereafter +CO_2), and (2) three nutrient levels, nutrient ambient conditions at the sampling moment (hereafter Amb), enrichment with Saharan dust at a final concentration of 30 μg P L^{-1} (hereafter Dust), and inorganic nutrient enrichment mimicking the input of P adsorbed to dust (hereafter NP) (Fig. 1). Microcosms were not replicated as they were run only to raise the seston that

was subsequently used to test herbivorous growth rates in fully replicated bioassays that pursued true estimates of error (Hurlbert, 1984). To achieve −CO_2 conditions, half of the microcosms were aerated with standard air using an air pump to provide natural atmospheric partial pressure of ca. 400 ppm. For +CO_2 conditions, half of microcosms received aeration from a gas tank containing a mixture of gases resembling atmospheric air with a high partial pressure of 750 ppm CO_2 to accommodate with predictions of the RCP8.5 scenario of the Intergovernmental Panel on Climate Change (IPCC, 2013). The microcosms were aerated for 10 s every 60 s as recommended by Urabe and Waki (2009), using a time-controlled electrovalve. The Saharan dust was collected *in situ* in the Moroccan region of Merzouga (Tafilalet, Morocco; 31°06′00″ N, 3°59′24″ W). Before addition, the soil was sieved with a nested column with wire mesh cloth of 1 mm and 100 μm and dust was collected on a pan underneath the nest of sieves. The collected particles were then winnowed near a tilted glass, and particles adhering to the glass were gently collected with a fine brush. The size of the collected sample ranged between 1 and 10 μm (Leitz Fluovert FS, Leica, Wetzlar, Germany), which are in the range of the mean particle size recorded in heavy deposition events of the Mediterranean region (Guieu et al., 2010; Pey et al., 2013). The sealed plastic containers and glass in contact with the soil were previously cleaned using 0.2 M HCl acid bath and Milli-Q water. The amount of dust added was calculated
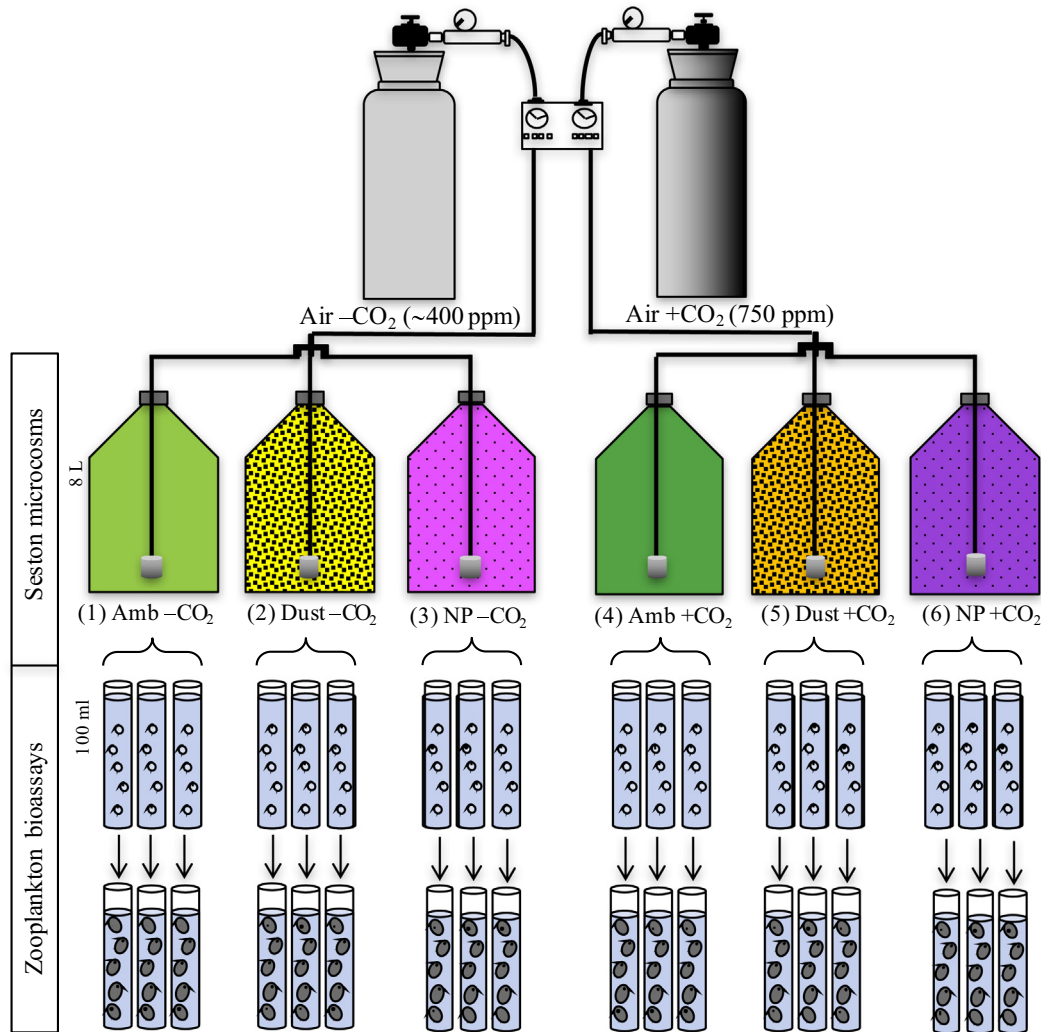

Fig. 1. Scheme illustrating the experiments conducted in the three lakes studied. Grey color denotes air enriched with CO_2. Microcosms were not nutrient amendment (green colored bags, Amb), received dust (yellow-orange bags, Dust) or inorganic nutrients (purple bags, NP), completing 6 treatments indicated by numbers in brackets. The zooplankton bioassays were performed in triplicate for each of the 6 treatments, yielding a total of 18 zooplankton glass tubes with 5 daphnids per tube. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

according to previous P-release experiments in the laboratory where 4.1 mg L^{-1} of dust released 0.97 ± 0.17 μM P (González-Olalla et al., 2017). By adding the reported amount of dust, we were able to mimic an event of high deposition as observed in the surface water layer of mountain lakes of the Iberian Peninsula (Villar-Argaiz et al., 2002; Carrillo et al., 2008) and the Western Mediterranean region (Lekunberri et al., 2010). Inorganic nutrients for the NP treatments received P (as NaH$_2$PO$_4$). These microcosms were also added with N (as NH$_4$NO$_3$) to give a final N:P molar ratio of 30, resembling the mean nutrient concentrations observed for atmospheric depositions events by Morales–Baquero et al. (2006). The comparison between the dust- and inorganic NP treatments allowed for the evaluation of the effect of the dust particles *per se* on the dynamics of the plankton community.

2.4. Daphnia growth bioassays

One day after the setup of the microcosms, coupled-growth bioassays were initiated using *D. pulicaria* as a model organism. The strain of *D. pulicaria* originated from a single clone maintained under constant laboratory conditions in the Institute of Water Research (University of Granada, Spain). At the start of the experiments, five *D. pulicaria* neonates collected within 24 h of hatching were placed into individual glass tubes that each contained 100 mL of the experimental food suspension (Fig. 1). Five subsamples of the daphnid were collected to determine initial body weight using a Mettler ultramicrobalance (±0.1 μg, Mettler, Germany). The coefficient of variation (CV = standard deviation / mean × 100) among weighed samples rarely exceeded 10%. Three replicates were run for all experimental treatments in a growth chamber under the same temperature and light conditions as the microcosm room. Every day, individuals were transferred to clean tubes containing fresh experimental food suspension and twice a day the water in the tubes was gently stirred with a pipette to prevent algal settlement. After five to six days, daphnids were rinsed in distilled water, pipetted into preweighed aluminium cups, which were reweighed after 24 h at 60°C. Somatic growth rate (*g*) was calculated as follow:

$$g = [\ln (M_t) - \ln (M_o)]/t$$

where M_t is the final body mass, M_o is the initial body mass and *t* is the time (in days) at which animals were collected for their weight.

2.5. Chemical and food quantity/quality determinations

Water from the initial composite lake sample and from each microcosm was collected for chemical (total [TP] and dissolved [TDP] P), and biological (Chlorophyll *a* [Chl *a*], seston C, N and P) determinations at the start of the zooplankton bioassays (day 1), and every two days for a total of three samples for each experimental treatment. Seston was collected by filtering a volume of 100–200 mL onto precombusted (550°C) glass-fiber filters (Whatman GF/F). Seston C and N in the filters was determined using an elemental analyzer (Perkin Elmer 2400 CNH), and samples for TP, TDP and seston P were persulfate-digested and analyzed as soluble reactive P applying the acid molybdate technique (APHA, 2012). Chl *a* was measured fluorimetrically (LS55 Perkin Elmer fluorometer) following the methodology of Jeffrey and Humphrey (1975). The carbonate-bicarbonate system in the incubation enclosures (water pH, free CO$_2$, carbonate and bicarbonate) was monitored throughout the experiments using an automatic titrator (905 Titrando, Metrohm) equipped with TiAmo v.2.0. Major cation analyses in the dust were performed by Inductively Coupled Plasma Optical Emission Spectroscopy (ICP-OES) using a Perkin Elmer Optima 8300. All samples were run in duplicate.

2.6. Remote sensing data

As a measure of the aerosol content in the troposphere, we used the aerosol index (AI) produced by the Giovanni online data system (NASA Goddard Earth Sciences Data and Information Services Center; Bhartia, 2012) as a proxy for the potential impact of dust inputs into the studied systems (Mladenov et al., 2011). Available AI data spans four decades of almost uninterrupted daily measurements by three NASA monitoring instruments: TOMS Nimbus-7 (1979–1993), TOMS Earth Probe (1996–2004) and OMI (2004–2017). The area analyzed (−3.67 W, 36.92 N, -2.52 E, 37.25 N) comprised the three studied lakes. Positive values of AI represent absorbing aerosols, and values >1 were considered to represent high deposition days (Cabrerizo et al., 2016). We counted the number of high deposition days for each year. The mean AI intensity of a given year was calculated by dividing the sum of the intensitites for all days with AI >1 by the number of high deposition days for that year.

2.7. Data analyses

Differences in seston (C and C:P ratio) among microcosms due to CO$_2$, Dust and NP were assessed by paired *t*-test using the three samples collected during the growth assays. We calculated the magnitude of the single effect of these assayed factors as:

Single effect (%) = (var$_{single}$−cont)/(cont) × 100

where the cont represents samples under Amb-CO$_2$ in all cases, and var$_{single}$ represents: (i) Amb + CO$_2$ for CO$_2$ effect, Dust-CO$_2$ for Dust effect, and NP-CO$_2$ for NP effect (Table 3). The joint effect magnitudes of CO$_2$ and Dust or CO$_2$ and NP on seston C and seston C:P ratios were calculated as:

Joint effects (%) = (var$_{multiple}$−cont)/(cont) × 100

where the cont and var$_{multiple}$ represents, respectively, samples under: (i) Dust-CO$_2$ and Dust+CO$_2$ for CO$_2$ and Dust; and ii) NP-CO$_2$ and NP + CO$_2$ for CO$_2$ and NP.

For the growth bioassays, the effects of CO$_2$, nutrients (independent variables), and their interaction on *D. pulicaria* growth (dependent variable) were tested by two-way analysis of variance (ANOVA). When significant interactive effects were found, differences among treatments were tested by *post hoc* Newman-Keuls' tests. Simple Least-Squared Regression analysis (LSR) was used to test the effects of (1) time on AI frequency and intensity, and (2) seston C and C:P ratios on the growth of *D. pulicaria*. We first performed the zooplankton growth regression analysis examining the effects of seston C and seston C:P for all our lake data. To assess the possible impact of the nutrient treatment on our analysis, we also carried out regression analysis grouping the data according to the nutrient level (Amb, Dust or NP). When regressions were significant, a homogeneity-of-slopes model (ANCOVA) was used to test the effect of nutrient treatment (categorical factor) across seston composition (continuous predictor variable) on *Daphnia* growth rate (dependent variable). Data were checked for normal distribution of the residuals (Kolmogorov-Smirnov test) and homocedasticity (Levene's test) to ensure that the assumptions of parametric tests were met and, values were log transformed when these conditions were not met. All the statistical analyses were run using IBM SPSS Statistics v.24.

3. Results

3.1. Lake physical, chemical, and biological conditions

Several physical, chemical, and biological characteristics varied among the ecosystems studied (Table 1). For example, differences in

Table 1
Physical and chemical variables measured at the beginning of the experiments in the three lakes studied. Values are means of three depths evenly spaced at the deepest station of the lake.

Variable	Lake		
	Dulce	Archidona	Cogollos
Maximum depth (m)	3.56	10	2.5
T (°C)	20.7	21.6	8.8
$K_{d\ 305}$ UVB (m^{-1})	17.4	9.9	2.3
$K_{d\ 380}$ UVA (m^{-1})	6.3	2.3	3.8
K_d PAR (m^{-1})	1.4	0.6	1.1
pH	7.0	7.9	7.5
Alkalinity (mEq L^{-1})	18.5	13.7	0.5
Total phosphorus (µg L^{-1})	39.8	25.9	15.2
Total dissolved phosphorus (µg L^{-1})	20.7	13.0	0.70
Chlorophyll a (µg L^{-1})	1.9	3.3	3.7
Seston C (µM)	47.7	54.2	16.3
Seston C:P (molar)	78.5	99.2	51.7
Seston C:N (molar)	9.5	9.6	6.2

Table 2
Chemical composition of the dust used in the enriched treatments. Values are in percentage of dust-dry mass (% dust DM) and total concentrations in the dust-enriched treatments (µM). Values are means of two replicates ±1SD. Elements not reported for the ICP-OES were below the detection limit.

Cation/anion	Method	% dust DM	Dust treatment (µM)
Al	ICP-OES[a]	4.9 ± 1.1	7.4 ± 1.6
Ca	"	7.2 ± 3.9	7.40 ± 4.0
Fe	"	2.1 ± 0.5	1.52 ± 0.4
K	"	2.4 ± 0.2	2.54 ± 0.2
Mg	"	2.0 ± 1.2	3.37 ± 2.1
Mn	"	0.04 ± 0.01	0.03 ± 0.01
Na	"	0.3 ± 0.1	0.51 ± 0.2
S	"	5.9 ± 0.3	7.52 ± 0.4
P	SPECT[b]	0.73	0.97

[a] ICP-OES: Inductively Coupled Plasma Optical Emission Spectroscopy (Perkin-Elmer Optima 8300).
[b] SPECT: Spectrophotometry (Perkin-Elmer Lambda 40).

surface-to-bottom temperature ranged from 25 to 13 °C in Lake Archidona (Fig. 2E), while the more shallow Dulce and Cogollos lakes had uniform temperature profiles with mean water temperatures of 20.7 and 8.8 °C, respectively (Fig. 2 D, F). According to the vertical attenuation coefficients for radiation, Cogollos was the clearest lake, followed by Archidona and Dulce (Fig. 2A, B, C). Also, a wide range in seston C was shown by these three lakes, ranging from ca. 16 µM in Lake Cogollos to ~50 µM in Lake Dulce and 54 µM in Lake Archidona (Table 1). TP varied between 15.2 µg L^{-1} in Lake Cogollos to ~40 µg L^{-1} in Lake Dulce. According to these values, the trophic state of the lakes varied from a high-mesotrophic state of Lake Dulce to the more oligotrophic conditions of Lake Cogollos. Nonetheless, the lowest Chl a concentration of 1.9 µg L^{-1} in Lake Dulce (Table 1), suggests a lower contribution of algal carbon to the seston fraction in this ecosystem.

3.2. Dust composition and trends

Table 2 contains information about the chemistry of the dust added in the experiments. Total element concentrations decreased as follow: Ca, S, Al, K, Fe, Mg, Na and Mn. Concentration of other trace elements were below the detection limits.

The dust in this study was similar in composition to that of previous reports for dry deposited material across the Mediterranean basin (e.g., Herut et al., 2016).

Aerosol deposition data since 1979 revealed a remarkable tendency for the higher occurrence of these events (Fig. 3A). Despite the notable interannual variation, AI frequency increased threefold over the past four decades (LSR: AI frequency$_{1979-2017}$ = 0.88 × Time - 1713, r = 0.52, p = 0.001, n = 35; Fig. 3A). Although, AI intensity did not show significant temporal changes over this period ($p > 0.05$; Fig. 3B), it particularly increased throughout the period of 2007–2017 exceeding
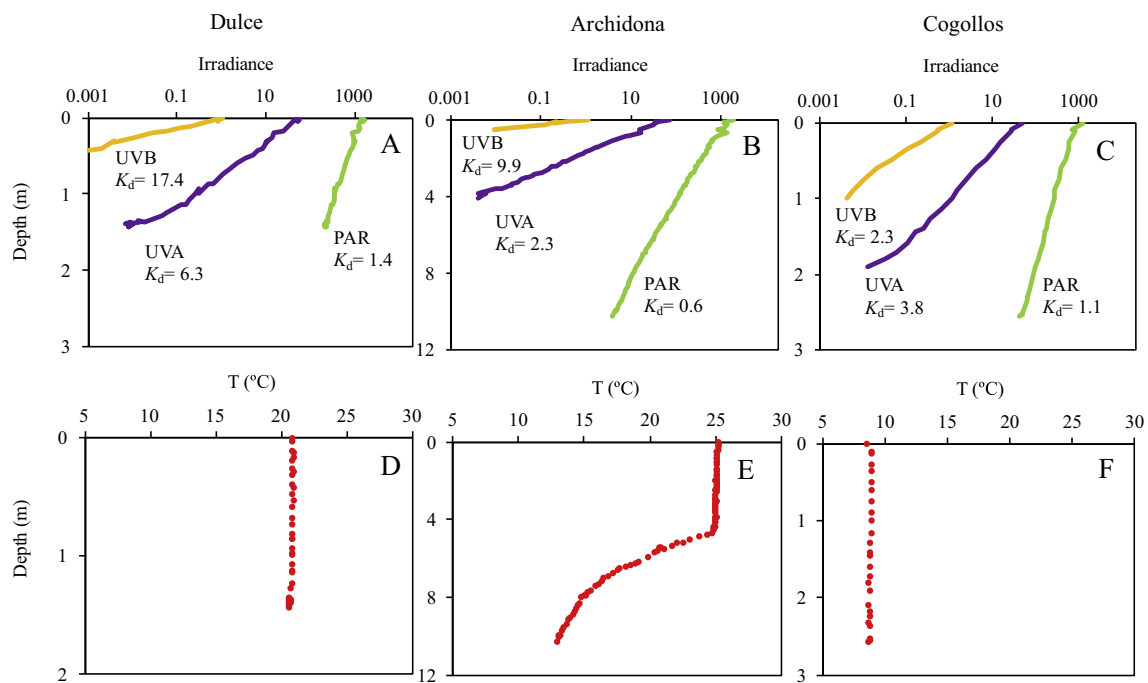

Fig. 2. Irradiance and temperature plots for lakes (A, D) Dulce, (B, E) Archidona, and (C, F) Cogollos. Diffuse attenuation coefficients (Kd) were determined from the slope of the linear regression of the natural logarithm of downwelling irradiance vs. depth for discrete wavelengths within each region of the solar spectrum: UVB (305 nm), UVA (380 nm) and PAR (400–800 nm). Irradiance units are µW cm^{-2} nm^{-1} for UVB and UVA and µE m^{-2} s^{-1} for PAR.
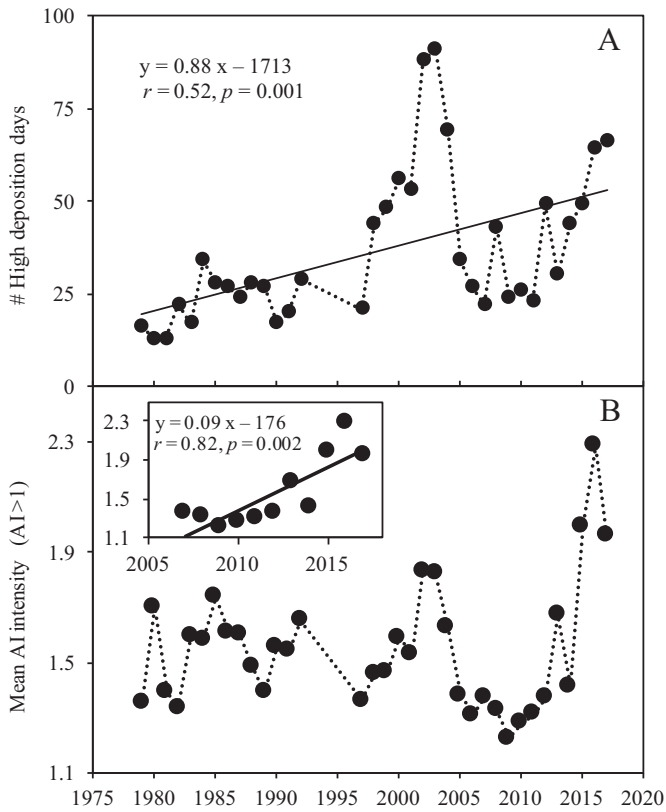
Fig. 3. Interannual trends in the number of high deposition days (A) and the mean intensity of high deposition days (B) during the 1979–2017 period. The inset shows the intensity of high deposition days for the 2007–2017 period. Lines are best fits from least-square regressions.

unprecedent values of 2 (LSR: AI intensity$_{2007–2017}$ = 0.99 × Time – 176, r = 0.82, p = 0.002, n = 11; Fig. 3B).

3.3. Experimental effects on microcosms: pH and free CO$_2$

Fig. 4 shows pH and free CO$_2$ trends in the microcosms for the three lakes studied. The enrichment with CO$_2$ in lakes Dulce and Archidona

increased the concentration of this free molecule in the water (Fig. 4D, E), which shifted the reaction towards increased the bicarbonate and reduced the carbonate in the water (data not shown). Consequently, the water pH fell by about 0.3 units in the +CO$_2$ treatments (Fig. 4A, B). Conversely, the effects of enriching with CO$_2$ were not that clear in Lake Cogollos, where free CO$_2$ in the water slightly decreased and the pH barely increased in all treatments (Fig. 4C, F). The unexpected increase in pH due to CO$_2$ in Cogollos microcosms could be explained by the slight increase in the water temperature in comparison to that of the lake with carry-on consequences for the rate of photosynthesis and water pH, as well as by the relatively low buffer capacity of this lake located in a siliceous watershed.

3.4. Experimental effects on microcosms: seston C and C:P ratio

The experimental manipulation of CO$_2$, dust, and nutrients affected the quantity and quality of the seston that was subsequently used to test for the effects of herbivorous growth. Seston C generally increased in response to dust and NP enrichment in Dulce and Archidona lakes (Table 3, Fig. 5A, B). However, the CO$_2$ did not increase seston C unless supplemented with dust and NP in these two lakes. Under these circumstances seston C exceeded 83 μM (1000 μg C L^{-1}), a threshold above which *Daphnia* growth may no longer be constrained by food availability (Lampert and Muck, 1985). In contrast, experimental manipulation did not significantly affect seston C in Lake Cogollos, except for the slight increase observed for the +NP + CO$_2$ treatment (Table 3, Fig. 5 C). Altogether, the experimental manipulation with the enrichment of CO$_2$ and nutrients generated a food-quantity gradient in terms of seston C that, at the end of the microcosm incubations, ranged from 36 μM in the Amb-CO$_2$ treatment in Lake Cogollos to 169 μM in the NP + CO$_2$ treatment in Lake Dulce.

Response to experimental manipulation was not that clear for seston C:P ratios. Thus, while seston C:P ratios appeared higher under +CO$_2$ in Lake Dulce, the effects were not statistically significant (Table 3, Fig. 5D). Likewise, seston C:P generally decreased in response to the addition of dust and NP in Archidona and Cogollos lakes, but these effects were only significant for the +NP-CO$_2$ treatment in Lake Archidona (Table 3, Fig. 5E, F). Mean seston C:P ratios for all treatments were well under the threshold of 350, below which zooplankton growth could shift from P to C limitation (Becker and Boersma, 2003). The food quality seston C:P gradient for all study sites ranged from 51 in
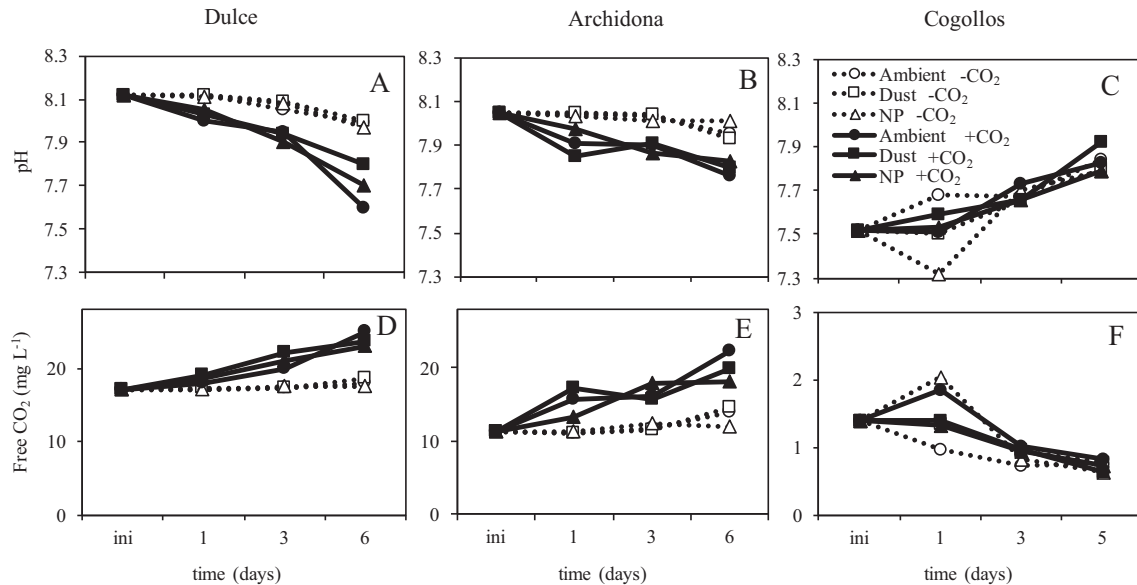

Fig. 4. Water pH and free CO$_2$ values in the microcosms of llakes (A, D) Dulce, (B, E) Archidona and (C, F) Cogollos. Values are single measurements for the CO$_2$ (−CO$_2$ and + CO$_2$) and nutrient (ambient, dust, and NP) treatments. Label 'ini' in the x-axis corresponds with the initial value.

Table 3
Single and joint effects of CO_2, dust, and NP on seston C and C:P ratio in the microcosms for the three lakes studied tested by paired t-tests.

Effect	Dulce				Archidona				Cogollos			
	Seston C		Seston C:P		Seston C		Seston C:P		Seston C		Seston C:P	
	Δ[a]	p[b]	Δ[a]	p[b]	Δ[a]	p[b]	Δ[a]	p[b]	Δ[a]	p[b]	Δ[a]	p[b]
CO_2	−1.6	n.s.	75.4	n.s.	−1.2	n.s.	−26.9	n.s.	−17.5	n.s.	−16.4	n.s.
Dust	10.8	*	13.2	n.s.	−1.8	n.s.	−14.4	n.s.	−14.3	n.s.	−39.9	n.s.
NP	34.8	*	−29.7	n.s.	55.0	*	−47.7	*	20.4	n.s.	−51.8	n.s.
CO_2 and Dust	62.9	**	84.6	n.s.	37.9	*	−11.5	n.s.	−0.9	n.s.	−9.5	n.s.
CO_2 and NP	75.6	**	41.5	n.s.	−13.1	n.s.	−15.9	n.s.	11.7	n.s.	31.2	n.s.

[a] Δ expresses the magnitude (as percentage) and sign (−, inhibitory; no sign, stimulatory) of the effects calculated for the means of three temporal replicates.
[b] Significance level (n.s. not significant, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

Table 4
Effects on *Daphnia pulicaria* growth rate of CO_2, nutrient enrichment and their interaction in the zooplankton bioassays, tested by two-way ANOVA.

Lake	Effect	df[a]	SS[b]	F-ratio[c]	p[d]	PV[e]
Dulce	CO_2	1	0.021	104.96	***	22.6
	Nutrients	2	0.044	110.98	***	47.3
	$CO_2 \times$ nutrients	2	0.026	65.36	***	28.0
	Error	12	0.002			2.2
Archidona	CO_2	1	0.005	9.78	**	7.0
	Nutrients	2	0.055	57.29	***	77.5
	$CO_2 \times$ nutrients	2	0.003	5.59	*	7.0
	Error	12	0.006			8.5
Cogollos	CO_2	1	0.00001	1.42	n.s.	2.4
	Nutrients	2	0.00028	22.70	***	76.7
	$CO_2 \times$ nutrients	2	0.00001	0.70	n.s.	2.4
	Error	11	0.00007			18.6

[a] Degrees of freedom.
[b] Sums of Squares.
[c] F-test results.
[d] Significance level (n.s. not significant, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).
[e] Percentage variance (PV) calculated as sums of squares of treatments: total sums of sums of squares.

the NP-CO_2 treatment in Lake Dulce to 359 in the Amb-CO_2 treatment in Lake Archidona.

3.5. CO_2, dust and NP effects on Daphnia growth rate

We used the seston suspensions raised after CO_2, dust, and nutrient manipulations to test for their effects on *D. pulicaria* growth rate. The results of the ANOVA showed that the nutrient treatments explained the highest percentage of the variance (between 47 and 78%) in *D. pulicaria* growth in all lakes (Table 4, Fig. 6), whereas CO_2 had no significant effect on Lake Cogollos and contributed only with an additional 23% in Lake Dulce and 7% in Lake Archidona. Because of the significant $CO_2 \times$ nutrients effects in the latter lakes (Table 4), differences in the response among treatments were further tested using *post hoc* tests (Fig. 6). These comparisons indicated that the CO_2 had no effect under ambient conditions, but stimulated growth after the amendment with dust ($\Delta \sim 54$ and 21% in Dulce and Archidona, respectively) and nutrients ($\Delta \sim 20$ and 43% in Dulce and Archidona, respectively) (Fig. 6A,
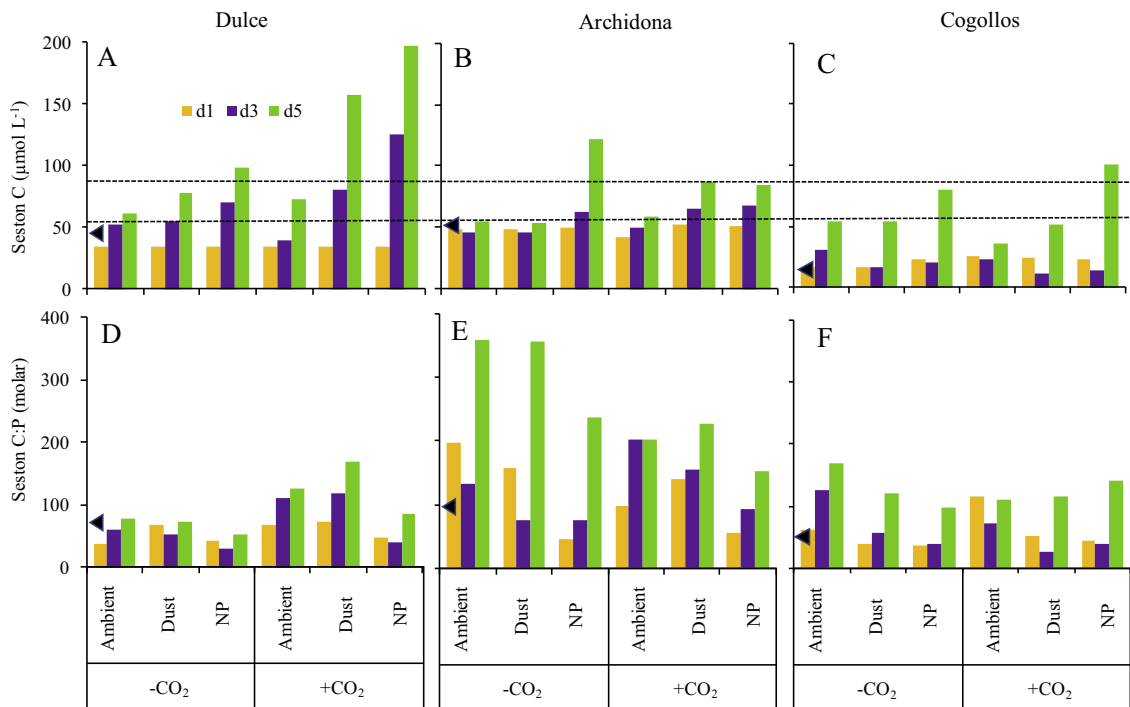

Fig. 5. Seston C and C:P ratios under CO_2 (-CO_2 and + CO_2) and nutrient (ambient, dust, and NP) treatments for each of the three sampling days (d1, d3, and d5) during *Daphnia pulicaria* growth bioassays. Horizontal dashed lines indicate seston C limitation thresholds for *D. pulicaria* maximum growth at 42 and 83 µM seston C (Lampert and Muck, 1985). Arrows next to the y-axis correspond with initial values. Scale bars represent the mean of seston C and C:P values of two laboratory pseudoreplicates. Statistical comparison among treatments is shown in Table 3.
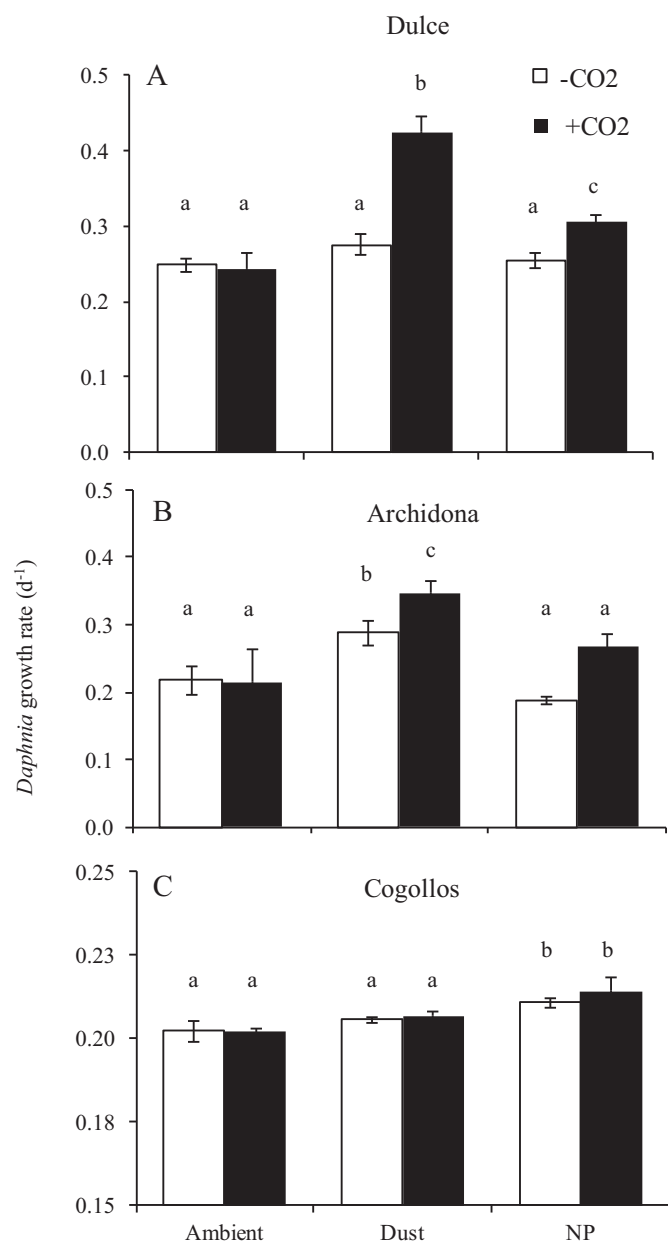
Fig. 6. Effects of CO_2 and nutrient treatments on *Daphnia pulicaria* growth in lakes (A) Dulce, (B) Archidona, and (C) Cogollos. Values represent the mean for three replicates ±1 SD. Significant differences among treatments are denoted by different case letters according to post hoc Newman-Keuls' test. Note the different scale on the Y-axis in the (C) panel.

B). In contrast, only NP augmented the growth of *D. pulicaria*, although by <5% in Lake Cogollos (Fig. 6C).

In the analysis for all the data across all study sites, seston C was the only variable that was positively related to *Daphnia* growth rate, although the correlation was not strong (LSR: y = 0.003 x + 0.138, r = 0.58, p = 0.011, n = 18; Fig. 7). In contrast, the regression analysis using nutrient treatment as a grouping factor yielded considerably better *Daphnia* growth predictions despite the reduction in number of cases (see regression model fits for each nutrient treatment in Fig. 7). Differences in the slopes between Dust and Amb treatments (analysis of covariance: $F_{1,8}$ = 14,79, p = 0.005) and between Dust and NP treatments (analysis of covariance: $F_{1,8}$ = 44.1, p < 0.001) indicated that *Daphnia* growth rate was most sensitive to dust enrichment.

4. Discussion

This study responds to the current demand for field research on the joint effects of CO_2 and nutrients at the primary producer-consumer interphase. Our experimental results do not support expectations from first hypothesis that *Daphnia* growth rate would be increased by CO_2 enrichment. The cause for the negligible effect of increased CO_2 on zooplankton growth is a question of great interest as CO_2 can simultaneously affect primary producers in both direct and indirect manners. Indirectly, CO_2 could increase pCO_2 in the water and shift the carbonate buffer system towards more acidic conditions, which can affect phytoplankton communities and in turn their herbivorous consumers. However, the fact that water maintained its pH above 7 even after CO_2 enrichment indicates that the studied lakes were well-buffered systems, perhaps due to continuous supply of nutrients from dry and wet atmospheric depositions. Because zooplankton were not significantly affected in any of the two lakes where supplementation with CO_2 resulted in decreased pH, growth-rate differences between *D. pulicaria* in this study cannot be attributed to acidic conditions caused by elevated CO_2 levels. Directly, CO_2 might influence phytoplankton stoichiometry and biomass, and therefore the food quality and quantity for herbivorous consumers, respectively (Riebesell et al., 2007; Verschoor et al., 2013; Schoo et al., 2013). However, the single effect of high CO_2 levels in this study had no significant impact on the seston C or C:P ratio, both of which remained low (see Fig. 5, Table 3). Similar effect of high pCO_2 levels in accentuating phytoplankton nutrient limitation have been previously documented in chemostat experiments (Verspagen et al., 2014). Under the prevailing scarcity of nutrients for phytoplankton, rising CO_2 concentrations would likely have a minor impact on zooplankton growth, presumably because seston C did not surpass the general range of 42–83 μM, below which *D. pulicaria* remains food limited (Lampert and Muck, 1985). An alternative explanation for the low growth of *D. pulicaria* derives from the "stoichiometric knife-edge" hypothesis, which predicts a negative effect of an excessively P-rich food on herbivore consumers (Elser et al., 2005). In a more recent study using three species of *Daphnia*, Elser et al. (2016) showed that animals grew more slowly when C:P of food was <120, a very similar ratio to that of Amb + CO_2 treatments in Dulce and Cogollos lakes (Fig. 5D, F). Unlike many northern hemisphere (Sterner et al., 2008) or Andean lakes with relatively high C:P ratios (Balseiro et al., 2007; Laspoumaderes et al., 2013; Villar-Argaiz et al., 2018b), seston in southern Mediterranean lakes is typically P-rich with characteristically low C:P ratios below 300 (Villar-Argaiz et al., 2002; Carrillo et al., 2017; this study). Food C:P imbalances due excessive P content could conceivably have an under-recognized role in affecting *Daphnia* growth in many Mediterranean P-rich lakes.

In support of our second hypothesis, the growth rate of *D. pulicaria* was strongly stimulated under the joint addition of CO_2 and NP or Dust in Dulce and Archidona lakes, but by the sole addition of NP in the more oligotrophic lake of Cogollos. These results raise the questions of what caused the observed $CO_2 \times$ nutrient interaction, and why these effects occurred in only two of the three lakes studied. Because CO_2 and nutrients often drive plant stoichiometry in opposite directions, but productivity in the same direction (Reich et al., 2010), we might forecast that the scarcity of one of these factors in nature could limit primary production and as a consequence restrain the quantity of food for consumer herbivores. We conclude this is true of the lakes studied, where food-quantity constraints on zooplankton growth could have prevailed over those of food quality with seston C:P consistently below the 300 threshold reported by Becker and Boersma (2003). In line with these expectations, the CO_2 did not support higher zooplankton growth rates in lakes Dulce and Archidona unless supplemented with Dust or NP, which increased seston as a food source for zooplankton. The fact that Lake Cogollos responded only to the single effects of greater inorganic NP could be associated with the more oligotrophic conditions for this lake. It is widely accepted that under ultra- and oligotrophic
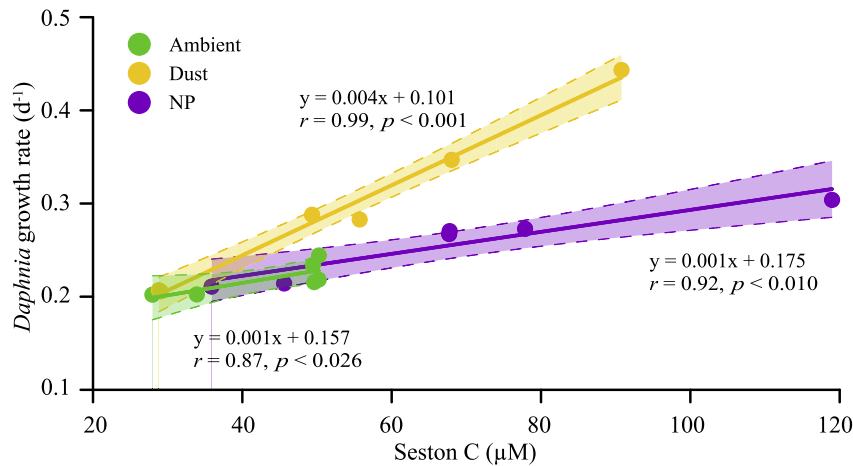
Fig. 7. Relationship between seston C and *Daphnia pulicaria* growth rate for each nutrient treatment (ambient, dust and NP) in this study. Seston C values represent the mean for all sampling dates. Equation and regression parameters for each nutrient treatment are included in the Figure. Dashed lines indicate 95% interval confidence for each regression. Model equation and statistics for all data was y = 0.003 x + 0.138 ($r = 0.58$; $p = 0.011$, $n = 18$).

conditions, bacteria compete efficiently with phytoplankton for nutrients (Currie, 1984), possibly restraining seston C below or barely above subsistence levels for *Daphnia* (see thresholds by Lampert and Muck in Fig. 5C). Thus, our findings are consistent with the numerous observations that report that *Daphnia* growth is limited primarily by C in nutrient-poor lakes (e.g., Persson et al., 2007).

In agreement with these arguments, the joint analysis of our data for the three lakes studied showed that zooplankton growth was only positively correlated with seston C, corroborating our finding that food-quality effects were negligible compared to the more prominent impact of food quantity. We found that this prediction improved when observations were grouped according to the enriched treatments, and that the response was strongest for dust (see the steepest slope in the regression of Fig. 7). A worthwhile question that emerges is why did enrichment with dust favor *Daphnia* growth over that with inorganic nutrients? One likely explanation is that nutrients adsorbed onto dust are available to microorganism uptake at lower but more continuous rates than are inorganic nutrients. Support for this explanation comes from field observations in a high mountain lake of southern Spain where, after large deposition events, the steadily increase in dissolved nutrients frequently precedes strong algal blooms (Villar-Argaiz et al., 2002). Also, different fertilization experiments in Mediterranean coastal waters, have shown a delayed increase in Chl *a* (and primary production) when enriched with dust relative to inorganic P (Lekunberri et al., 2010; Pitta et al., 2017). It is also possible that Saharan dust stimulated bacterial growth (Guieu et al., 2014), and the potential ingestion rate of bacteria by flagellate phagotrophic algae as a food source for zooplankton (Tsagaraki et al., 2017; Tsiola et al., 2017). Finally, another possible explanation is that, in addition to macro-nutrients, dust provides with high amounts of micro-nutrients that could limit primary production (Rahav et al., 2016) or affect, such as in the case of Ca, the growth and survival of *Daphnia* (Tan and Wang, 2009). Thus, our dust enrichment provided with a Ca supply of ca. 300 μg L^{-1} (Table 2) could help surpass the threshold of 0.1–0.5 mg Ca L^{-1} reported for the survival of a *Daphnia* species (Hessen et al., 2000). Because the effects of low Ca on *Daphnia* growth rate are more severe during the first days after hatching (Hessen et al., 2000), the low growth of the young could well be a symptom of Ca deficiency in treatments not supplemented by dust. Atmospheric inputs are not only a significant source of Ca to both terrestrial to aquatic ecosystems, but they can become the only Ca source in silicious watersheds in the southwestern Mediterranean region (Pulido-Villena et al., 2006). Also, dust can be an indispensable source of trace metals such as iron (Fe), which can limit photosynthetic activity in many freshwater environments and up to one third of the world's oceans (Schoffman et al., 2016). While the biochemical role of

dust deposition on aquatic and terrestrial ecosystems is increasingly recognized (Gallisai et al., 2014), more work is needed to determine how and the rate at which nutrients adsorbed onto dust became available to aquatic organisms and to establish how this alters growth and elemental composition of the living biomass.

5. Conclusions

The finding of this study that seston and *Daphnia* growth rate did not respond to the single effect of CO_2 enrichment indicates that there are other factors limiting zooplankton growth. This result support the idea that zooplankton, in the oligo- to mesotrophic lakes considered in our study with characteristically low seston C:P, may not be critically sensitive to rising CO_2 levels. The effects of CO_2 in these lakes were instead largely mediated by nutrient availability, such that it was only under the simultaneous synergistic effect of inorganic NP or dust when CO_2 boosted zooplankton growth by stimulating seston growth rather than lowering C:nutrient ratios. Because food-quality effects should be more pronounced under high food quantity (Sterner, 1997), the expectation of more prominent CO_2 effects under the nutrient-replete conditions of eutrophic and hypereutrophic systems needs to be tested directly. Broad-scale analogous studies on different species of zooplankton using natural seston with characteristically higher C:P ratios would be immensely valuable, and would presumably give a more accurate assessment of how lake food webs will globally respond to the joint effects of CO_2 and nutrients. However, the effects of dust may not be confined to lakes but pervasive to marine environments. Our results suggest that the synergistic $CO_2 \times$ dust effects are expected to be more intense in the Mediterranean region, where the intensity and frequency of atmospheric intrusions have steadly increased over the last four decades (Bullejos et al., 2010; Beklioglu et al., 2010; Klingmüller et al., 2016; this study).

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