1 **Prokaryotic and eukaryotic microbial community responses to N and P nutrient addition in**
2 **oligotrophic Mediterranean coastal waters: novel insights from DNA metabarcoding and**
3 **network analysis**

4 **Ioulia Santi^{a,b}, Anastasia Tsiola^{a,b}, Panagiotis D. Dimitriou^b, Stilianos Fodelianakis^c, Panagiotis**
5 **Kasapidis^d, Nafsika Papageorgiou^b, Daniele Daffonchio^c, Paraskevi Pitta^{a*}, Ioannis Karakassis^b**

6 ^a Institute of Oceanography, Hellenic Centre for Marine Research (HCMR), PO Box 2214, Heraklion
7 71003, Crete, Greece

8 ^b Department of Biology, University of Crete, Voutes Campus, Heraklion 71003, Crete, Greece

9 ^c Biological & Environmental Sciences & Engineering Division, King Abdullah University of Science
10 & Technology, Thuwal 23955-6900, Saudi Arabia

11 ^d Institute of Marine Biology, Biotechnology & Aquaculture, Hellenic Centre for Marine Research,
12 PO Box 2214, 71003 Heraklion, Crete, Greece

13 * Corresponding author: Paraskevi Pitta. E-mail address: vpitta@hcmr.gr. Postal address: Hellenic
14 Centre for Marine Research, PO Box 2214, Heraklion 71003, Greece

15

ABSTRACT

The effects of the abrupt input of high quantities of dissolved inorganic nitrogen and phosphorus on prokaryotic and eukaryotic microbial plankton were investigated in an attempt to simulate the nutrient disturbances caused by eutrophication and climate change. Two nutrient levels were created through the addition of different quantities of dissolved nutrients in a mesocosm experiment. During the developed blooms, compositional differences were found within bacteria and microbial eukaryotes, and communities progressed towards species of faster metabolisms. Regarding the different nutrient concentrations, different microbial species were associated with each nutrient treatment and community changes spanned from the phylum to the operational taxonomic unit (OTU) level. Network analyses revealed important differences in the biotic connections developed: more competitive relationships were established in the more intense nutrient disturbance and networks of contrasting complexity were formed around species of different ecological strategies. This work highlights that sudden disturbances in water column chemistry lead to the development of entirely different microbial food webs with distinct ecological characteristics.

KEYWORDS

Plankton, bacteria, microbial eukaryotes, nutrients, ecological networks, coastal water, mesocosm.

1. INTRODUCTION

Nutrient discharge into the marine environment due to human activities have increased in quantity and frequency over time (Hallegraeff, 2010). At the same time, other phenomena such as severe precipitations and floods, which also result in nutrient discharge into the sea (Madsen et al., 2014), have intensified due to anthropogenic climate change (Hirabayashi et al., 2013). The

effects of direct human activities on the marine environment have been studied for decades (Doney, 2010), with a recent emphasis on, for example, wastewater influxes (Powley et al., 2016), urban inputs (Richa et al., 2017) and enrichment by aquaculture (Tsagaraki et al., 2013). Nonetheless, the ecological response of marine systems to the massive and abrupt nutrient addition connected to climate change phenomena (Krishnamurthy et al., 2010) remains an open issue.

With the frequency of intense climate phenomena increasing globally (Hirabayashi et al., 2013), the response by oligotrophic systems, which constitute the vast majority of the global oceans (Morel et al., 2010), is of major interest (Lejeusne et al., 2010). The Mediterranean Sea, one of the most oligotrophic marine areas in the world (Krom et al., 2003), is thought to be sensitive to nutrient supply changes (Karydis and Kitsiou, 2012) but the response of such ecosystems to massive and sudden nutrient release remains, to our knowledge, uncertain. Additionally, in the Mediterranean basin, the intensity of both Saharan dust deposits and riverine influxes related to climate change is escalating (Powley et al., 2017), thus total external nutrient enrichment is growing.

In the marine environment, among the dissolved nutrients, nitrogen and phosphorus are fundamentally connected to autotrophic biomass production and their cycles implicate several microbial communities. Dissolved nitrogen and phosphorus input in an equilibrated marine system will, most probably, result in a phytoplankton bloom (Bracken et al., 2015; Thyssen et al., 2014). In both natural and induced bloom events, large micro-autotrophs, such as diatoms, are known to benefit rapidly from the elevated nutrients, followed by populations of slower metabolic dynamics (Lin et al., 2016; Yoshimura et al., 2014). Following the classic approach of nutrient addition events, recent studies have expanded our knowledge of plankton ecology, demonstrating, for example, that the composition of the bacterial community depends on the

63 presence of different types of dissolved organic matter (DOM) molecules (Teeling et al., 2012) and
64 selective feeding may occur among protists (Martinez-Garcia et al., 2012). These recent insights,
65 in combination with the different metabolic capabilities of species, imply that the response of
66 some plankton communities to nutrient addition may have remained undetected.

67 In this context, we aimed to revisit the well-studied topic of the impact of nutrient input into the
68 marine environment but from a different angle by causing a sudden and extremely high nutrient
69 disturbance and subsequently investigating the responses of plankton from oligotrophic habitats
70 to this drastic change in nutrient levels. This study simultaneously examines the bacterial and the
71 microbial eukaryote community and focuses on both the composition and the biomass of the
72 different plankton components. The abrupt increase in nutrient concentrations attempts to
73 simulate events such as severe precipitations directly enhancing marine nutrient concentrations
74 and land floods leading to enormous riverine inputs in the sea (Madsen et al., 2014). Our
75 experiment was carried out in large mesocosm enclosures as they are considered the most
76 representative experimental approach for studying natural plankton responses with reduced
77 small-scale experimental errors (Mostajir et al., 2013).

78 Assuming that a sudden nutrient increase would result in high plankton biomass, the goals of this
79 experiment are: 1) to test if nutrient addition results in diversity changes and, more specifically,
80 changes in prokaryotic and eukaryotic plankton communities; 2) to test whether the different
81 quantities of nutrients would lead to changes of plankton community composition; 3) to define
82 the succession between the different microbial populations under the specific nutrient loads; and
83 4) to check differences, if any, in the relationships among microbes between the two nutrient
84 quantities given that interactions between community members play a vital role in the temporal
85 alteration of plankton under changed conditions. To our knowledge, the results of extreme
86 climate events on plankton have not been comprehensively studied let alone when examining

several communities simultaneously or by taking into account relationships between specific plankton members; therefore, we believe there is room for a thorough and up-to-date study of nutrient effects on marine plankton.

2. MATERIALS AND METHODS

2.1 Experimental design and nutrient manipulation

The nutrient enrichment experiment took place at the CRETACOSMOS mesocosm facilities in autumn 2014 for 58 days. The experiment was part of a joint research activity with an additional benthic-pelagic coupling focus and therefore sediment was deployed together with marine water. The overall experimental and sampling design are described elsewhere in detail (Dimitriou et al., 2017a, 2017b). Briefly, the marine water used for the experiment was collected from a coastal eastern Mediterranean site (35° 20' 05.14" N; 25° 16' 50.44" E) from 2 m depth. After seawater was transferred into the mesocosms (1500 l seawater volume per mesocosm), bilayer lids of Plexiglas and nylon-mesh were placed on each mesocosm unit. Initial samples prior to nutrient manipulation were collected and then, later that day (Day-0), nutrients were added. Nutrient amendment was performed in triplicate for the two different manipulated treatments; "Control" mesocosms, without any nutrient addition, were also deployed in triplicate. After nutrient addition, the N:P ratio was intended to rise 1.5 and 2 times higher for "Low" and "High" nutrient treatments, respectively; the final nutrient concentration and N:P ratio of the amendment are presented in Table 1. Freshly prepared solutions of KH_2PO_4 and KNO_3 at appropriate concentrations were used for the nutrient amendment. Intense stirring to ensure the homogeneous distribution of nutrients in each mesocosm was applied immediately after nutrient addition. For this study, surface (1 m) mesocosm water was sampled in all cases. Water

temperature and sunlight illuminance were continuously monitored during the experiment using HOBO data loggers (UA-002-64 ONSET Corporation) at 2 min intervals.

2.2 Chemical variables and chlorophyll-a measurements

Samples for dissolved nutrient and chlorophyll-a analyses were collected daily until Day-3, and every three days following Day-3. Analyses of dissolved nutrient and oxygen concentration were performed directly after sampling. Phosphate was measured as described by Rimmelin and Moutin (2005), nitrate, nitrite, silicate and dissolved oxygen as described in Strickland and Parsons (1972) and ammonium according to Ivančič and Degobbis (1984). Detection limits for phosphate, nitrate, nitrite, silicate, oxygen and ammonium were $8 \cdot 10^{-4}$, 0.017, 0.017, 0.025, 3 and 0.019 μM , respectively.

Water samples for chlorophyll-a were successively filtered on 10, 5, 2 and 0.2 μm polycarbonate membranes using a gentle vacuum. Chlorophyll-a concentration was measured fluorometrically, according to Yentsch and Menzel (1963), following a 90%-acetone extraction. Total chlorophyll-a values were calculated by summing the corresponding fractionated values. The 10 μm membrane accounted for the micro-autotrophs ($>10 \mu\text{m}$), summed values from the 2 μm and 5 μm membranes together accounted for the nano-autotrophs (2-5 μm), while the 0.2 μm membrane represented the pico-photosynthesizers (0.2-2 μm).

2.3 Biomass determination of plankton populations

Samples for plankton enumeration and biomass calculations were collected daily until Day-3 and every three days following Day-3. The enumeration of picoplankton was performed using a FACSCalibur™ flow cytometer (Becton Dickinson) equipped with a 488-nm argon laser. Samples were fixed with 0.5% glutaraldehyde, formerly 0.2 μm -filtered for particle removal, and the protocols of Marie et al. (1999) and Gasol and Del Giorgio (2000) were applied for the analyses.

Fixation was allowed for 30 minutes in the dark and at 4°C. The nucleic acid stain SYBR Green I (Molecular Probes, USA) was used for the heterotrophic prokaryotes (HP) analysis at 4X final concentration. Autotrophic populations were enumerated and distinguished based on their autofluorescence; cyanobacteria of the genus *Synechococcus* were distinguished from other pico-autotrophs due to their distinct phycoerythrin and chlorophyll-a fluorescence. Yellow-green microspheres of 1 µm diameter were added as an internal reference during both analyses. The flow rate of the cytometer was measured daily before and after the analysis. The biomass of heterotrophic prokaryotes and *Synechococcus* was calculated by using the conversion factors 20 fg C and 250 fg C per cell, respectively (Lee Fuhrman, 1987; Kana Gliber, 1987).

For the enumeration of heterotrophic and autotrophic nanoflagellates (HNFs and ANFs), samples were fixed with 1.8% formaldehyde buffered with sodium tetraborate decahydrate and filtered for particle removal through 0.45 µm filters. Fixation was allowed for one hour in the dark and at 4°C. Subsequently, samples were stained with 0.2 mg l⁻¹ DAPI (4,6-diamidino-2-phenylindole) for 10 minutes and filtered through 0.6 µm black polycarbonate membranes (Porter and Feig, 1980). Filters were mounted on glass slides and stored at -20°C until analysis. Enumeration was performed using epifluorescence microscopy under UV-excitation. Autotrophic nanoflagellates were distinguished from heterotrophic ones by the red fluorescence of their chloroplasts, observed under blue light excitation; at least 50 fields were counted for each sample. Nanoflagellates were separated into four size classes (<2 µm, 2-5 µm, 5-10 µm and >10 µm) using an ocular micrometer. An ellipsoid shape was assumed for nanoflagellates and the biovolume was calculated separately for each size class using the measured dimensions. Biomass was calculated using the conversion factor 183 fg C µm⁻³ described by Caron et al. (1995).

Samples for microplankton community biomass were fixed with 2% acid lugol solution and stored at 4°C in the dark until analysis. Prior to analysis, subsamples of 100 ml were left to settle for

about 24 hours in an Utermöhl chamber (Utermöhl, 1931) and were subsequently analyzed with an inverted microscope. Cells larger than 10 µm were identified to the genus level, and the dimensions of each cell were measured using image analysis software. The geometry of individual cells was assigned according to Hillebrand et al. (1999) and Olenina et al. (2006); biovolume was calculated and subsequently the content of carbon in each cell was calculated using coefficients described by Davidson et al. (2002) and Putt and Stoecker (1989).

2.4 DNA metabarcoding analysis

Six liters of water were collected every six days and pre-filtered through a 200 µm mesh net; subsequently, biomass for molecular analyses was collected on 0.2 µm polyethersulfone membranes through filtration. Membrane filters were stored at -80°C until further processing. DNA was extracted and purified using the PowerWater DNA isolation kit (MoBio Laboratories, USA) according to the manufacturer's instructions, with a minor modification at the elution step. DNA was double-eluted to increase concentration, initially using the manufacturer's buffer and then immediately afterwards using PCR-grade water. For the bacterial community, the V3-V4 16S rRNA gene region was amplified by PCR using the primers described by Klindworth et al. (2012) and modified by Apprill et al. (2015). For the eukaryotic community, the V9 region of the 18S rRNA gene was targeted using the specific primers described in Amaral-Zettler et al. (2009). The two-step parallel multiplexing method (Shokralla et al., 2015) was used prior to sequencing and therefore the specific primers were equipped with a tail complementary to the second PCR primers, which additionally contained short index sequences (for post-sequencing sample identification) and flow cells adaptors for Illumina MiSeq. The bacterial amplicons of the first PCR were purified using the illustra ExoProStar PCR and Sequence reaction kit while the SequelPrep Normalization kit (Thermo Scientific, USA) was used for the second PCR product purification and normalization; in both cases, the manufacturer's instructions were followed. Sequencing of the

16S rRNA gene amplicon was performed with an Illumina MiSeq platform at the Biological Core Lab (KAUST, Saudi Arabia). Products from 18S gene PCR and the subsequent second-step-PCR were cleaned using the magnetic beads Agencourt AMPure XP (Beckman Coulter, USA). An equimolar amount of each sample was pooled for sequencing using the MiSeq Reagent Kit v2 on Illumina MiSeq platform at the Institute of Marine Biology Biotechnology and Aquaculture of HCMR, Greece. The processing of data after sequencing is described in detail in the SI. Metrics of sequencing data as well as diversity indices are presented in Table S1. Raw sequences were submitted to the ENA-GenBank under the study accession number PRJEB25787.

2.5 Data analysis and statistics

The effect of treatment and time on each nutrient and chlorophyll-a concentration, as well as on the biomass of each population (i.e. *Synechococcus*, ANF, Bacillariophyta, HP, HNF, Ciliophora, Dinoflagellata), was tested using mixed-effect linear models individually for each response variable; mesocosm identity (i.e. mesocosm unit) was considered as the random effect (more information available in the SI). Permutational multivariate analysis of variances (PERMANOVA) was applied to test if plankton communities of different treatments and/or experimental times were dissimilar at the OTU level. The same analysis was performed to examine the separation of the experiment into time periods based on the dissolved nutrients and chlorophyll-a concentrations. Multi-level pattern analysis was performed in order to associate specific important OTUs to the treatments (indicator species analysis: De Cáceres et al., 2010; more information available in SI).

In order to study the overall ecological web in each treatment and to disentangle the biotic-biotic interactions and relationships, network analysis based on the extended local similarity analysis (eLSA) algorithm (Durno et al., 2013; Ruan et al., 2006; Xia et al., 2011) was performed (more

information available in SI). For an additional focus on the biotic relationships formed under the different nutrient conditions, two specific OTUs were selected for the calculation of subnetworks resulting from the overall networks. Specifically, a *Pelagibacter* and a Rhodobacteraceae OTU were selected due to their contrasting ecological strategies (oligotrophy versus copiotrophy, respectively), which are well documented in the literature (for example: Teeling et al., 2016; Chafee et al., 2018). To choose between the several *Pelagibacter* and Rhodobacteraceae OTUs of this study, the ones found significant by the indicator species analysis (Fig. S6) were selected.

Significance level was set to 5% in all cases. All statistical analyses and data processing were performed with R v 3.4.2 (R: A language and environment for statistical computing; <https://www.R-project.org/>). Details for the data analysis and statistics, including mixed model construction, statistical designs and network constructions, can be found in the SI section.

3. RESULTS

3.1 Bloom development and progress throughout the experiment

Measurements of physicochemical variables during the experiment are presented in Fig. 1 and Fig. S1. Concentrations of the added nutrients (phosphate and nitrate) were higher at “High” than at “Low” treatment and showed similar behavior over time (Fig. 1A-B). Dissolved oxygen concentration notably increased after Day-6 in both nutrient treatments (Fig. 1F); conversely, nitrite concentration progressively increased with time until Day-12 and then decreased (Fig. 1C). For phosphate, nitrate and nitrite, the effect of treatment was significant ($p < 0.05$).

Total chlorophyll-a showed two different peaks per nutrient treatment, indicating phytoplankton blooms (Fig. 2A). The first peak appeared on Day-3 at both nutrient treatments and was of a similar chlorophyll-a concentration. The second chlorophyll-a peak was noted on Day-9 for treatment “Low” and on Day-12 for “High”; this was also the highest chlorophyll-a peak during

the experiment (treatment “High”, Day-12). Based on chlorophyll-a levels (Fig. 2A) and the concentration of nutrients and oxygen (Fig. 1), the experiment was separated into three distinct time periods. The first period (“prior bloom”) included Day-0 and Day-1 and reflected the initial conditions (Fig. 1-2). During Day-2 to Day-6 (“bloom 1”) and Day-9 to Day-15 (“bloom 2”), added nutrients were progressively depleted, as shown in Table 2. Concentrations of phosphate, nitrate, nitrite, dissolved oxygen and chlorophyll-a differed significantly among the three time periods ($F_{\text{perm}}=27.12$, $p<0.05$). The three phases (“prior bloom”, “bloom 1”, “bloom 2”) were synchronized between nutrient treatments (“Low” and “High”), with bloom events starting and ending at the same time.

The values of water-column variables after Day-15 can be found in the SI section (Fig. S1-S6). According to chlorophyll-a concentration, two additional bloom events took place, one during Day-18 to Day-21 in both treatments and one during Day-24 to Day-51 only in treatment “High”. However, after Day-15 the deviation of measurements within treatments was greater than the deviation between treatments for several biotic variables (Fig. S2-S5). Therefore, detailed results concerning biotic variables (i.e. biomass of plankton populations) after Day-15 will not be extensively discussed but can be found in the SI section.

3.2 Compositional differences in the bacterial community

Metabarcoding community composition was analyzed on Day-0, Day-6 and Day-12. Bacterial community composition on Day-0 was similar among treatments at the phyla (Fig. 3A), genera (Fig. 4A) and OTU levels (Fig. S6). PERMANOVA analysis on the bacterial OTUs showed significant differences among the communities due to treatment ($F_{\text{perm}}=1.15$, $p<0.05$) and time ($F_{\text{perm}}=3.93$, $p<0.05$). When treatment effect was divided into specific factors, “Control” was found to be significantly different from both “Low” and “High” ($F_{\text{perm}}=1.31$, $p<0.05$); in addition, the two

nutrient treatments were found to be significantly different ($F_{\text{perm}}=1.00$, $p<0.05$). Indeed, nMDS analysis depicted the distinct separation of Day-0 from Day-6 and Day-12 communities (Fig. S8A).

Phylum Bacteroidetes increased on Day-6 in all treatments (Fig. 3A); however, the relative abundance of Bacteroidetes groups varied between “Control”, “Low” and “High” (Fig. 4A). Notably, the genus *Flaviicola* showed higher relative abundance in “Low” in comparison to “High” and “Control”, while unass. Cryomorphaceae and unass. Flavobacteriales were relatively more abundant in both nutrient treatments than in “Control” (Fig. 4A). On Day-6, Proteobacteria dominated the community in “Low” by far (more than 75% of the relative abundance; Fig. 3A). The groups *Alteromonas*, *Pseudoalteromonas*, unass. Alphaproteobacteria, unass. Oceanospirillaceae and unass. Rickettsiales noted increased percentages in “Low”, whereas *Congretilbacter* and unass. Rhodobacteraceae were relatively more abundant in “High” than in “Low” or “Control” (Fig. 4A). Additionally on Day-6, Actinobacteria were more abundant in “High” than in “Low” by more than 10% (Fig. 3A), with the group *candidatus* Aquiluna noting the highest percentages (Fig. 4A), and the phyla Firmicutes and Planctomycetes showing higher percentages in “Control” in comparison to the two nutrient treatments (Fig. 3A).

Bacterial community partition to the different phyla was rather similar for the three treatments on Day-12 (Fig. 3A). However, Proteobacteria contributed approximately 15% more in “Control”; *Pelagibacter* and unass. Alphaproteobacteria seemed to be responsible for this (Fig. 4A). Bacteroidetes in “Low” (*Flavobacterium*, unass. Cryomorphaceae, unass. Flavobacteraceae) and Cyanobacteria (*Synechococcus*) in “High” accounted for the respective percentages in “Control”. Moreover, the percentage of Planctomycetes increased in both nutrient treatments on Day-12 (Fig. 3A).

According to the indicative species analysis for the bacterial community, treatments were found to be significantly associated to several OTUs on Day-0 (Fig. S6). In treatment “Low” alone, only one OTU, belonging to the genera *Saprospira*, was found to be significant (Fig. S6C). On the other hand, there were several OTUs significantly associated to “High” (Fig. S6D) and, additionally, numerous OTUs characteristic of both “Low” and “High” treatments (Fig. S6E).

3.3 Compositional differences in the microbial eukaryote community

Likewise, PERMANOVA on the eukaryotic OTUs showed significant differences in communities among treatments ($F_{\text{perm}}=1.04$, $p<0.05$) and due to time ($F_{\text{perm}}=3.85$, $p<0.05$). When treatment effect was further divided, “Control” was significantly different from both treatments ($F_{\text{perm}}=1.14$, $p<0.05$) and, further, the two treatments were dissimilar ($F_{\text{perm}}=0.04$, $p<0.05$). NMDS analysis confirmed the distinct separation of the Day-0, Day-6 and Day-12 communities (Fig. S8B).

A characteristic change in community composition on Day-6 was the elimination of Bacillariophyta and Ciliophora and the dominance of Dinoflagellata in all three treatments, but with higher percentages of Dinoflagellata over Bacillariophyta in the “Low” and “High” treatments (Fig. 3B, 4B). Particularly, the dinoflagellates *Amoebophrya*, *Gyrodiniellum*, *Scrippsiella* and unass. Dinophyceae increased in relative abundance in the nutrient treatments (Fig. 4B). Haptophytes emerged on Day-6 in all mesocosms but with a much stronger presence in the “High” treatment (Fig. 3B). On Day-6, Cryptophytes showed increased percentages only in “High” and “Low” (Fig. 3B), with the genus *Geminigera* dominating in relative abundance in the two nutrient treatments (Fig. 4B). Chlorophyta were present only in the “Low” treatment on Day-6 (Fig. 3B). On Day-12, the dominance of Dinoflagellata in all mesocosms was even more prominent than earlier (Fig. 3B, 4B). Indeed, unass. Dinophyceae and unass. Gymnodiniales noted particularly high percentages

(Fig. 4B). The phylum Labyrinthulomycetes appeared for the first time in both treatments on Day-12 (Fig. 3B).

Indicative species analysis found Ciliophora and Bacillariophyta OTUs to be significantly associated to “Control” and to Day-0 (Fig. S7A-B). Only one OTU belonging to the Dinoflagellata *Scrippsiella* was found characteristic for the “Low” treatment (Fig. S7C). For “High”, several indicative Dinoflagellata OTUs were found (Fig. S7D). There were also OTUs significantly associated with both nutrient treatments (Fig. S7E). Notably, the four *Scrippsiella* OTUs found to be significant for the two nutrient treatments differed from the one associated to the “Low” treatment (Fig. S7C, S7E).

3.4 Plankton responses during the first bloom (“bloom 1”; Day-2-Day-6)

At the beginning of the first bloom (Day-2), in treatment “Low”, the contribution of nano-autotrophs to total chlorophyll-a increased over that of the micro-autotrophs, which kept decreasing until the end of this bloom (Fig. 2C). In “High”, the contribution of nano- and micro-autotrophs was reversed: the contribution of the nano-autotrophs decreased and then increased at the termination of the bloom while the opposite was the case for micro-autotrophs. Regarding pico-autotrophs, their contribution to total chlorophyll-a increased only at the end of the first bloom (Day-6) in both nutrient treatments (Fig. 2B-D).

For heterotrophic prokaryotes (HP) and *Synechococcus* biomass, no differences between “Control” and nutrient treatments were found during the first bloom (Fig. 5A, 5D). There was a significant effect of treatment on bacterial productivity ($\chi^2(6)=7.13$, $p < 0.05$) and, in fact, bacterial productivity was significantly higher in both nutrient treatments compared to the “Control” ($b=0.00001$, $t(14)=2.90$, $p < 0.05$, $r=0.61$; Fig. S5). A sudden decline in biomass at the termination of the first bloom was obvious for HP populations (Day-6; Fig. 5D).

During this bloom, ANF biomass increased in both nutrient treatments, in contrast to the “Control” (Fig. 5B). The effect of treatment was significant for this group ($\chi^2(6)=6.88$, $p<0.05$) throughout the experiment. ANF biomass peaked on Day-3, like chlorophyll-a, and decreased on Day-6 in both nutrient treatments, with peak values in “High” being 6-fold higher than those in “Low”. Concerning the biomass of the respective heterotrophs, no significant differences were found for HNFs among treatments throughout the experiment. HNF biomass peaked at the same time as ANFs (Day-3) in both treatments, with an important decrease of their biomass (95% for “High” and 85% for “Low”) at the end of this bloom (Fig. 5E).

An increase in the biomass of Ciliophora was measured only on Day-2-Day-3 in treatment “High” (Fig. 5F); this may explain why the effect of treatment on Ciliophora biomass was not significant. Dinoflagellata biomass increased rapidly at the end of the first bloom (Day-6) in the “High” treatment (Fig. 5G). In the case of Dinoflagellata, the interaction of time and treatment was significant ($\chi^2(9)=16.02$, $p<0.05$). When comparing “High” to “Low” and “Control”, there were significant differences in Dinoflagellata biomass over time ($b=0.0035$, $t(12)=3.74$, $p<0.05$, $r=0.73$), indicating that the increased biomass through time found in the “High” treatment was significantly higher than in “Low” and “Control”. Bacillariophyta biomass decreased in all treatments for the duration of this bloom (Fig. 5C).

3.5 Plankton responses during the second bloom (“bloom 2”; Day-9-Day-15)

During the second bloom, the contribution of nano-autotrophs to total chlorophyll-a was higher in comparison to the previous bloom period; in “Low” it remained relatively stable (39-43%) during this period whereas in “High” it increased by more than 10% from Day-9 to Day-12 and then decreased again towards Day-15 (Fig. 2B-D). During this bloom the contribution of micro-autotrophs to total chlorophyll-a increased with time in both treatments (Fig. 2B-D). Concerning

the contribution of pico-autotrophs to total chlorophyll-a, it differed between the treatments, decreasing in “Low” and fluctuating in “High”.

At the beginning of the second bloom (Day-9), ANFs and *Synechococcus* showed increased biomass in both treatments (Fig. 5A-B). ANF biomass peaked on Day-9, with “High” presenting more than double the values in “Low”, while *Synechococcus* peaked later, on Day-12. As mentioned earlier (“bloom 1”), for ANFs the main effect of treatment was significant throughout the experiment. For *Synechococcus*, there were significant interaction effects of time and treatment ($\chi^2(2)=9.81$, $p<0.05$). Further, this significant difference was found for both “High” and “Low” when the interaction effect was broken down to contrasts ($b=0.002$, $t(50)=3.09$, $p<0.05$, $r=0.4$).

HPs were found to have a slightly increased biomass in both nutrient treatments on Day-9, after which their biomass decreased (Fig. 5D). For treatment “High”, HP biomass was found to be nearly double than in “Low”; however, only the interaction between treatment and time was significant ($\chi^2(9)=18.26$, $p<0.05$), indicating that HP biomass in “High” and “Low” over the days was significantly different to “Control” ($b=-0.0035$, $t(50)=-3.43$, $p<0.05$, $r=0.44$) and different between the two nutrient treatments ($b=0.0071$, $t(50)=3.68$, $p<0.05$, $r=0.46$).

HNFs peaked on Day-9 but decreased after that, like HP biomass (Fig. 5D). No significant differences among treatments were found for HNFs throughout the experiment (Fig. 5E). Likewise for Dinoflagellata biomass, the interaction effect of experimental time and treatment was significant ($\chi^2(9)=16.02$, $p<0.05$), with a peak in biomass on Day-12 in both treatments (Fig. 5G). Bacillariophyta biomass did not differ significantly between “Control” and nutrient treatments.

3.6 Relationships and interactions between plankton communities

361 The biotic relationship that emerged in the three treatments were examined using network
362 analysis. All three networks were formed around OTUs that were either exclusively found or
363 strongly present on Day-0 (Fig. S9) and the topological parameters of the three networks were of
364 similar values (Table S2).

365 Positive network links between autotrophs and between grazers (Table 3A i-ii; i.e. Autotr. $\leftrightarrow$
366 Autotr. and Grazer $\leftrightarrow$ Grazer) showed lower percentages while positive connections from
367 autotrophs and/or grazers to HP populations (Table 3A iv-v; i.e. Autotr. $\rightarrow$ HP and Grazer $\rightarrow$ HP
368 positive connections) showed higher percentages in “High”. Additionally, higher percentages of
369 negative relationships among the same trophic groups (Table 3B i-ii; i.e. Autotr. $\leftrightarrow$ Autotr. and
370 Grazer $\leftrightarrow$ Grazer) were also found in “High”.

371 The structure of the *Pelagibacter* and Rhodobacteraceae subnetworks, constructed in order to
372 further examine the biotic relationships, appeared fundamentally different (Fig. 6 and Table 4).
373 *Pelagibacter* participated in more relationships in the “High” network while Rhodobacteraceae
374 showed an opposite pattern and was found to participate in substantially fewer connections.
375 *Pelagibacter* in “Control” showed positive interactions with another *Pelagibacter*, a *Bathycoccus*
376 and a *Flavobacterium* OTU while, due to the increased number of connections in the “High”
377 network, specific edges were not visualized; however, only 4.2% of these relationships were
378 negative. Accordingly, Rhodobacteraceae participated in numerous relationships in “Control”
379 while in network “High” the Rhodobacteraceae OTU of interest directly participated only in
380 negative relationships. Treatment “Low” showed in both cases an intermediate structure
381 between the “Control” and “High” subnetworks.

382 Reverse patterns between *Pelagibacter* and Rhodobacteraceae subnetworks were found for most
383 of the topological parameters. The *Pelagibacter* network “High” showed the highest connectivity

(average no. of neighbors and density) and transitivity (which measures the probability for tight local connections) and the lowest likelihood for unevenness (heterogeneity and centralization) in comparison to “Control” and “Low”, while Rhodobacteraceae network “High” showed low connectivity and transitivity but the highest betweenness and the longest average path lengths. Furthermore, network “Control” of Rhodobacteraceae had the lowest betweenness and shortest average path lengths.

4. DISCUSSION

In this study we investigated the consequences of sudden large nutrient loads on plankton originating from an oligotrophic habitat, and further examined how plankton communities are affected by different levels of nutrient input. Our results showed that different levels of nutrient addition induced both quantitative and qualitative changes, thereby modifying the structure of prokaryotic and eukaryotic microbial plankton communities.

4.1 Nutrient addition altered the prokaryotic and eukaryotic community composition

Contrary to previous mesocosm experiments with nutrient addition (Baltar et al., 2016; S Fodelianakis et al., 2014; Øvreås et al., 2003), in this study we did find important changes in community composition at phyla, genera and OTU levels (Fig. 3-4 and S6-7). This difference in results could be due to the fact that the above-mentioned surveys investigated the effect of a lower nutrient amount compared to the present study. On the other hand, in Ly *et al.* (2014), where a high nutrient amount was added, important eukaryotic community composition variations were noted. Further, biomass and activity rates were altered in all the above-mentioned cases, as in the present experiment. Therefore, it seems that compositional changes depend on the level of nutrient disturbance whereas biomass and activity changes emerge even under mild nutrient disturbances. The compositional differences between the two nutrients levels

found in this study further support this notion and indicate that the level of nutrient disturbance is related to specific changes in community structure.

Overall, a transition from less active to fast-growing species with rapid metabolisms was expected as a result of nutrient addition (Chafee et al., 2018; Lin et al., 2016). The prokaryotic community was altered after nutrient addition, and different bacteria, favored by nutrient surplus, were associated with the nutrient treatments after Day-0 (Fig. S6). For instance, species like *Pelagibacter*, characteristic of oligotrophic conditions and adapted to less-dynamic lifestyles (for example, Teeling et al., 2016), were significant on Day-0 but were not after the nutrient manipulation. The disadvantage of *Pelagibacter* with nutrient addition has been reported elsewhere (Fodelianakis et al., 2014; Sebastián et al., 2012a). Bacteroidetes were importantly enhanced but did not seem to vary between nutrient treatments. Members of this phylum are typically found during blooms (Pinhassi et al., 2004; Teeling et al., 2016; Teira et al., 2010) and are favored by phytoplankton-derived organic matter (Teeling et al., 2012).

With time, the eukaryotic community shifted from Bacillariophyta- to Dinoflagellata-dominated, while Haptophytes also appeared. When an environmental disturbance occurs, the balance usually shifts towards more dynamic groups, such as Dinoflagellata and Haptophytes, that are capable of both autotrophic and heterotrophic growth (Lin et al., 2016). In the present case, Dinoflagellata and Haptophytes increased in all mesocosms regardless of the nutrient addition, and it was the phylum Cryptophytes that appeared to have increased in connection to nutrient addition. The shift towards smaller autotrophs (Haptophytes, Chrysophytes, Cryptophytes) after chronic eutrophication has been reported (Suikkanen et al., 2013); nevertheless, the shift in this case was quicker (from Day-0 to Day-6) and towards one phylum (Cryptophytes).

4.2 Different nutrient quantities caused different compositional changes in plankton

Notably, only treatment “High” was found to sustain increased bacterial populations distinctive of rapid metabolic rates, fast-growth capacities and efficient nutrient uptake, such as Rhodobacteraceae and Flavobacteriaceae (Chafee et al., 2018; Teeling et al., 2016). Bacterial community composition change towards members of the Roseobacter clade, which have higher dissolved nutrient requirements, has been reported for oligotrophic communities after phosphate input (Sebastián et al., 2012). Another major difference between nutrient treatments was the strong presence of Actinobacteria in treatment “High”. Several studies on natural and laboratory populations (Ballen-Segura et al., 2017; Jezbera et al., 2005; Pernthaler and Amann, 2005) report that members of the Actinobacteria escape HNF and ciliated grazers, either due to their small size (Jezbera et al., 2005) or because of gram+ cell wall properties (Jousset, 2012). Therefore, we may assume that the increased numbers of Actinobacteria in treatment “High” could be related to their escaping from massive grazing, especially because the decline in biomass at that time (Day-6) suggests grazing of plankton populations.

Interestingly, differentiation between the nutrient treatments was also found at the OTU level of the eukaryotic community, with different Dinoflagellata representative of each treatment (Fig. S7) and differences in relative abundance among treatments (Fig. 5). Different nutrient quantities seem to have been responsible for the subtle compositional differences among treatments; the different metabolic capabilities and rates of species allowed some to prevail under lower, and others under higher, nutrient conditions. Furthermore, a phylum-level alteration in the eukaryotic community was also evident due to Chlorophytes, which were only present in the “Low” treatment on Day-6.

4.3 Succession of plankton populations and food web responses after extremely high nutrient addition

Previous studies have shown that autotrophic communities differ along different trophic gradients: cyanobacteria (picoplankton) dominate at oligo-mesotrophic conditions and, alternatively, larger eukaryotic algae prevail in eutrophic states (Bec et al., 2011). In the current experiment, autotrophic nanoflagellates prevailed initially when nutrient concentrations peaked (Day-2-3) while all autotrophic groups (pico-, nano- and micro-) were favored later when nutrient concentrations were still elevated but nutrients had been partially consumed (after Day-6). Osmotrophs responded to both nutrient addition levels but the response magnitude varied between the two treatments; this fact dictated the observed differences in community composition (Fig. 3-4) and ecological relationships (section 4.5; Fig. 6; Table 4-5) in these two treatments.

Even though nutrient availability was greater in treatment “High”, during the first bloom chlorophyll-a increase was, curiously, similar to “Low”. Except for nutrient availability, there are other factors regulating nutrient uptake (cell size and shape, nutrient uptake affinity, rate of transport mechanisms), especially when nutrients are in excess (Lin et al., 2016); it seems that in the case of treatment “High”, autotrophs reached their maximum nutrient uptake capacity and consequently the upper limit of photosynthesis. The instant response of HNFs to nutrient change (Day-3) and the decrease in all plankton populations at the end of the first blooming period (Day-6) were common under both nutrient conditions. However, Ciliophora responded by an increase in biomass only in treatment “High” and were later succeeded by Dinoflagellata. Regardless of the trophic role of Ciliophora and Dinoflagellata, a clear succession pattern in micro-eukaryotes was found only under the higher nutrient conditions.

The situation during the second bloom was similar between treatments; all autotrophic groups were present but pico- and nano-autotrophs dominated chlorophyll-a. This is the case of a classic bottom-up control with pico- and nano-, followed by micro-autotrophs, competing for nutrient

uptake (Fuchs and Franks, 2010). In the “Low” treatment, this bloom continued with micro-autotrophs contributing increasingly to chlorophyll-a and elevated biomass as both Bacillariophyta and Dinoflagellata populations were enhanced. On the other hand, in the “High” treatment, the bloom continued with ANFs prevailing in chlorophyll-a contribution but not showing a particularly high biomass, which implies that their population was heavily grazed.

Heterotrophic prokaryotes (HP) were greatly favored under “High” nutrient addition; nutrient availability to picoplankton, as well as the massive biomass decline possibly resulting in DOM enrichment at the end of the previous bloom, were probable reasons for the enhanced HP population during the second bloom. Since during the first bloom the “High” treatment retained a higher overall biomass, the biomass loss at the termination of the bloom would have resulted in more DOM both in terms of quantity and quality, inducing thus the different HP response between treatments. Later in this bloom, low HP biomass with high bacterial productivity indicates a top-down control of this community in both treatments (Lynam et al., 2017).

4.4 Later responses of the water column variables

After Day-15, changes in the water chemistry and plankton biomass did not show any discernible grouping pattern related to the applied treatments. Since external events were kept at a minimum and there were no methodological alterations (change in protocols, instruments, users), it is reasonable to assume that even though until Day-15 nutrient addition was the major driver of observed changes, after that day stochastic processes induced the changes in plankton communities. Despite this unpredictable variability, a conspicuous difference between the two treatments was established; the decrease of the *Synechococcus* population below the detection limit in all replicates of the higher nutrient addition treatment.

4.5 Biotic interactions and ecological links between plankton communities

The overall OTU eLSA networks appeared similar in structure and topology among the three treatments. However, there were important discrepancies in the connections among the trophic groups; these discrepancies in trophic connections percentages (Table 3) strongly suggest that different biotic-biotic relationships and interactions were created after the nutrient addition of different quantities. In fact, contrasting ecological links arose between the two nutrient addition levels: 1) positive connections among the same trophic groups (Table 3A i-ii) that suggest mutual growth of populations (Faust and Raes, 2012) were stronger under the lower nutrient addition; in contrast to 2) more negative connections among the same trophic groups (Table 3B i-ii), which indicate increased competition (Faust and Raes, 2012), were developed under the higher nutrient conditions. As for positive relationships between different trophic groups (Table 3A iv-v), in the “High” nutrient treatment, the benefit of HPs by other groups was almost 10% more than in “Low”. This interaction is most probably related to the enhanced DOM release caused by the increased growth of autotrophs and/or grazers in the “High” nutrient treatment.

In addition to biotic connections between trophic groups, when focusing deeper on biotic connections around specific OTUs (*Pelagibacter* and Rhodobacteraceae), the two subnetworks were structured contrastingly (Fig. 6). Of the three *Pelagibacter* subnetworks, “High” was the most highly connected in terms of both overall and local connectivity. The reverse case was true for Rhodobacteraceae: the subnetwork “Control” was the most highly connected in comparison to “Low” and “High”. In both cases (*Pelagibacter* and Rhodobacteraceae), nutrient addition of different levels led to the formation of different ecological networks and indeed the amount of nutrients was substantial for the development of microbial inter-connections.

It has been argued that highly connected ecological networks are more robust and resilient (Dunne et al., 2002) and that robustness increases with connectivity irrespective of specific population abundance and biomass (Dunne et al., 2002; Genitsaris et al., 2016). In our data

Rhodobacteraceae appeared to form a more complex and robust network under no nutrient disturbance. Considering the rapid metabolism and competitive nature of this group (Chafee et al., 2018; Teeling et al., 2016), we suggest that given nutrient surplus, it outcompeted others, established its niche and no longer interacted with many microbes, as indicated by the low number of Rhodobacteraceae connections in subnetwork “High”. However, the low robustness of subnetwork “High”, combined with the fact that it arose only with certain changes in water chemistry (nutrient input), made the established ecological relationships weak and therefore likely to rapidly alter due to external influences. The several negative relationships found between Rhodobacteraceae and other autotrophs under both nutrient addition conditions are additional indications of the highly competitive life-strategy Rhodobacteraceae may have followed. On the other hand, *Pelagibacter* participated in a more robust ecological subnetwork in the highest nutrient addition. Bacteria in this group are known for slow metabolic capabilities and low, though stable, population sizes (Chafee et al., 2018). With nutrient surplus, this species did not seem to benefit from the extra nutrients due to its low metabolic capabilities but rather maintained its population by participating in a complex, highly connected network.

5. CONCLUSIONS & SUMMARY

The sudden high-concentration nutrient pulse caused blooms and plankton biomass elevations as expected; at the same time, diversity within plankton communities also changed and alterations were obvious even at the phyla level. Notably, differences in community composition were detected even between the different nutrient levels. Nano-autotrophs, rather than micro-autotrophs, were the first to take advantage of the nutrient addition.

Biotic relationships among populations varied significantly between the different nutrient concentrations. Increased competition connections characterized populations under higher

nutrient surplus whereas with a lower nutrient amount, populations appeared to co-exist. Further, distinctly different ecological networks were structured around specific bacteria under the different nutrient conditions, thus marking the diverse response of microorganisms to nutrient surpluses of different amounts.

To summarize, nutrient addition induced changes in community structure, biotic relationships and, consequently, important alterations in the entire plankton web function. The input of a high nutrient amount related to climate phenomena introducing nutrients in the marine environment, affected several aspects of the marine plankton community and changed not only the biomass and activity but also the composition and biotic links of populations.

FUNDING

This work was funded by the European Union and Greek national funds through the Operational Program "Education and Lifelong Learning" of the National Strategic Reference Framework (NSRF) – ARISTEIA II (HYPOXIA project, No 5381), and by King Abdullah University of Science and Technology (KAUST) through baseline funding to DD. The authors declare no conflict of interest. All authors have approved the final submitted manuscript.

ACKNOWLEDGEMENTS

This work was funded by the European Union and Greek national funds through the Operational Program "Education and Lifelong Learning" of the National Strategic Reference Framework (NSRF) – ARISTEIA II (HYPOXIA project, No 4705), and by King Abdullah University of Science and Technology (KAUST) through baseline funding to DD. We are grateful to the participants of the mesocosm experiment for their significant help during experimental setup and sampling: S. Diliberto, C. Frangoulis, A. Geropoulos, V. Kalogeropoulou, M. Kanellopoulou, M. Moraitis, and Tsikopoulou I. Special thanks are attributed to E. Dafnomili, M. Kagiorgi and S. Zivanovic for

assistance with dissolved nutrients analyses and G. Piperakis for inspiring technical assistance. J.B. Kristoffersen and C. Pavloudi are greatly acknowledged for valuable advising during molecular analyses.

AUTHOR CONTRIBUTIONS

IK, PP, NP, PDD designed the experiment. PP, IS, PDD, NP organized the experiment. PDD measured dissolved oxygen. NP measured photosynthetic pigments. IS performed plankton biomass determination analyses. AT measured bacterial productivity. IS, AT, SF, PK, DD performed molecular analyses. IS, SF, PK, DD performed bioinformatics workflow. IS interpreted and statistically analyzed the data. IS, AT, PP, IK wrote the manuscript with input from other authors.

REFERENCES

1. Amaral-Zettler, L.A., McCliment, E.A., Ducklow, H.W., Huse, S.M., 2009. A method for studying protistan diversity using massively parallel sequencing of V9 hypervariable regions of small-subunit ribosomal RNA Genes. *PLoS One* 4, e6372. <https://doi.org/10.1371/journal.pone.0006372>
2. Apprill, A., McNally, S., Parsons, R., Weber, L., 2015. Minor revision to V4 region SSU rRNA 806R gene primer greatly increases detection of SAR11 bacterioplankton. *Aquat. Microb. Ecol.* 75, 129–137. <https://doi.org/10.3354/ame01753>
3. Ballen-Segura, M., Felip, M., Catalan, J., 2017. Some Mixotrophic Flagellate Species Selectively Graze on Archaea. *Appl. Environ. Microbiol.* 83, e02317-16. <https://doi.org/10.1128/AEM.02317-16>
4. Baltar, F., Palovaara, J., Unrein, F., Catala, P., Horňák, K., Šimek, K., Vaqué, D., Massana, R., Gasol, J.M., Pinhassi, J., 2016. Marine bacterial community structure resilience to changes in protist predation under phytoplankton bloom conditions. *ISME J.* 10, 568–581. <https://doi.org/10.1038/ismej.2015.135>
5. Bec, B., Collos, Y., Souchu, P., Vaquer, A., Lautier, J., Fiandrino, A., Benau, L., Orsoni, V., Laugier, T., 2011. Distribution of picophytoplankton and nanophytoplankton along an anthropogenic

- eutrophication gradient in French Mediterranean coastal lagoons. *Aquat. Microb. Ecol.* 63, 29–45.
<https://doi.org/10.3354/ame01480>
6. Bracken, M.E.S., Hillebrand, H., Borer, E.T., Seabloom, E.W., Cebrian, J., Cleland, E.E., Elser, J.J.,
 Gruner, D.S., Harpole, W.S., Ngai, J.T., Smith, J.E., 2015. Signatures of nutrient limitation and co-
 limitation: Responses of autotroph internal nutrient concentrations to nitrogen and phosphorus
 additions. *Oikos* 124, 113–121. <https://doi.org/10.1111/oik.01215>
 7. Caron, D.A., Dam, H.G., Kremer, P., Lessard, E.J., Madin, L.P., Malone, T.C., Napp, J.M., Peele, E.R.,
 Roman, M.R., Youngbluth, M.J., 1995. The contribution of microorganisms to particulate carbon and
 nitrogen in surface waters of the Sargasso Sea near Bermuda. *Deep Sea Res. Part I Oceanogr. Res.*
Pap. 42, 943–972. [https://doi.org/http://dx.doi.org/10.1016/0967-0637\(95\)00027-4](https://doi.org/http://dx.doi.org/10.1016/0967-0637(95)00027-4)
 8. Chafee, M., Fernández-Guerra, A., Buttigieg, P.L., Gerdtts, G., Eren, A.M., Teeling, H., Amann, R.L.,
 2018. Recurrent patterns of microdiversity in a temperate coastal marine environment. *ISME J.* 12,
 237–252. <https://doi.org/10.1038/ismej.2017.165>
 9. Davidson, K., Roberts, E.C., Gilpin, L.C., 2002. The relationship between carbon and biovolume in
 marine microbial mesocosms under different nutrient regimes. *Eur. J. Phycol.* 37, 501–507.
<https://doi.org/10.1017/S096702620200389X>
 10. De Cáceres, M., Legendre, P., Moretti, M., 2010. Improving indicator species analysis by combining
 groups of sites. *Oikos* 119, 1674–1684. <https://doi.org/10.1111/j.1600-0706.2010.18334.x>
 11. Dimitriou, P.D., Papageorgiou, N., Geropoulos, A., Kalogeropoulou, V., Moraitis, M., Santi, I.,
 Tsikopoulou, I., Pitta, P., Karakassis, I., 2017a. A novel mesocosm setup for benthic-pelagic coupling
 experiments. *Limnol. Oceanogr. Methods* 15, 349–362. <https://doi.org/10.1002/LOM3.10163>
 12. Dimitriou, P.D., Papageorgiou, N., Geropoulos, A., Kalogeropoulou, V., Moraitis, M., Santi, I.,
 Tsikopoulou, I., Pitta, P., Karakassis, I., 2017b. Benthic pelagic coupling in a mesocosm experiment:
 Delayed sediment responses and regime shifts. *Sci. Total Environ.* 605–606, 637–645.
<https://doi.org/10.1016/J.SCITOTENV.2017.06.239>
 13. Doney, S.C., 2010. The growing human footprint on coastal and open-Ocean biogeochemistry.
Science (80-.). <https://doi.org/10.1126/science.1185198>

14. Dunne, J.A., Williams, R.J., Martinez, N.D., 2002. Network structure and biodiversity loss in food webs: Robustness increases with connectance. *Ecol. Lett.* 5, 558–567. <https://doi.org/10.1046/j.1461-0248.2002.00354.x>
15. Durno, W.E., Hanson, N.W., Konwar, K.M., Hallam, S.J., 2013. Expanding the boundaries of local similarity analysis. *BMC Genomics* 14, S3. <https://doi.org/10.1186/1471-2164-14-S1-S3>
16. Faust, K., Raes, J., 2012. Microbial interactions: from networks to models. *Nat Rev Micro* 10, 538–550.
17. Fodelianakis, S., Papageorgiou, N., Pitta, P., Kasapidis, P., Karakassis, I., Ladoukakis, E.D., 2014. The Pattern of Change in the Abundances of Specific Bacterioplankton Groups Is Consistent across Different Nutrient-Enriched Habitats in Crete. *Appl. Environ. Microbiol.* 80, 3784–3792. <https://doi.org/10.1128/aem.00088-14>
18. Fodelianakis, S., Pitta, P., Thingstad, T., Kasapidis, P., Karakassis, I., Ladoukakis, E., 2014. Phosphate addition has minimal short-term effects on bacterioplankton community structure of the P-starved Eastern Mediterranean. *Aquat. Microb. Ecol.* 72, 98–104. <https://doi.org/10.3354/ame01693>
19. Fuchs, H.L., Franks, P.J.S., 2010. Plankton community properties determined by nutrients and size-selective feeding. *Mar. Ecol. Prog. Ser.* <https://doi.org/10.2307/24875175>
20. Gasol, J.M., Del Giorgio, P.A., 2000. Using flow cytometry for counting natural planktonic bacteria and understanding the structure of planktonic bacterial communities. *Sci. Mar.* 64, 197–224. <https://doi.org/10.3989/scimar.2000.64n2197>
21. Genitsaris, S., Monchy, S., Breton, E., Lecuyer, E., Christaki, U., 2016. Small-scale variability of protistan planktonic communities relative to environmental pressures and biotic interactions at two adjacent coastal stations. *Mar. Ecol. Prog. Ser.* 548, 61–75. <https://doi.org/10.3354/meps11647>
22. Hallegraeff, G.M., 2010. Ocean climate change, phytoplankton community responses, and harmful algal blooms: A formidable predictive challenge. *J. Phycol.* 46, 220–235. <https://doi.org/10.1111/j.1529-8817.2010.00815.x>
23. Hillebrand, H., Dürselen, C.-D., Kirschtel, D., Pollingher, U., Zohary, T., 1999. Biovolume Calculation for Pelagic and Benthic Microalgae. *J. Phycol.* 35, 403–424. <https://doi.org/10.1046/j.1529-8817.1999.3520403.x>

- 648 24. Hirabayashi, Y., Mahendran, R., Koirala, S., Konoshima, L., Yamazaki, D., Watanabe, S., Kim, H., Kanae,
649 S., 2013. Global flood risk under climate change. *Nat. Clim. Chang.* 3, 816–821.
650 <https://doi.org/10.1038/nclimate1911>
- 651 25. Ivančič, I., Degobbis, D., 1984. An optimal manual procedure for ammonia analysis in natural waters
652 by the indophenol blue method. *Water Res.* 18, 1143–1147. [https://doi.org/10.1016/0043-](https://doi.org/10.1016/0043-1354(84)90230-6)
653 [1354\(84\)90230-6](https://doi.org/10.1016/0043-1354(84)90230-6)
- 654 26. Jezbera, J., Hornak, K., Simek, K., 2005. Food selection by bacterivorous protists: insight from the
655 analysis of the food vacuole content by means of fluorescence in situ hybridization. *FEMS Microbiol.*
656 *Ecol.* 52, 351–363.
- 657 27. Jousset, A., 2012. Ecological and evolutive implications of bacterial defences against predators.
658 *Environ. Microbiol.* 14, 1830–1843. <https://doi.org/10.1111/j.1462-2920.2011.02627.x>
- 659 28. Kana, T.M., Glibert, P.M., 2000. Effect of irradiances up to 2000 $\text{IaE m}^{-2} \text{s}^{-1}$ on marine *Synechococcus*
660 WH7803--I. Growth, pigmentation, and cell composition. *Deep. Res.* 34, 479–495.
- 661 29. Karydis, M., Kitsiou, D., 2012. Eutrophication and environmental policy in the Mediterranean Sea: A
662 review. *Environ. Monit. Assess.* <https://doi.org/10.1007/s10661-011-2313-2>
- 663 30. Klindworth, A., Pruesse, E., Schweer, T., Peplies, J., Quast, C., Horn, M., Glöckner, F.O., 2012.
664 Evaluation of general 16S ribosomal RNA gene PCR primers for classical and next-generation
665 sequencing-based diversity studies. *Nucleic Acids Res.* <https://doi.org/10.1093/nar/gks808>
- 666 31. Krishnamurthy, A., Moore, J.K., Mahowald, N., Luo, C., Zender, C.S., 2010. Impacts of atmospheric
667 nutrient inputs on marine biogeochemistry. *J. Geophys. Res.* 115, G01006.
668 <https://doi.org/10.1029/2009JG001115>
- 669 32. Krom, M.D., Groom, S., Zohary, T., 2003. The Eastern Mediterranean In: *The Biogeochemistry of*
670 *Marine Systems*. Blackwell Publishing, Oxford.
- 671 33. Lee Fuhrman, J. A., S., 1987. Relationships between Biovolume and Biomass of Naturally Derived
672 Marine Bacterioplankton. *Appl. Environ. Microbiol.* 53, 1298–1303.
- 673 34. Lejeusne, C., Chevaldonné, P., Pergent-Martini, C., Boudouresque, C.F., Pérez, T., 2010. Climate
674 change effects on a miniature ocean: the highly diverse, highly impacted Mediterranean Sea. *Trends*

- 675 Ecol. Evol. <https://doi.org/10.1016/j.tree.2009.10.009>
- 676 35. Lin, S., Litaker, R.W., Sunda, W.G., 2016. Phosphorus physiological ecology and molecular
677 mechanisms in marine phytoplankton. *J. Phycol.* 52, 10–36. <https://doi.org/10.1111/jpy.12365>
- 678 36. Ly, J., Philippart, C.J.M., Kromkamp, J.C., 2014. Phosphorus limitation during a phytoplankton spring
679 bloom in the western Dutch Wadden Sea. *J. Sea Res.* <https://doi.org/10.1016/j.seares.2013.12.010>
- 680 37. Lynam, C.P., Llope, M., Möllmann, C., Helaouët, P., Bayliss-Brown, G.A., Stenseth, N.C., 2017.
681 Interaction between top-down and bottom-up control in marine food webs. *Proc. Natl. Acad. Sci. U.*
682 *S. A.* 114, 1952–1957. <https://doi.org/10.1073/pnas.1621037114>
- 683 38. Madsen, H., Lawrence, D., Lang, M., Martinkova, M., Kjeldsen, T.R., 2014. Review of trend analysis
684 and climate change projections of extreme precipitation and floods in Europe. *J. Hydrol.* 519, 3634–
685 3650. <https://doi.org/10.1016/j.jhydrol.2014.11.003>
- 686 39. Marie, D., Partensky, F., Vaulot, D., Brussaard, C., 1999. Enumeration of phytoplankton, bacteria, and
687 viruses in marine samples, in: Robinson, J.P., Darzynkiewicz, Z., Dean, P.N., Orfao, A., Rabinovitch,
688 P.S., Stewart, C.C., Tanke, H.J., Wheelless, L.L. (Eds.), *Current Protocols in Cytometry*. John Wiley &
689 Sons, New York, pp. 1–15.
- 690 40. Martinez-Garcia, M., Brazel, D., Poulton, N.J., Swan, B.K., Gomez, M.L., Masland, D., Sieracki, M.E.,
691 Stepanauskas, R., 2012. Unveiling in situ interactions between marine protists and bacteria through
692 single cell sequencing. *ISME J.* 6, 703–707.
693 <https://doi.org/http://www.nature.com/ismej/journal/v6/n3/supinfo/ismej2011126s1.html>
- 694 41. Morel, A., Claustre, H., Gentili, B., 2010. The most oligotrophic subtropical zones of the global ocean:
695 Similarities and differences in terms of chlorophyll and yellow substance. *Biogeosciences* 7, 3139–
696 3151. <https://doi.org/10.5194/bg-7-3139-2010>
- 697 42. Mostajir, B., Le Floch, E., Mas, S., Pete, R., Parin, D., Nougier, J., Foulland, E., Vidussi, F., 2013. A
698 new transportable floating mesocosm platform with autonomous sensors for real-time data
699 acquisition and transmission for studying the pelagic food web functioning. *Limnol. Oceanogr.*
700 *Methods* 11, 394–409. <https://doi.org/10.4319/lom.2013.11.394>
- 701 43. Olenina, I., Hajdu, S., Edler, L., Andersson, A., Wasmund, N., Busch, S., Göbel, J., Gromisz, S., Huseby,

- S., Huttunen, M., Jaanus, A., Kokkonen, P., Ledaine, I., Niemkiewicz, E., 2006. Biovolumes and size-classes of phytoplankton in the Baltic Sea. *HELCOM Balt. Sea Environ. Proc.* No160, 144pp.
44. Øvreås, L., Bourne, D., Sandaa, R.-A., Casamayor, E.O., Benlloch, S., Goddard, V., Smerdon, G., Haldal, M., Thingstad, T.F., 2003. Response of bacterial and viral communities to nutrient manipulations in seawater mesocosms. *Aquat. Microb. Ecol.* 31, 109–121. <https://doi.org/10.3354/ame031109>
45. Pernthaler, J., Amann, R., 2005. Fate of Heterotrophic Microbes in Pelagic Habitats: Focus on Populations. *Microbiol. Mol. Biol. Rev.* 69, 440–461. <https://doi.org/10.1128/mmbr.69.3.440-461.2005>
46. Pinhassi, J., Sala, M.M., Havskum, H., Peters, F., Guadayol, Ò., Malits, A., Marrasé, C., 2004. Changes in bacterioplankton composition under different phytoplankton regimens. *Appl. Environ. Microbiol.* 70, 6753–6766. <https://doi.org/10.1128/AEM.70.11.6753-6766.2004>
47. Porter, K.G., Feig, Y.S., 1980. The use of DAPI for identifying and counting aquatic microflora. *Limnol. Oceanogr.* <https://doi.org/10.4319/lo.1980.25.5.0943>
48. Powley, H.R., Cappellen, P. Van, Krom, M.D., 2017. Nutrient Cycling in the Mediterranean Sea: The Key to Understanding How the Unique Marine Ecosystem Functions and Responds to Anthropogenic Pressures, in: Fuerst-Bjelis, B. (Ed.), *Mediterranean Identities - Environment, Society, Culture*. InTech, pp. 47–77. <https://doi.org/10.5772/intechopen.70878>
49. Powley, H.R., Dürr, H.H., Lima, A.T., Krom, M.D., Van Cappellen, P., 2016. Direct Discharges of Domestic Wastewater are a Major Source of Phosphorus and Nitrogen to the Mediterranean Sea. *Environ. Sci. Technol.* 50, 8722–8730. <https://doi.org/10.1021/acs.est.6b01742>
50. Putt, M., Stoecker, D.K., 1989. An experimentally determined carbon : volume ratio for marine “oligotrichous” ciliates from estuarine and coastal waters. *Limnol. Oceanogr.* <https://doi.org/10.4319/lo.1989.34.6.1097>
51. Richa, K., Balestra, C., Piredda, R., Benes, V., Borra, M., Passarelli, A., Margiotta, F., Saggiomo, M., Biffali, E., Sanges, R., Scanlan, D.J., Casotti, R., 2017. Distribution, community composition and potential metabolic activity of bacterioplankton in an urbanized Mediterranean Sea coastal zone. *Appl. Environ. Microbiol.* 83, AEM.00494-17. <https://doi.org/10.1128/AEM.00494-17>

- 729 52. Rimmelin, P., Moutin, T., 2005. Re-examination of the MAGIC method to determine low
730 orthophosphate concentration in seawater. *Anal. Chim. Acta* 548, 174–182.
731 <https://doi.org/10.1016/j.aca.2005.05.071>
- 732 53. Ruan, Q., Dutta, D., Schwalbach, M.S., Steele, J.A., Fuhrman, J.A., Sun, F., 2006. Local similarity
733 analysis reveals unique associations among marine bacterioplankton species and environmental
734 factors. *Bioinformatics* 22, 2532–2538. <https://doi.org/10.1093/bioinformatics/btl417>
- 735 54. Sebastián, M., Pitta, P., González, J.M., Thingstad, T.F., Gasol, J.M., 2012. Bacterioplankton groups
736 involved in the uptake of phosphate and dissolved organic phosphorus in a mesocosm experiment
737 with P-starved Mediterranean waters. *Environ. Microbiol.* 14, 2334–2347.
738 <https://doi.org/10.1111/j.1462-2920.2012.02772.x>
- 739 55. Shokralla, S., Porter, T.M., Gibson, J.F., Dobosz, R., Janzen, D.H., Hallwachs, W., Golding, G.B.,
740 Hajibabaei, M., 2015. Massively parallel multiplex DNA sequencing for specimen identification using
741 an Illumina MiSeq platform. *Sci. Rep.* 5, 9687. <https://doi.org/10.1038/srep09687>
- 742 56. Smith, V.H., Schindler, D.W., 2009. Eutrophication science: where do we go from here? *Trends Ecol.*
743 *Evol.* 24, 201–207. <https://doi.org/http://dx.doi.org/10.1016/j.tree.2008.11.009>
- 744 57. Strickland, J.D.H., Parsons, T.R., 1972. A Practical Handbook of Seawater Analysis. *A Pract. Handb.*
745 *seawater Anal.* 167, 185. <https://doi.org/10.1002/iroh.19700550118>
- 746 58. Suikkanen, S., Pulina, S., Engström-Öst, J., Lehtiniemi, M., Lehtinen, S., Brutemark, A., 2013. Climate
747 Change and Eutrophication Induced Shifts in Northern Summer Plankton Communities. *PLoS One* 8,
748 e66475. <https://doi.org/10.1371/journal.pone.0066475>
- 749 59. Teeling, H., Fuchs, B.M., Becher, D., Klockow, C., Gardebrecht, A., Bennke, C.M., Kassabgy, M., Huang,
750 S., Mann, A.J., Waldmann, J., Weber, M., Klindworth, A., Otto, A., Lange, J., Bernhardt, J., Reinsch, C.,
751 Hecker, M., Peplies, J., Bockelmann, F.D., Callies, U., Gerdt, G., Wichels, A., Wiltshire, K.H., Glöckner,
752 F.O., Schweder, T., Amann, R., 2012. Substrate-Controlled Succession of Marine Bacterioplankton
753 Populations Induced by a Phytoplankton Bloom. *Science (80-.).* 336, 608–611.
754 <https://doi.org/10.1126/science.1218344>
- 755 60. Teeling, H., Fuchs, B.M., Bennke, C.M., Krüger, K., Chafee, M., Kappelmann, L., Reintjes, G.,

- Waldmann, J., Quast, C., Glöckner, F.O., Lucas, J., Wichels, A., Gerdt, G., Wiltshire, K.H., Amann, R.L., 2016. Recurring patterns in bacterioplankton dynamics during coastal spring algae blooms. *Elife* 5. <https://doi.org/10.7554/eLife.11888>
61. Teira, E., Martínez-García, S., Calvo-Díaz, A., Morán, X.A.G., 2010. Effects of inorganic and organic nutrient inputs on bacterioplankton community composition along a latitudinal transect in the Atlantic Ocean. *Aquat. Microb. Ecol.* 60, 299–313. <https://doi.org/10.3354/ame01435>
62. Thyssen, M., Grégori, G.J., Grisoni, J.M., Pedrotti, M.L., Mousseau, L., Artigas, L.F., Marro, S., Garcia, N., Passafiume, O., Denis, M.J., 2014. Onset of the spring bloom in the northwestern Mediterranean Sea: Influence of environmental pulse events on the in situ hourly-scale dynamics of the phytoplankton community structure. *Front. Microbiol.* 5, 387. <https://doi.org/10.3389/fmicb.2014.00387>
63. Tsagaraki, T.M., Pitta, P., Frangoulis, C., Petihakis, G., Karakassis, I., 2013. Plankton response to nutrient enrichment is maximized at intermediate distances from fish farms. *Mar. Ecol. Prog. Ser.* 493, 31–42. <https://doi.org/10.3354/meps10520>
64. Utermöhl, von H., 1931. Neue Wege in der quantitativen Erfassung des Planktons. (Mit besondere Berücksichtigung des Ultraplanktons). *Verhandlungen der Int. Vereinigung für Theor. und Angew. Limnol.* 5, 567–595.
65. Xia, L.C., Steele, J.A., Cram, J.A., Cardon, Z.G., Simmons, S.L., Vallino, J.J., Fuhrman, J.A., Sun, F., 2011. Extended local similarity analysis (eLSA) of microbial community and other time series data with replicates. *BMC Syst. Biol.* 5, S15. <https://doi.org/10.1186/1752-0509-5-S2-S15>
66. Yentsch, C.S., Menzel, D.W., 1963. A method for the determination of phytoplankton chlorophyll and phaeophytin by fluorescence. *Deep. Res. Oceanogr. Abstr.* 10, 221–231. [https://doi.org/10.1016/0011-7471\(63\)90358-9](https://doi.org/10.1016/0011-7471(63)90358-9)
67. Yoshimura, T., Nishioka, J., Ogawa, H., Kuma, K., Saito, H., Tsuda, A., 2014. Dissolved organic phosphorus production and decomposition during open ocean diatom blooms in the subarctic Pacific. *Mar. Chem.* 165, 46–54. <https://doi.org/10.1016/j.marchem.2014.08.003>

TABLES

Table 1: Initial nutrient conditions and nutrient amendment at the “Low” and “High” treatments. Concentrations were measured prior (Day-0), and, immediately after manipulation. Concentrations of nitrate and nitrite, as dissolved N, and phosphate, as dissolved P, were used for the N:P ratio calculations. Averaged and standard deviation values, calculated from the three replicated mesocosms, are shown.

	Prior manipulation	After manipulation	
		Low	High
NO ₃ ⁻ (μM)	0.09 ± 0.01	26.02 ± 1.32	55.98 ± 0.29
PO ₄ ³⁻ (μM)	0.03 ± 0.00	5.59 ± 0.86	8.56 ± 0.86
N:P	3.2 ± 0.52	4.7 ± 0.70	6.5 ± 0.03

Table 2: Nutrient flux with time for the three treatments (“Control”, “Low” and “High”) calculated from the slope between two succeeding time points. Values are in μM per day with negative sign indicating consumption of nutrients.

		Day-2→Day-3	Day-3→Day-6	Day-6→Day-9	Day-9→Day-12	Day-12→Day-15
NO_3^-	Control	0.07	0.02	-0.02	-0.02	0.00
	Low	-3.41	-0.59	-3.83	-1.08	0.22
	High	-8.87	-0.37	-5.94	-2.83	-0.14
PO_4^{3-}	Control	0.02	0.02	-0.02	-0.01	0.00
	Low	-0.20	-0.13	-0.83	-0.04	-0.01
	High	-0.50	-0.22	-0.79	-0.42	-0.22

826

827

828

Table 3: Connections among the different trophic groups as calculated from network analysis based on the eLSA algorithm. Percentages of the total positive/negative connections within each network ("Control", "Low" and "High") are presented. Connections that represented less than 0.5% are not shown here.

	Control	Low	High
A. Positive Connections (%)			
i. Autotr. $\leftrightarrow$ Autotr.	33.5	38.7	27.5
ii. Grazer $\leftrightarrow$ Grazer	26.4	26	13.2
iii. HP $\leftrightarrow$ HP	2.5	2.8	5.3
iv. Autotr. $\rightarrow$ HP	19.6	17.5	27.3
v. Grazer $\rightarrow$ HP	17.4	14.2	26.1
B. Negative Connections (%)			
i. Autotr. $\leftrightarrow$ Autotr.	29.3	17.5	54.3
ii. Grazer $\leftrightarrow$ Grazer	1	2.4	15.1
iii. Grazer $\rightarrow$ HP	54.2	66.4	20.1
iv. Grazer $\rightarrow$ Autotr.	6.2	6.4	9.4
v. Parasites $\rightarrow$ any organism	9.1	6.4	0.5

829

830

831

832

833

834

835

836

837

838

839

840

841

842

843

844

845

846

847

848

Table 4: Topological parameters characterizing the *Pelagibacter* and *Rhodobacteraceae* ecological subnetworks.

	<i>Pelagibacter</i>			<i>Rhodobacteraceae</i>		
	Control	Low	High	Control	Low	High
Nodes	7	12	94	13	9	6
Edges	12	44	4371	75	30	13
Negative edges (%)	58.3	29.5	4.2	45.3	26.7	38.5
Diameter	2	2	1	2	2	2
<i>Connectivity</i>						
Average No of Neighbors	3.43	7.33	93	11.54	6.67	4.33
Density	0.28	0.33	0.50	0.48	0.42	0.43
<i>Likelihood for unevenness</i>						
Heterogeneity	0.21	0.13	0	0.007	0.062	0.041
Centralization	0.25	0.18	0	0.02	0.09	0.08
<i>Small-world properties</i>						
Transitivity	0.73	0.82	1	0.97	0.89	0.87
Betweenness	0	0.08	0	0.008	0.033	0.040

849

850

851 **FIGURES**

852

853 Figure 1: Mean concentration (± standard deviation) of dissolved inorganic nutrients (A.-E.) and dissolved oxygen (F.)
 854 throughout the experiment. G-H: Temperature and sunlight illuminance measured continuously during the experiment.

855

856

857

858

859

860

861

862

Figure 2: A: Temporal change of chlorophyll-a concentration throughout the experiment. Averaged values and standard deviation error bars, calculated from the three replicated mesocosms, are presented. B-D: contribution of the different size fractions to total chlorophyll-a concentration at the different treatments.

863

864

865

866 Figure 3: Composition of bacterial (A) and eukaryotic (B) communities presented as relative abundance of each phylum.
867 Relative abundance was calculated as percentage of final total reads per sample. Percentages correspond to averaged
868 values of each treatments' replicates. Bacterial phylum Proteobacteria is analyzed to the respective classes: Alpha-,
869 Beta-, Delta- and Gamma-Proteobacteria. Category "Other" comprises the sum of phyla with relative abundance less
870 than 5% at all cases. For bacterial community those phyla were: Acidobacteria, Armatimonadetes, Bacteria-ZB3,
871 Chlamydiae, Chlorobi, Dependientiae, Elusimicrobia, Fusobacteria, Gracilibacteria, Lentisphaerae, Marinimicrobia,
872 Microgenomates, Omnitrphica, Parcubacteria, unass. Bacteria, Verrucomicrobia and Tenericutes. For eukaryotic
873 community those phyla were: Amoebozoa, Bigyra, Cercozoa, Hyphochytriomycota, Ochrophyta, Oomycota and
874 Rhodophyta. C: Control, L: Low, H: High.

875

A. Bacterial Community - Genera

B. Eukaryotic Community - Genera

Figure 4: Relative abundance heatmaps of the most representative bacterial (A) and eukaryotic (B) phyla. The phyla that noted more than 10% relative abundance at least once during the experiment were selected and the OTUs of each phylum were summed to the genus level. Taxonomic affiliation at the lowest possible level for each genus group, is shown. The color scale represents the relative abundance calculated as percentage of final total reads per sample. Percentages correspond to averaged values of each treatments' replicates. C: Control, L: Low, H: High.

Figure 5: Biomass of the different planktonic groups throughout the experiment. Averaged values and standard deviation error bars, calculated from the three replicated mesocosms, are presented. A: *Synechococcus* biomass, B: Autotrophic nanoflagellate biomass, C: Bacillariophyta biomass, D: Heterotrophic prokaryote biomass, E: Heterotrophic nanoflagellate biomass, F: Ciliophora biomass and G: Dinoflagellata biomass. In G. the y-axis is broken for better visualization from 0.035 to 0.099 mg C ml^{-1} ; however no measured values are hidden because of y-axis break.

Figure 6: Network diagrams for two selected OTUs. Nodes represent OTUs; connections (edges) have been calculated with the eLSA algorithm; dashed lines correspond to negative relationships. Only significant relationships ($p < 0.05$) with $-0.66 > \text{LSA} > 0.66$ are presented. Square symbol is used for the bacterial and circle for eukaryotic OTUs. The *Pelagibacter* (A-C) and *Rhodobacteraceae* (D-F) OTUs around which the networks were build, are represented by the star symbol. Different colors represent the different taxonomic groups. The high number of total edges in network C did not allow clear visualization of individual edges.

The response of microbial communities of oligotrophic coastal water to inorganic N and P additions: Novel insights from prokaryote and eukaryote metabarcoding and network analysis.

Ioulia Santi^{a,b}, Anastasia Tsiola^{a,b}, Panagiotis D. Dimitriou^b, Stilianos Fodelianakis^c, Panagiotis Kasapidis^d, Nafsika Papageorgiou^b, Daniele Daffonchio^c, Paraskevi Pitta^{a*}, Ioannis Karakassis^b

^a Institute of Oceanography, Hellenic Centre for Marine Research (HCMR), PO Box 2214, Heraklion 71003, Crete, Greece

^b Department of Biology, University of Crete, Voutes Campus, Heraklion 71003, Crete, Greece

^c Biological & Environmental Sciences & Engineering Division, King Abdullah University of Science & Technology, Thuwal 23955-6900, Saudi Arabia

^d Institute of Marine Biology, Biotechnology & Aquaculture, Hellenic Centre for Marine Research, PO Box 2214, 71003 Heraklion, Crete, Greece

* Corresponding author: Paraskevi Pitta. E-mail address: vpitta@hcmr.gr. Postal address: Hellenic Centre for Marine Research, PO Box 2214, Heraklion 71003, Greece

SUPPLEMENTARY INFORMATION

Water column chemical variables

Sampled seawater for particulate organic carbon (POC) and nitrogen (PON) determination was filtered on pre-combusted glass fiber filters and stored at -20°C until analysis. Analysis was performed as described in Hedges and Stern (1984).

Bacterial biomass productivity rate

Biomass production from heterotrophic bacteria was determined using the ³H-leucine protocol according to Kirchmann *et al.* (1986) with incorporating the later modifications of Smith and Azam (1992).

Sequence data processing

Raw sequences from the 16S rRNA sequencing were processed using UPARSE v82 (Edgar, 2013) algorithm and QIIME v1.93 (Caporaso *et al.*, 2012). Paired-reads were assembled with fastq-join algorithm of QIIME (Caporaso *et al.*, 2012) allowing for maximum 10% dissimilar alignment with an overlap of at least 50 nucleotides. Only sequences with mean Phred quality score higher than 20 were kept. After primers were trimmed, UPARSE (Edgar, 2013) was used for de-replication and operational taxonomic units (OTUs) clustering at >97% similarity and de novo chimera detection. An additional chimera control using Gold database (<https://gold.jgi.doe.gov/>) was performed. Taxonomy was assigned with QIIME (Caporaso *et al.*, 2012) using reference datasets from Greengenes (<http://greengenes.secondgenome.com/>) with the default uclust method and with at least 6 true reference database hits.

For the 18S rRNA dataset, raw sequence reads were processed using the OBITools metabarcoding software suite (Boyer et al., 2016). Sequences were trimmed to a median Phred quality score higher than 40, paired-reads were assembled with at least 50 nucleotides overlapping and pairs of higher than 40 alignment quality score were selected. Length filter was applied so that, only sequences of length between 80 and 200 bp were selected. Sequences were de-replicated with subsequent removal of sequences occurring only once in the dataset. PCR and sequencing errors, as well as chimeras, were removed using the obiclean algorithm of OBITools that kept sequences not having related sequences counting more than 5% of their count. Clustering to OTUs was performed using the SWARM (v2), a stepwise deterministic aggregation algorithm (Mahé et al., 2015). Taxonomy was assigned with the ecotag algorithm (Boyer et al., 2016) using NCBI (<https://www.ncbi.nlm.nih.gov/taxonomy>) as taxonomic reference. Reference sequences were acquired from the EMBL-EBI databank (<https://www.ebi.ac.uk/>) and, subsequently, the 18S amplicon was targeted with *in silico* ecoPCR (Ficetola et al., 2010) using the primers used for the actual PCR (Amaral-Zettler et al., 2009).

Finally, both datasets were refined by blank correction, removal of false positive results due to random index swap (Wangenstein and Turon, 2016), minimal abundance filtering (removal of OTUs with less than 5 reads), and contaminant removal (Wangenstein and Turon, 2016). Archaea sequences were removed from the 16S dataset since specific primers did not target them. Because we were interested only in nano- and micro- protists from the eukaryotic dataset, Metazoa and Fungi sequences were removed. Samples with fewer than 10,000 final reads were deleted from both datasets. Metrics of sequencing data, as well as, diversity indices are presented in Table S1. In text, sometimes the term species is used; however this is only for reasons of clarity and, in fact, OTUs are meant.

Data analysis and statistics

For the mixed-effect linear model, mesocosm identity (i.e. mesocosm unit) was considered as the random effect, thus accounting for a within-mesocosm nested design, while treatment, experimental day and their interaction were the fixed factors; maximum likelihood fit was used for the estimations. Each model was progressively build with the inclusion of a single predictor each time starting from a baseline with no predictors; the variances of the models were compared and the model that significantly improved the fit most was selected for each response variable. Planned contrasts were set for the treatments in order to identify the obtained differences (control versus nutrient treatments and “High” versus “Low”). Effect size (r) was calculated when contrasts found significant differences.

In the PERMANOVA, the data were considered nested within mesocosm identity (i.e. mesocosm unit) and Bray-Curtis dissimilarity matrices were produced from the datasets. Planned orthogonal contrasts were set to test from which treatment level the differences were caused (control versus nutrient treatments and “High” versus “Low”). NMDS scaling was performed at three dimensions and the minimum number of iterations was set to 20. The number of permutations was 999 for all permutational analyses. For PERMANOVA and nMDS analysis, data were square-root transformed. For the multi-level pattern analysis, samples for Day-0 were considered as one

977 separate group reflecting the situation prior to manipulation (De Cáceres et al., 2010). Only
978 indicator value indices higher than 89% and with p-values<0.05 are presented.

979 A separate network analysis was applied for each nutrient treatment and the control, with time
980 points being the different experimental days. To our knowledge, LSA (Ruan et al., 2006) is the only
981 algorithm that can account for time lags between the different samples. Equal number of the
982 most abundant, the most common and the most variable OTUs among samples were included in
983 the analysis. The selection of OTUs that participated in the analysis was recommended by several
984 peer-viewed publications (for example: Gilbert *et al.*, 2011; Fuhrman *et al.*, 2015). Only significant
985 positive (eLSA>0.66) and significant negative (eLSA<-0.66) associations were considered for the
986 network construction. Concerning the specific relationships encompassed in the networks, we
987 calculated the number of relationships between specific groups according to their metabolic
988 potentials as described in literature (for example: Genitsaris *et al.*, 2016). Therefore, taxonomic
989 groups were assigned to specific trophic group as follows: 1) autotrophs: Bacillariophyta,
990 Chlorophyta, Cyanobacteria, Ochrophyta, Rhodobacterales (Alphaproteobacteria), Rhodophyta,
991 Rhodospirillales (Alphaproteobacteria); 2) mixotrophs: Ciliophora, Cryptophytes, Dinoflagellata,
992 Haptophytes; 3) grazers: Amoebozoa, Bigyra; 4) heterotrophic prokaryotes: Actinobacteria,
993 Alphaproteobacteria (except Rhodobacterales and Rhodospirillales), Bacteroidetes,
994 Betaproteobacteria, Gammaproteobacteria, Planctomycetes; 5) parasites: Cercozoa,
995 Hyphochytriomycota, Labyrinthulomycetes, Oomycota. Mixotrophs were included in
996 relationships corresponding to both autotrophs and grazers.

997 The prokaryotic and eukaryotic datasets were processed individually at all cases, due to the
998 differences at the dataset's acquisition. Package nlme v3.1-131 (Pinheiro et al., 2018) was used
999 for the mixed-model approach, package vegan v2.4-4 (Oksanen et al., 2017) for the multivariate
1000 community methods (PERMANOVA, nMDS, Mantel) and the indicpecies (De Cáceres and
1001 Legendre, 2009) package was used for the indicator species analysis. Network analysis was
1002 conducted with the igraph package (Csárdi and Nepusz, 2006).

Table S1: Sequence processing statistics (number of final sequences and OTUs) and alpha diversity estimations: Shannon-Wiener's diversity (H) and Pielou's evenness (J) indices, in the bacterial and eukaryotic datasets. Averaged and standard deviation values calculated from the replicated mesocosms are presented. In parenthesis is the number of replicates used for the calculations in each case after the removal of samples with less than 10,000 final reads at the end of sequences processing.

		Final No of spequences	No of OTUs	Shannon-Wiener Index (H)	Piellou's Index (J)		
Bacteria	Day-0	Control (3)	57428 ± 4085	137 ± 11	3.1 ± 0.3	0.7 ± 0.1	
		Low (2)	36657 ± 2902	142 ± 2	3.0 ± 0.2	0.6 ± 0.1	
		High (2)	102470 ± 523	147 ± 8	3.2 ± 0.1	0.7 ± 0.0	
	Day-6	Control (2)	25595 ± 1916	117 ± 11	2.9 ± 0.3	0.7 ± 0.1	
		Low (2)	29985 ± 5870	104 ± 27	3.0 ± 0.1	0.7 ± 0.1	
		High (2)	27336 ± 693	195 ± 57	3.0 ± 0.2	0.6 ± 0.1	
	Day-12	Control (3)	30729 ± 3275	182 ± 5	2.7 ± 0.2	0.5 ± 0.0	
		Low (3)	20648 ± 3241	173 ± 6	2.7 ± 0.6	0.5 ± 0.1	
		High (2)	13110 ± 1199	165 ± 6	2.9 ± 0.1	0.5 ± 0.0	
	Eukaryotes	Day-0	Control (3)	56456 ± 4655	304 ± 33	2.6 ± 0.2	0.4 ± 0.0
			Low (3)	65711 ± 4021	340 ± 13	2.8 ± 0.5	0.4 ± 0.1
			High (2)	59549 ± 3607	335 ± 10	2.9 ± 0.3	0.5 ± 0.1
Day-6		Control (2)	12908 ± 739	82 ± 38	3.5 ± 0.4	0.7 ± 0.0	
		Low (3)	12027 ± 1801	79 ± 7	3.5 ± 0.3	0.7 ± 0.1	
		High (2)	33113 ± 842	432 ± 72	4.2 ± 0.3	0.7 ± 0.1	
Day-12		Control (3)	26927 ± 3628	320 ± 24	3.0 ± 0.5	0.5 ± 0.1	
		Low (3)	39115 ± 1357	326 ± 84	2.5 ± 0.5	0.4 ± 0.2	
		High (2)	50056 ± 2393	404 ± 24	2.6 ± 0.3	0.4 ± 0.0	

Table S2: Topological parameters characterizing the three ecological networks in figure S9.

	Control	Low	High
Nodes	215	198	198
Edges	6406	5786	5805
Negative edges (%)	8.8	5.2	7.8
Diameter	17	19	24
<i>Connectivity</i>			
Average No of Neighbors	59.59	58.44	58.64
Density	0.14	0.15	0.30
<i>Likelihood for unevenness</i>			
Heterogeneity	0.03	0.02	0.03
Centralization	0.11	0.11	0.12
<i>Small-world properties</i>			
Transitivity	0.98	0.98	0.98
Betweenness	0.01	0.008	0.02
Average path length	1.26	1.27	1.28

1009

1010

1011

1012

1013 Figure S1: Concentration of dissolved inorganic nutrients (A.-E.) and dissolved oxygen (F.) from Day-15 until
 1014 the end of the experiment. Averaged values and standard deviation error bars, calculated from the three
 1015 replicated mesocosms, are presented. G-H: Temperature and sunlight illuminance measured continuously
 1016 from Day-15 until the end of the experiment.

1017

1018

1019 Figure S2: Chlorophyll-a concentration from Day-15 to the end of the experiment. Averaged values and
 1020 standard deviation error bars, calculated from the three replicated mesocosms, are presented.

1021

1022

1023 Figure S3: Ratio of particulate organic carbon to nitrogen (POC:PON) throughout the experiment.

1024

Figure S4: Biomass of the different planktonic groups from experimental day-15 until experiments' termination. Averaged values and standard deviation error bars, calculated from the three replicated mesocosms, are presented.

Figure S5: Bacterial productivity rate in μg carbon per ml per hour during the experiment.

Bacterial Community - OTUs (indicative)

A. D0 (Control, Low, High)

B. Control (during blooms)

C. Low (during blooms)

D. High (during blooms)

E. Low & High (during blooms)

Figure S6: Significantly associated bacterial OTUs to the different treatments according to the indicator species analysis. Taxonomic association at the lowest possible level for each OTU, is shown. Only OTUs that noted indicator value indices higher than 89% and with p-values<0.05 are presented. Relative abundance calculated as percentage of final total reads per sample. A: Relative abundance of OTUs significantly associated to Day-0 throughout the experiment; Day-0 was not separated into treatments since PERMANOVA did not reveal any significant differences among treatments. B: Relative abundance of OTUs significantly associated to "Control" throughout the experiment. C: Relative abundance of OTUs significantly associated to treatment "Low" throughout the experiment. D: Relative abundance of OTUs significantly associated to treatment "High" throughout the experiment. E: Relative abundance of OTUs significantly associated to both nutrient treatments ("Low" & "High") throughout the experiment.

Eukaryotic Community - OTUs (indicative)

A. D0 (Control, Low, High)

B. Control (during blooms)

C. Low (during blooms)

D. High (during blooms)

E. Low & High (during blooms)

1045

1046 Figure S7: Significantly associated eukaryotic OTUs to the different treatments according to the indicator
 1047 species analysis. Taxonomic association at the lowest possible level for each OTU, is shown. Only OTUs that
 1048 noted indicator value indices higher than 89% and with p-values<0.05 are presented. Relative abundance
 1049 calculated as percentage of final total reads per sample. A: Relative abundance of OTUs significantly
 1050 associated to Day-0 throughout the experiment; Day-0 was not separated into treatments since
 1051 PERMANOVA did not reveal any significant differences among treatments. B: Relative abundance of OTUs
 1052 significantly associated to "Control" throughout the experiment. C: Relative abundance of OTUs
 1053 significantly associated to treatment "Low" throughout the experiment. D: Relative abundance of OTUs
 1054 significantly associated to treatment "High" throughout the experiment. E: Relative abundance of OTUs
 1055 significantly associated to both nutrient treatments ("Low" & "High") throughout the experiment.

A. Bacterial Community - OTUs (nMDS)

B. Eukaryotic community - OTUs (nMDS)

Figure S8: Non-metric multidimensional scaling (nMDS) for the bacterial (A) and the eukaryotic (B) OTUs. Different symbols are used for the different time periods, while color corresponds to treatments. Samples belonging to prior effect period and control samples from each bloom period are represented grouped. Bray-Curtis dissimilarity matrices were used in both cases. Stress values are noted on the plots.

Figure S9: Network diagram for the three treatments “Control” (A), “Low” (B), “High” (C). Nodes represent OTUs. Connections (edges) have been calculated with the eLSA algorithm; dashed lines correspond to negative relationships. Only significant relationships ($p < 0.05$) with $-0.66 > \text{LSA} > 0.66$ are presented. Square symbol is used for the bacterial and circle for eukaryotic OTUs. Different colors represent the different taxonomic groups. Concurrent from time-shifted relationships are not discriminated, as the focus is on the interactions developed in each treatment.

REFERENCES

1. Amaral-Zettler, L.A., McCliment, E.A., Ducklow, H.W., Huse, S.M., 2009. A method for studying protistan diversity using massively parallel sequencing of V9 hypervariable regions of small-subunit ribosomal RNA Genes. *PLoS One* 4, e6372. <https://doi.org/10.1371/journal.pone.0006372>
2. Boyer, F., Mercier, C., Bonin, A., Le Bras, Y., Taberlet, P., Coissac, E., 2016. obitools: A unix-inspired software package for DNA metabarcoding. *Mol. Ecol. Resour.* 16, 176–182. <https://doi.org/10.1111/1755-0998.12428>
3. Caporaso, J.G., Lauber, C.L., Walters, W.A., Berg-Lyons, D., Huntley, J., Fierer, N., Owens, S.M., Betley, J., Fraser, L., Bauer, M., Gormley, N., Gilbert, J.A., Smith, G., Knight, R., 2012. Ultra-high-throughput microbial community analysis on the Illumina HiSeq and MiSeq platforms. *ISME J.* 6, 1621–1624. <https://doi.org/http://www.nature.com/ismej/journal/v6/n8/supinfo/ismej20128s1.html>
4. Csárdi, G., Nepusz, T., 2006. The igraph software package for complex network research. *InterJournal Complex Syst.* 1695, 1–9. <https://doi.org/citeulike-article-id:3443126>
5. De Cáceres, M., Legendre, P., 2009. Associations between species and groups of sites: Indices and statistical inference. *Ecology* 90, 3566–3574. <https://doi.org/10.1890/08-1823.1>
6. De Cáceres, M., Legendre, P., Moretti, M., 2010. Improving indicator species analysis by combining groups of sites. *Oikos* 119, 1674–1684. <https://doi.org/10.1111/j.1600-0706.2010.18334.x>
7. Edgar, R.C., 2013. UPARSE: Highly accurate OTU sequences from microbial amplicon reads. *Nat. Methods* 10, 996–998. <https://doi.org/10.1038/nmeth.2604>
8. Ficetola, G.F., Coissac, E., Zundel, S., Riaz, T., Shehzad, W., Bessière, J., Taberlet, P., Pompanon, F., 2010. An In silico approach for the evaluation of DNA barcodes. *BMC Genomics* 11, 434. <https://doi.org/10.1186/1471-2164-11-434>
9. Fuhrman, J.A., Cram, J.A., Needham, D.M., 2015. Marine microbial community dynamics and their ecological interpretation. *Nat. Rev. Microbiol.* 13, 133–146. <https://doi.org/10.1038/nrmicro3417>
10. Genitsaris, S., Monchy, S., Breton, E., Lecuyer, E., Christaki, U., 2016. Small-scale variability of protistan planktonic communities relative to environmental pressures and biotic interactions at two adjacent coastal stations. *Mar. Ecol. Prog. Ser.* 548, 61–75. <https://doi.org/10.3354/meps11647>
11. Gilbert, J.A., Steele, J.A., Caporaso, J.G., Steinbrück, L., Reeder, J., Temperton, B., Huse, S., McHardy, A.C., Knight, R., Joint, I., Somerfield, P., Fuhrman, J.A., Field, D., 2011. Defining seasonal marine microbial community dynamics. *ISME J.* 6, 298–308. <https://doi.org/10.1038/ismej.2011.107>
12. Hedges, J.I., Stern, J.H., 1984. Carbon and nitrogen determinations of carbonate-containing solids. *Limnol. Oceanogr.* <https://doi.org/10.4319/lo.1984.29.3.0657>
13. Ivančič, I., Degobbis, D., 1984. An optimal manual procedure for ammonia analysis in natural waters by the indophenol blue method. *Water Res.* 18, 1143–1147. [https://doi.org/10.1016/0043-1354\(84\)90230-6](https://doi.org/10.1016/0043-1354(84)90230-6)
14. Kirchman, D., Newell, S., Hodson, R., 1986. Incorporation versus biosynthesis of leucine: implications for measuring rates of protein synthesis and biomass production by bacteria in marine systems. *Mar. Ecol. Prog. Ser.* 32, 47–59. <https://doi.org/10.3354/meps032047>
15. Mahé, F., Rognes, T., Quince, C., de Vargas, C., Dunthorn, M., 2015. Swarm v2: highly-scalable and high-resolution amplicon clustering. *PeerJ* 3, e1420. <https://doi.org/10.7717/peerj.1420>

- 1118 16. Oksanen, J., Blanchet, F.G., Friendly, M., Kindt, R., Legendre, P., McGlinn, D., Minchin,
1119 P.R., O'Hara, R.B., Simpson, G.L., Solymos, P., Stevens, M.H.H., Szoecs, E., Wagner, H., 2017.
1120 vegan: Community Ecology Package.
- 1121 17. Pinheiro, J., Bates, D., DebRoy, S., Sarkar, D., R Core Team, 2018. lme: Linear and
1122 Nonlinear Mixed Effects Models.
- 1123 18. Ruan, Q., Dutta, D., Schwalbach, M.S., Steele, J.A., Fuhrman, J.A., Sun, F., 2006. Local
1124 similarity analysis reveals unique associations among marine bacterioplankton species and
1125 environmental factors. *Bioinformatics* 22, 2532–2538.
1126 <https://doi.org/10.1093/bioinformatics/btl417>
- 1127 19. Smith, D.C., Azam, F., 1992. A simple, economical method for measuring bacterial
1128 protein synthesis rates in seawater using 3H-leucine 1. *Mar. Microb. Food Webs* 6, 107–114.
- 1129 20. Wangenstein, O.S., Turon, X., 2016. Metabarcoding Techniques for Assessing
1130 Biodiversity of Marine Animal Forests, in: *Marine Animal Forests*. Springer International
1131 Publishing, Cham, pp. 1–29. <https://doi.org/10.1007/978-3-319-21012-4>

1132

1133