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Nitrogen and phosphorus limitation of oceanic microbial growth during spring in the Gulf of Aqaba

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ABSTRACT: Bioassay experiments were performed to identify how growth of key groups within the microbial community was simultaneously limited by nutrient (nitrogen and phosphorus) availability during spring in the Gulf of Aqaba's oceanic waters. Measurements of chlorophyll *a* (chl *a*) concentration and fast repetition rate (FRR) fluorescence generally demonstrated that growth of obligate phototrophic phytoplankton was co-limited by N and P and growth of facultative aerobic anoxygenic photoheterotropic (AAP) bacteria was limited by N. Phytoplankton exhibited an increase in chl *a* biomass over 24 to 48 h upon relief of nutrient limitation. This response coincided with an increase in photosystem II (PSII) photochemical efficiency (F_v/F_m), but was preceded (within 24 h) by a decrease in effective absorption cross-section (σ_{PSII}) and electron turnover time (τ). A similar response for τ and bacterio-chl *a* was observed for the AAPs. Consistent with the up-regulation of PSII activity with FRR fluorescence were observations of newly synthesized PSII reaction centers via low temperature (77K) fluorescence spectroscopy for addition of N (and N + P). Flow cytometry revealed that the chl *a* and thus FRR fluorescence responses were partly driven by the picophytoplankton (<10 µm) community, and in particular *Synechococcus*. Productivity of obligate heterotrophic bacteria exhibited the greatest increase in response to a natural (deep water) treatment, but only a small increase in response to N and P addition, demonstrating the importance of additional substrates (most likely dissolved organic carbon) in moderating the heterotrophs. These data support previous observations that the microbial community response (autotrophy relative to heterotrophy) is critically dependent upon the nature of transient nutrient enrichment.

KEY WORDS: Phytoplankton · Aerobic anoxygenic photoheterotroph · Bacterial · Fast repetition rate fluorometry · Chlorophyll *a* · Nutrient limitation · Gulf of Aqaba

INTRODUCTION

Upper open-ocean ecosystems contain an incredibly diverse array of eukaryotic and prokaryotic microbes that are fueled by autotrophic and heterotrophic pathways. Much of their growth is sustained by recycling of inorganic nutrients and nitrogen fixation; however, periodic injections of new nutrients from deep water, via uplift of the thermocline from passing eddies (Falkowski et al. 1991, McGillicuddy et al. 2007) and solitons (Holligan et al. 1985), or aerosol deposition (Paerl et al. 2002, Camargo & Alonso 2006, Chen et al.

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2007) significantly perturb the status quo. A rapid increase in microbe biomass quickly follows these nutrient injections with significant implications for not only the flux of organic carbon from near-surface to deeper waters (e.g. Tanaka et al. 2007) but also for the net sequestration of inorganic carbon from the atmosphere (e.g. Mills et al. 2008).

The Gulf of Aqaba, Eilat (24 km wide, 800 to 1800 m deep) is one of 2 large gulfs in the Red Sea, located to the east of the Sinai Peninsula and west of the Arabian mainland. Oligotrophic oceanic waters of the Gulf are characterized by deep winter mixing and intense summer stratification; consequently, these waters experience strong seasonal changes of the predominant limiting factor for microbial growth (Lindell & Post 1995, Stambler 2006, Mackey et al. 2009). During winter the Gulf is subjected to benthic injections of nitrogen that maintain the nitrogen: phosphrous ratio (N:P) close to the 'Redfield ratio' (N:P = 16) (Häse et al. 2006). At this time, eukaryotic algae dominate but growth is limited by light availability with deep mixing (Lindell & Post 1995, Stambler 2005, Al-Najjar et al. 2007). Water column stratification initiated during the spring traps nutrients in the high-light surface waters resulting in phytoplankton blooms, typically cyanobacteria and diatoms (Lindell & Post 1995, Al-Najjar et al. 2007). As spring progresses into summer, the phytoplankton community becomes increasingly dominated by picoeukaryotes and prochlorophytes (Lindell & Post 1995, Al-Najjar et al. 2007, Stambler 2006). Stratification minimizes deep-water injections of nitrogen into near-surface waters, and atmospheric loading of nutrients becomes an important determinant of nutrient availability (Chen et al. 2007). Estimates of N:P in the seawater-soluble fraction of dry deposition are well above 'Redfield' (ranging from 32 to 541), suggesting P limitation (Chen et al. 2007). Atmospheric inputs provide an important source of seawater-soluble iron (Chase et al. 2006), which appears to remain in excess of that required for microbial growth throughout the year (Chen et al. 2007). Thus, conditions in the Gulf during spring and summer appear to gravitate towards increasing P limitation for phytoplankton growth.

Experimental investigations of nutrient control upon microbial communities have often employed nutrient addition bioassays, and include the Gulf of Aqaba in both autumn and spring (Al-Qutob et al. 2002, Mackey et al. 2007, 2009) when communities were dominated by picoeukaryotes and prochlorophytes. These previous experiments demonstrated a community response following P but not N addition. The greatest community response was observed following a dual N and P addition suggesting that the microbial growth was thus co-limited by both nutrients (Mackey et al. 2009). Previous experiments followed only the phytoplankton biomass response to nutrient addition as cell or chlorophyll *a* (chl *a*) concentration. An increase was typically observed after >48 h and accompanied a shift in predominance towards cyanobacterial cells, i.e. an adaptive (taxonomic) response indicative of growth of opportunistic cells with a high nutrient affinity (Beardall et al. 2001, Davey et al. 2008). However, these observations do not consider the physiological responses that accompany or precede any change in phytoplankton abundance or the microbial community structure. Furthermore, changes in the phytoplankton structure alone do not provide any indication as to how other important microbial groups, such as heterotrophic aerobic anoxygenic photoheterotropic (AAP) bacteria that play key roles in nutrient cycling within open-ocean communities, simultaneously respond to the nutrient additions.

As part of the 8th Group for Aquatic Primary Productivity (GAP) International Workshop we used bioassay experiments to determine (1) the relative change of acclimation (physiology and growth) and of community structure for phototrophs in response to short term (<48 h) and longer term (>48 h) relief from nutrient limitation, and (2) the relative growth response of autotrophic, AAP and heterotrophic bacteria to combinations of nutrients. These experiments were performed during the spring when the water column began to stratify and become nutrient limited whilst still maintaining a relatively high diversity of picoeukaryotic and prokaryotic microbes.

MATERIALS AND METHODS

Bioassay sampling and experimentation. Bioassay experiments upon open-ocean microbial communities were performed following Moore et al. (2006, 2008). Surface water (0 to 10 m, 100 l) was collected pre-dawn from station A (29° 28′ N, 34° 55′ E) using 10 l Niskin bottles. The water column remained stratified with a thermocline between 50 and 100 m throughout the sampling period. Water above the thermocline exhibited higher temperature and chl *a* fluorescence and lower salinity (ca. 21.7°C, 37.2 instrument units, 40.5, 0 to 50 m mixed layer) compared to deeper water (ca. 21.0°C, 11.8 instrument units, 40.7, 300 m). Nutrient concentrations, except those for NO_2, were lower at 10 m (0.08 µmol l^{-1} NO_2, 0.10 µmol l^{-1} NO_3, 0.04 µmol l^{-1} PO_4 and 0.74 µmol l^{-1} $Si(OH)_4$) than for waters below the thermocline (mean ± SD for all samples between 150 and 300 m: 0.06 ± 0.01 µmol l^{-1} NO_2, 3.32 ± 0.04 µmol l^{-1} NO_3, 0.14 ± 0.01 µmol l^{-1} PO_4, 1.73 ± 0.04 µmol l^{-1} $Si(OH)_4$).

Water was immediately transported to shore and transferred to 18 acid-cleaned bottles (4 l). Each bottle was triple rinsed prior to filling. Triplicate filled bottles were then randomly selected for each of 5 treatments: a control (no addition); +N (1.0 µmol l^{-1} of NH_4^+ and 1.0 µmol l^{-1} NO_3^-, final concentration); +P (0.2 µmol l^{-1} NaH_2PO_4, final concentration); +N and P; and +deep water (DW). The +DW treatment refers to an addition of 'deep water' collected below the developing thermocline (150 to 300 m) and passed through a 0.2 µm filter to remove any algal cells. 10% (400 ml) of surface water was replaced with 400 ml of the DW. Final concentrations of nitrogen and phosphate added for the +DW treatment were 0.006 µmol l^{-1} NO_2, 0.33 µmol l^{-1} NO_3, and 0.014 µmol l^{-1} PO_4 to yield final concentrations in the bottles of 0.20 µmol l^{-1} (NO_2 + NO_3) and 0.04 µmol l^{-1} PO_4, i.e. a factor of 5 lower than in +N and +P treatments. Triplicate bottles were also filled for the initial (time zero) conditions. Bottles were then placed into one of 3 outdoor incubators connected in series and supplied with continuously flowing seawater pumped directly from the near shore. Temperature was monitored and the continuous flow was adjusted so that temperatures inside the incubators remained close to ambient (*in situ*) from the time of sampling.

Two bioassay experiments were performed: (1) a long-term (96 h), and (2) a short-term (48 h) experiment. These were started at dawn on the 2nd and 4th of April, respectively. The long-term experiment was analysed at 48 and 96 h whilst the short term experiment was analysed at 24 and 48 h. As such, both experiments had a common time point at which to compare any physiological and taxonomic responses to the treatments. Nutrients were re-supplied to the long term experiment at 48 h to the same final concentration, but in reduced volumes to compensate for the volume of water removed after the 48 h analysis, based on the assumption that all nutrients were exhausted. pH did not change significantly (<10%) in any treatments over the course of the short- and long-term experiments, indicating that inorganic carbon would not have been limiting. The analyses are detailed below.

Fast repetition rate (FRR) fluorescence. A custom built FRR fluorometer was used to examine both chl *a* fluorescence of the phototrophs (Kolber et al. 1998) and facultative photoheterotroph bacterio-chl *a* (Kolber et al. 2001). Bioassay sub-samples were dark acclimated for 50 to 60 min and processed randomly. A peristaltic pump was used to circulate each sample between the sub sample bottle and optical chamber of the fluorometer. In total 25 FRR fluorescence transients were made for each sample and for both chl *a* and bacterio-chl *a*. Each transient was fit with a biophysical model (Kolber et al. 1998, 2001) to yield physiological information under dark acclimated conditions. We used only information collected from the single turnover (ST) component of the transient (see Kolber et al. 1998), specifically: the maximum and minimum fluorescence yields (F_o, F_m, instrument units), the effective absorption cross section (σ_{PSII}, $Å^2$ $quanta^{-1}$) and minimum turnover time of electrons (τ, µs). The PSII photochemical efficiency (F_v/F_m, dimensionless) was calculated as $(F_m–F_o)/F_m$. Sample blanks were made and subtracted from the values of F_o and F_m (following Moore et al. 2006) prior to calculation of F_v/F_m. In the case of bacterio-chl *a*, the measurements for F_v/F_m and σ_{PSII} were not used since the signal to noise was deemed too low.

The simultaneous response of σ_{PSII} ($Å^2$ $quanta^{-1}$) and τ (µs) reveals important information about the photosynthetic response to the growth environment. A light saturation parameter (E_K, µmol photons m^{-2} s^{-1}) was estimated as $[1/(\tau \cdot \sigma)] \cdot 1.66 \times 10^8$, where the factor 1.66×10^8 accounts for the conversion of $Å^2$ to m^2, quanta to µmol quanta (photons) and µs to s (e.g. Moore et al. 2006). The actual value of E_K is dependent upon both the wavelength used to generate σ_{PSII} and which time constant associated with the FRR relaxation phase is used to describe τ (Kolber et al. 1998). Here, our FRR fluorescence values of σ_{PSII} are weighted by the blue light source whilst τ is from the first relaxation component of the entire ST relaxation phase and mostly due to re-oxidation of Q_A (primary electron acceptor quinone molecule, e.g. Robinson & Crofts 1983). For the purposes of our study we will consider only relative changes to these calculations of E_K, and employ the term $E_{K(FRR)}$

77K fluorescence emission spectroscopy. Samples (500 ml) were filtered onto GF/F (Whatman) filters. Each filter was cut to fit a sample holder that was placed in the glass optical dewar and filled with liquid nitrogen. After cooling, the sample was illuminated using a multispectral LED excitation source. This allows illumination to be set to one of 6 available LEDs in the spectral range 390 to 590 nm. The fluorescence emission of the sample was collected via an integrated lens and fiberoptics and detected using the Spectrometer SM-9000 (Photon Systems Instruments) for the whole hyperspectral range of the detector (190 to 1000 nm). The dark current of the instrument, which can introduce non-linearities in the fluorescence emission, was automatically subtracted before measurements. Spectra were integrated for 1s and each sample measured in triplicate. A blank (GF/F filter wetted with distilled water) was measured and subsequently subtracted from each sample measurement.

Flow cytometry. Samples were analyzed for picoplankton (cell diameter < 10 µm) community structure as described previously (Stambler 2006). Briefly, sample aliquots (1.8 ml) were fixed immediately at room

temperature in 20 µl of 25% glutaraldehyde (Sigma G-5882), deep-frozen in liquid nitrogen and kept at –80°C. All samples were analyzed within 1 mo by quick thawing for 1 to 2 min at 37°C and analyzed by flow cytometry (FACScan, Becton Dickinson) at room temperature (23 ± 2°C). Excitation was at 488 nm with an Argon laser. Red Chl *a* fluorescence (>630 nm) and orange phycoerythrin fluorescence (585 ± 15 nm) was quantified. Before running the samples, 0.93 µm beads (Polysciences) were added as an internal standard. Picoplankton biomass (carbon per unit volume) was also calculated from cell counts following Campbell et al. (1998): *Synechococcus* (the dominant cyanobacteria) as 175 fg C cell^{-1}, *Prochlorococcus* as 53 fg C cell^{-1}, and eukaryotes as 2100 fg C cell^{-1}.

Preserved samples from the long-term bioassay (at 96 h) were also run for bacterial enumeration. Total prokaryote abundance was determined from aliquots of 0.3 ml stained with a SYBR Green I (Molecular Probes) 10× solution (to a final dilution 1:1000 vol: vol) for 25 min in the dark and then run through the FACScan at 15 µl min^{-1} with 0.93 µm beads as an internal standard. Bacteria were detected in a plot of side scatter (SSC) versus green fluorescence (Fl1) (Gasol & del Giorgio, 2000).

Bacterial production (BP). In the short-term (48 h) experiment, we measured BP at 0, 24 and 48 h. We determined BP using the leucine uptake method (Simon & Azam 1989) as modified by Smith & Azam (1992). Aliquots of 30 nM ^{14}C-leucine were added to each of 4 replicate samples (1.7 ml), i.e. a concentration saturating for concentration-dependent incorporation experiments, in addition to 2 killed controls. Inoculated samples were incubated at ca. 20°C in the dark for 2 h during which time ^{14}C-leucine incorporation was linear. After the incubation, the reaction was terminated by the addition of 100% trichloroacetic acid (100 µl) and processed as described by Smith & Azam (1992). After 24 h in scintillation cocktail (Ultima Gold, Perkin Elmer), samples were counted with a Beckman scintillation counter. Leucine incorporation was converted to carbon production rate with a standard factor of 3.1 kg C mol^{-1} (Simon & Azam 1989).

Chlorophyll *a* (chl *a*) concentration and nutrient analyses. Chl *a* was determined fluorometrically in acetone extracted samples as previously described (Stambler 2006). Total oxidized nitrogen (nitrate and nitrite), soluble reactive phosphorus (SRP) and silicate concentrations were determined using colorimetric methods described by Hansen & Koroleff (1999), modified for a flow injection autoanalyzer (FIA, Lachat Instruments Model QuickChem 8000). SRP was preconcentrated before analysis by a factor of ca. 20 using the magnesium co-precipitation (MAGIC) method (Karl & Tien 1992, Mackey et al. 2009).

RESULTS

Short-term autotrophic response (24 to 48 h)

Synechococcus and picoeukaryote cells dominated the initial community biomass for this short-term bioassay (Fig. 1A-C). *Synechococcus* increased, whilst picoeukaryotes decreased, relative to their initial biomass for all treatments except +DW at 24 h (*Synechococcus* biomass decreased relative to the initial concentration in the +DW treatment at 24 h). *Synechococcus* biomass continued to increase for +N and +N and P, but decreased for the control, +P and +DW treatments, whilst picoeukaryote biomass continued to decrease for all treatments at 48 h.

The responses for chl *a* biomass were generally similar to those observed for cell biomass: chl *a* concentration increased in response to +DW, +N and +N and P treatments (Fig. 2A); however, the increase with +DW was short-lived (at +24 h only) (Fig. 1). The increase with +N and P, and to a much lesser extent +N, was highest after 48 h incubation; in contrast, chl *a* in both the control and +P treatment declined at 48 h.

As with chl *a* biomass, phytoplankton physiology exhibited clear differences between nutrient treatments (Fig 2B). The maximum PSII photochemical efficiency (F_v/F_m, dimensionless) increased with the +DW treatment at 24 h only and with the +N and P treatment at 48 h. Values of PSII effective absorption, σ_{PSII} (Å^2 quanta^{-1}), exhibited a systematic decrease for all treatments and in the control throughout the duration of incubation (Fig. 2C). This decrease likely reflected a photoacclimatory downregulation of light harvesting pigments where light levels within the incubator were on average higher than for surface waters. Values of σ_{PSII} also changed in response to specific nutrient treatments: σ_{PSII} was also lower in the +N, +N and P, and to a lesser extent, the +DW, than in the control and +P treatments at both 24 and 48 h time points. The response of the minimum electron turnover time, τ (µs), to the various nutrient treatments was opposite to that observed for σ_{PSII} (Fig. 2D), i.e. values of τ were higher for the control and +P, and to a lesser extent +DW, than for the +N and +N and P treatments at both time points.

Values for $E_{K(FRR)}$ (µmol photons m^{-2} s^{-1}) increased in the control at 48 h (257 ± 5, mean ± SE) relative to the initial value (224 ± 7) providing further evidence for acclimation to relatively higher light conditions within the incubators compared to *in situ* (initial) (data not shown). $E_{K(FRR)}$ also increased for +N and +N and P (396 ± 7) relative to the control, +DW (264 ± 4) and +P (253 ± 6) treatments at 48 h. Smaller antennae sizes yet faster turnover times appear to drive these higher values of $E_{K(FRR)}$, suggesting that nutri-

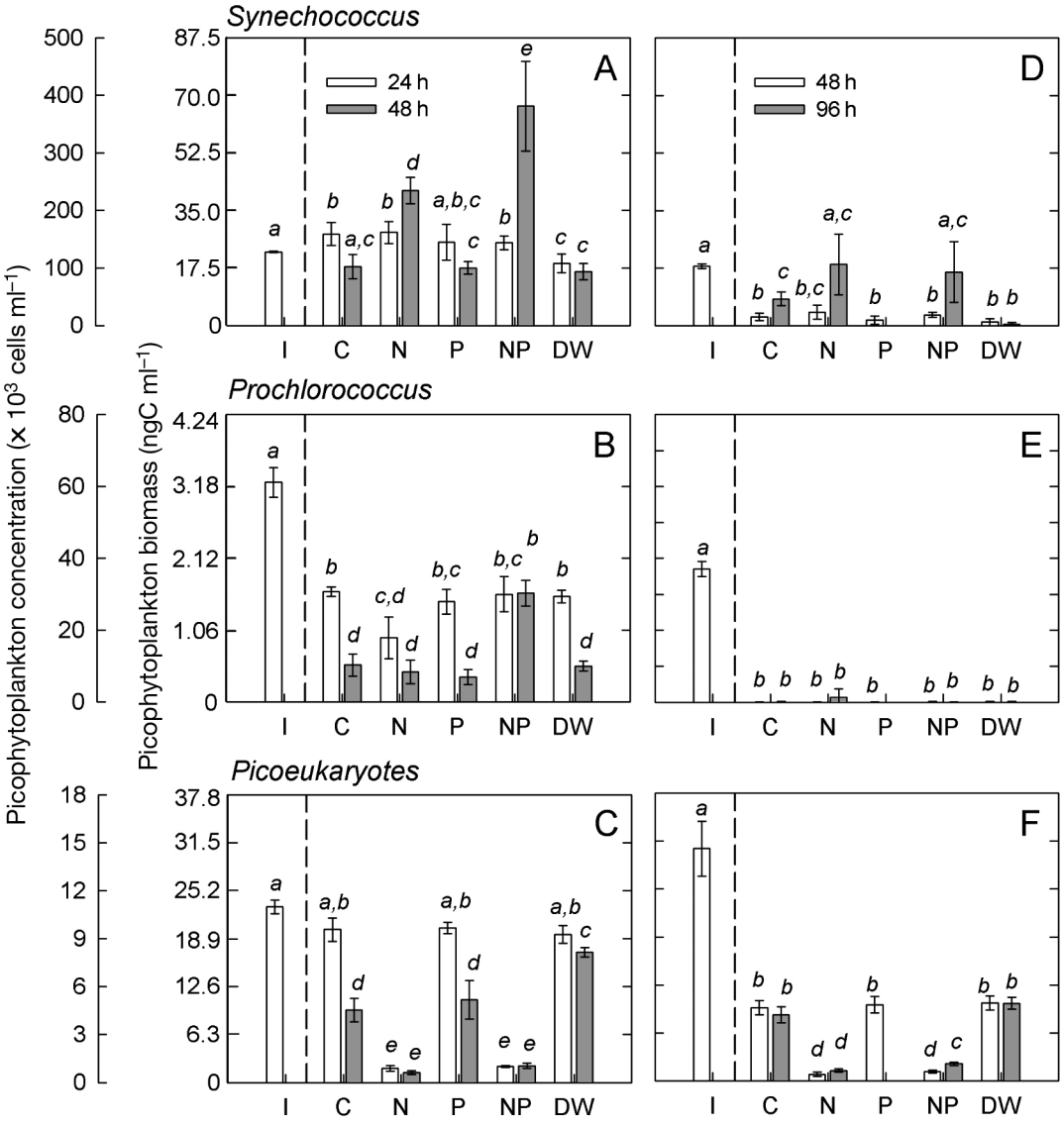

Fig. 1. Picophytoplankon cell concentration determined by flow cytometry and cell concentration converted to carbon biomass for all bioassay experiments: initial (I), control (C), +nitrogen (N), +phosphate (P), +nitrogen and phosphate (NP), and +deep water (DW) treatments (see 'Materials and methods: Bioassay sampling and experimentation'). Short-term (A–C, 48 h) and long-term (D–F, 96 h) bioassays are shown. Each bar represents the mean (±SE) from the triplicate bottles for each treatment. Letters indicate post hoc groupings following an ANOVA test between treatments for either the short- or long-term bioassay and for each fraction of the microbial community. Vertical dashed line separates initial data from experimental data for clarity

ent limitation affects both the light harvesting apparatus (light-dependent) and maximum electron turnover rate (light saturated) components of PSII (Q_A) activity.

AAP biomass and physiology also exhibited a clear response to the +N and +N and P treatments (Fig. 2E, F). An increase in bacterio-chl *a*, the magnitude of which was the same for both +N and +N and P treatments, was observed at 24 h, but bacterio-chl *a* did not increase further by 48 h. Values of τ decreased at 24 h but returned to initial values at 48 h.

Long-term autotrophic response (48 to 96 h)

Autotrophic community biomass was initially dominated by picoeukaryotes (Fig 1D-F); however, *Synechococcus* biomass increased with the +N and +N and P treatments by 96 h (but not at 48 h as observed in the short-term bioassay). In contrast, eukaryotes decreased with the +N and +N and P treatments at both 48 and 96 h, a response consistent with the short-term bioassay. As observed for the short-term bioassay, chl *a* concentrations increased in response to +N and P

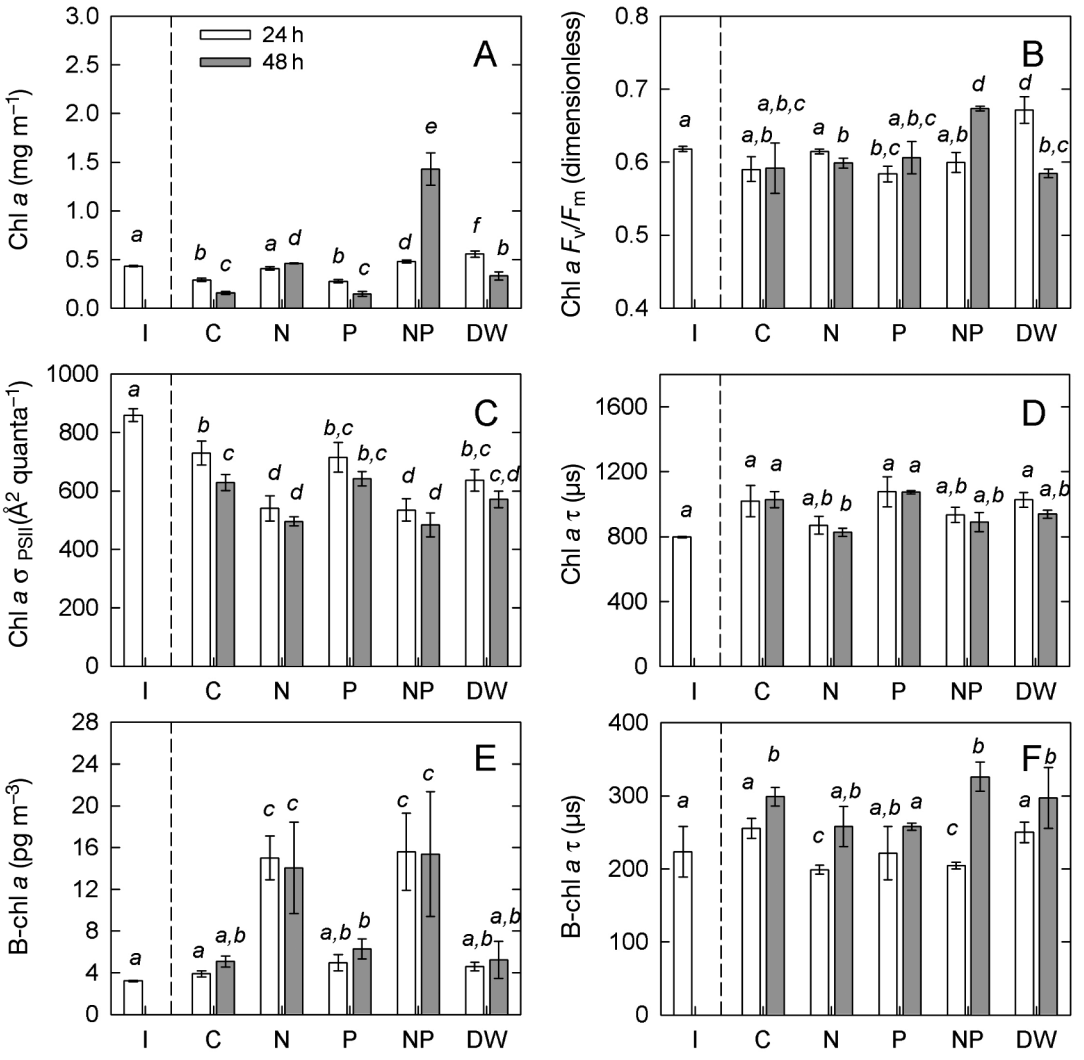

Fig. 2. Response for the short-term (48 h) bioassay of (A) chl *a* concentration and FRR fluorescence measurements of (B) chl *a* PSII maximum photochemical efficiency (F_v/F_m), (C) chl *a* PSII effective absorption cross section (σ_{PSII}), (D) chl *a* minimum turnover time of electrons (τ), (E) bacterio-chl *a* (B-chl *a*) concentration, and (F) B-chl *a* τ to initial and control, +nitrogen, +phosphate, +nitrogen and phosphate, and +deep water treatments (see 'Materials and methods: Bioassay sampling and experimentation'). See Fig. 1 for other details

but at 48 and 96 h. Chl *a* concentrations decreased in the +N treatments at both 48 and 96 h; however, these concentrations remained higher than for the control, +P and +DW treatments.

Physiological variables for this bioassay responded somewhat differently to the nutrient additions than observed for the short-term bioassay. Values for F_v/F_m for the +N and +DW treatment declined at 48 h relative to the initial, control, +P and +N and P. This decline continued for the +N treatment at 96 h (Fig. 3B). A slightly higher value for F_v/F_m was observed for the +N and P treatment at 96 h. Values of σ_{PSII} declined relative to the initial for +N and +N and P treatments at 48 h but declined for all treatments at 96 h (Fig 3C). Reciprocal changes for τ were again observed (Fig 3D); whilst all values decreased relative to an exceptionally high initial value, τ was lower for the +N and +N and P treatments than for the control, +P and +DW treatments. All values of τ subsequently increased at 96 h. In this bioassay experiment, estimates of $E_{K(FRR)}$ (µmol photons m^{-2} s^{-1}) again increased for the control (258 ± 8) at 48 h relative to the initial (137 ± 5); however, the value in the control did not increase further at 96 h. Also, $E_{K(FRR)}$ values for +N and +N and P treatments were higher (343 ± 7) than those for all other treatments (248 ± 7) at 96 h.

AAP (bacterio-chl *a*) biomass also increased with both +N and +N and P, and to a lesser extent +DW, treatments (Fig 3E) at 48 h; this biomass continued to

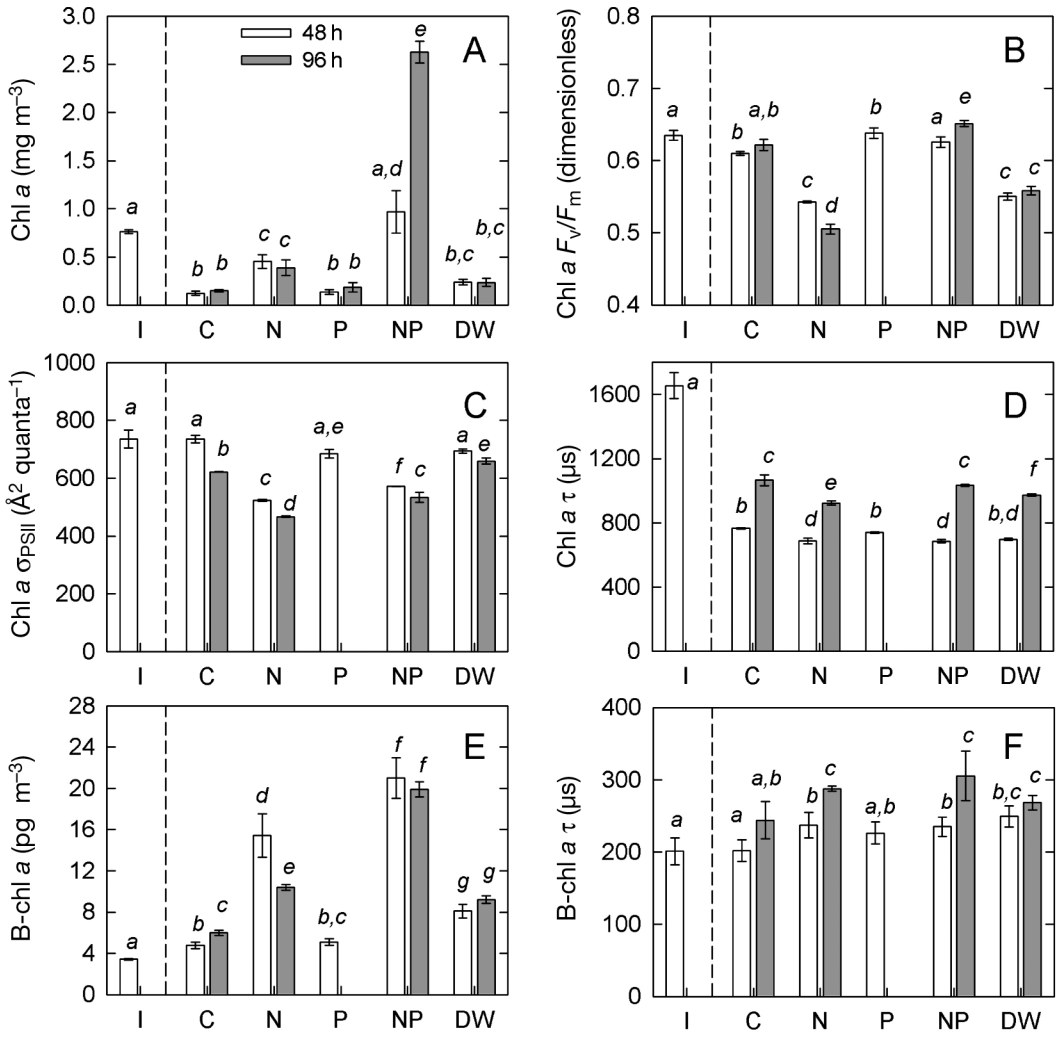

Fig. 3. Response for the long-term (96 h) bioassay of chl *a* and B-chl *a* parameters. See Fig. 2 for details

increase for the +N and P, but exhibited a small decrease for the +N treatment, by 96 h. Values for τ also increased in response to the +N and +N and P treatments but only at 96 h (Fig 3F).

77K spectra

Low temperature fluorescence emission spectra were used to further examine the response of the photosynthetic apparatus of the autotrophs. The band intensity of peak emission by PSII (ca. 685 nm) measured from all treatments for the 2 bioassays agreed well with corresponding FRR maximum fluorescence yields (F_m) (data not shown). Spectra recorded at the various sampling time points of both bioassays generally exhibited the same response to the different nutrient treatments (e.g. Fig. 4).

Emission spectra from control samples (and +P treatments, not shown) were characteristic for phytoplankton from oligotrophic environments (O. Prášil unpubl. data). Spectra were generally dominated by a single PSII emission band (centered at ca. 685 nm but with an associated minor satellite band at 745 nm), with some emission also observed for phycobilins (641 and 677 nm) and photosystem I (PSI) (707nm) (Fig. 4A,B). Deconvolution of the control spectra into these respective components (Fig. 4B) demonstrated that PSII contributed ca. 30 to 35% of the total (normalised) emission; nonetheless, there were clear differences between treatments. Following the +N and P (and +N, not shown) treatments, there was a significant increase in PSII emission band at 695 nm, relative to 685 nm, compared to the control. Only minor emission could be attributed to phycobilisomes (peak at 640 nm, area less than 1% of the total emission) or to PSI (emission at 707 nm, area 10 to 15%).

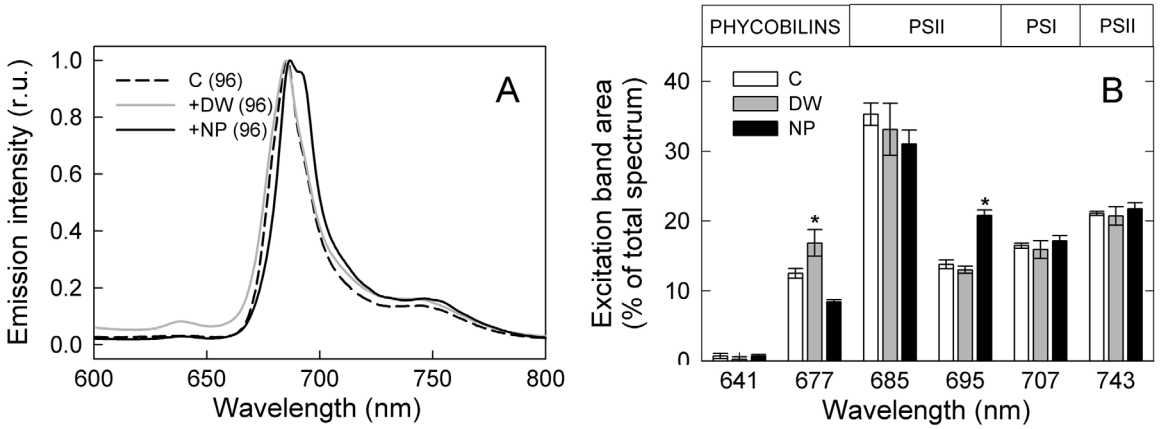

Fig. 4. (A) Example 77K fluorescence emission spectra from the long-term (96 h) bioassay for control (C), deep water (+DW) and +N and P (+NP) treatments at 96 h. Other treatments of +N or +P yielded the same spectra as +N and P and C, respectively, and have been omitted for clarity. Also, spectra have been normalized relative to the maximum peak height to demonstrate the relative spectral shifts. r.u.: relative units. (B) Deconvoluted mean (±SE) peak heights from triplicate bottles of each of the example treatments. Peak heights were chosen according to relevance to synthesis of PSII (685, 695, 743 nm) and PSI (707 nm) reaction centre proteins, and phycobilins (641, 677 nm) (see 'Results: 77K spectra'). * indicates significantly different values from ANOVA poc host tests, where an ANOVA was performed for all data (treatments) for each wavelengths

Heterotrophic bacteria

BP was determined only for the short-term bioassay. Relative to the initial samples, these data indicated a significant increase in response to the +DW treatment at 24 and 48 h, and also an increment in the +N and P treatment at 48 h (Fig. 5A). Unfortunately, we did not have flow cytometry data from the initial samples and so cannot determine whether this change at 24 h in productivity was accompanied by a change in bacterial cell number (bacterial biomass). However, BP does appear to contrast with observations of biomass and physiology for the phytoplankton, which largely responded to the +N and +N and P (but not +DW) treatments. Opportunistic samples for bacterial cell abundance were taken at 96 h from the long-term bioassay (Fig. 5B) and indicated that, despite a sustained response of bacterial productivity to +N and P or +DW (at least over 48 h), bacterial abundance was not higher in the +N and P treatment relative to the control and even appeared to decline in the +DW treatment.

DISCUSSION

Nutrient (co-)limitation of microbial growth

Responses of physiology (FRR fluorescence) and biomass (cell abundance) to the combination of nutrient additions here provide clear evidence for nutrient limitation and co-limitation of microbial productivity and growth. Co-limitation is most rigorously defined following Seppälä et al. (1999) as a significant response to

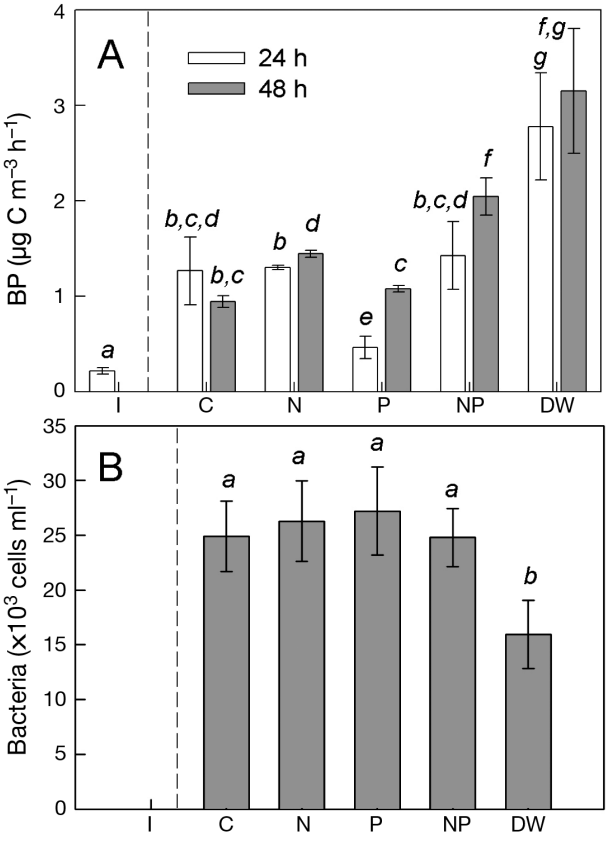

Fig. 5. Response of (A) bacterial productivity (BP) to initial and control, +nitrogen, +phosphate, +nitrogen and phosphate, and +deep water treatments (see 'Materials and methods: Bioassay sampling and experimentation') for the short-term (48 h) bioassay. (B) Bacterial counts corresponding to the 5 nutrient treatments at +96 h of the long-term bioassay. See Fig. 2 for other details

2 or more nutrients added in combination but not when these same nutrients are added individually; in contrast, sequential nutrient limitation defines an additive response to 2 or more nutrients. In the latter case, limitation by additional nutrients is induced by provision of the proximal limiting nutrient in stoichiometric excess to the cells' requirements, and thus is an artifact of the experiment.

Following Seppälä's definitions, chl *a* and F_v/F_m (at 48 h) demonstrated co-limitation by N and P whilst that of other physiological parameters (σ and τ for chl *a*) demonstrated sequential limitation by N and P, i.e. N was the proximal limiting nutrient. Co-limitation by N and P of the phytoplankton biomass is consistent with previous nutrient bioassays conducted in the Gulf of Aqaba in spring (Al-Qutob et al. 2002) and autumn (Mackey et al. 2009). We observed a transient response in chl *a* and F_v/F_m to the +DW treatment at 24 h. Here, the low concentrations of the N and P in the +DW treatment (compared to the other nutrient additions) were rapidly incorporated and exhausted (within 24 h). Variation in all parameters for the AAPs was consistent with sequential limitation by N and P.

The biomass response of picophytoplankton (<10 µm, Stambler 2006) in the 24 to 48 h bioassay was dominated by *Synechococcus*, again consistent with previous studies for oceanic microbial communities (Davey et al. 2008, Moore et al. 2008, Mackey et al. 2009). Abundance of *Prochlorococcus* and picoeukaryotes did not increase with +N (or +N and P) relative to the control suggesting that N limitation did not occur for all autotrophic fractions. In fact, picoeukaryotes exhibited a strong negative response to addition of +N and +N and P indicating that they could not compete for (or were inhibited by) high nutrient concentrations, and in any case were likely rapidly grazed or lysed. It is possible that the higher light in the incubators relative to *in situ* conditions (indicated by an increase in $E_{K(FRR)}$ in the control and P-limited treatments) may have favoured growth of *Synechococcus* over other picophytoplankton.

Whilst picoeukaryotes are known to be important to carbon cycling in the Gulf of Aqaba (Worden et al. 2004, Mackey et al. 2009), size-fractionated chl *a* measurements from surface waters, i.e. the initial conditions for the bioassays, demonstrated that only ca. 30 to 35% of all chl *a* passed through a 5 µm filter (V. Montecino pers. comm.) and thus potentially contributed to the picophytoplankton. Diatoms were frequently observed in the >5 µm fraction, and larger eukaryotes are prone to nutrient limitation in these waters when dominated by diatoms (Berninger & Wickham 2005). Larger eukaryotes not included in the flow cytometric analyses may have responded positively to the nutrient additions and contributed significantly to the increase in chl *a* in the bioassays.

We have some evidence for a response of larger eukaryotes in the +N and +N and P treatments: the increase in estimated picophytoplankton carbon biomass was much lower than the increase in chl *a* for +N (1.05- vs. 1.25-fold) and +NP (1.5- vs. 3.5-fold) over 48 h. The increase in carbon was accompanied by an increased numerical dominance by *Synechococcus* (from ca. 50 to 90%); however, to account for the chl *a* response, the *Synechococcus* would have required substantially higher values of chl *a* cell^{-1} (i.e. lower C cell^{-1} and/or high chl *a* C^{-1}) than the picoeukaryotes, the other dominant picophytoplankon fraction. This is unlikely since *Synechococcus* exhibits some of the lowest reported values of chl *a* cell^{-1} and chl *a* C^{-1} (see MacIntyre et al. 2002 and references therein). Also, the increased irradiance in the incubators relative to conditions *in situ* would not be conducive to a rise of chl *a* cell^{-1}. Therefore, the most likely explanation for these patterns is a positive biomass response of larger eukaryote fractions.

The phytoplankton response in the second bioassay experiment at 48 h and at 96 h was very different to that observed at the 48 h endpoint of the short-term bioassay. This is not surprising given the different initial composition of the community (Berninger & Wickham 2005, Moore et al. 2006, 2008). Initial chl *a* concentrations were higher and the estimated carbon biomass of the picophytoplankon was dominated by picoeukaryotes (ca. 65%), in contrast to the short-term bioassay. Chl *a* increased by a factor of 3 by 96 h; however, there was no evidence of a simultaneous increase in carbon biomass in any of the picophytoplankton fractions. Again, this implies the response of an unidentified, larger (>5 µm) fraction and/or an increase in chl *a* (cell)$^{-1}$. An increase in *Synechococcus* biomass was observed at 96 h compared to 48 h in the +N and +N and P treatments, perhaps indicating that any sole nutrient favouring larger eukaryotes, e.g. Si, may have become limiting to their growth, giving a competitive advantage to the smaller fractions. Unfortunately, without knowing the dynamics associated with larger cells, we cannot confirm these suggestions.

Autotroph photophysiology

FRR fluorescence and 77K spectroscopy provided evidence of how the phototrophic components of the microbial community adjusted physiologically (and in some cases adaptively). For the short-term bioassay, values of maximum PSII photochemical efficiency (F_v/F_m) increased in parallel with those of chl *a* for the +N and P (48 h) and +DW (24 h) treatments, consistent with active synthesis of PSII reaction centers upon relief of nutrient starvation (Kolber et al. 1988, Moore et al.

2006). The lack of response of F_v/F_m for the +N treatments may provide further evidence of P limitation in these bottles (Moore et al. 2008). The +N and +N and P bottles each had a reduction of both σ_{PSII} and τ at 24 h; in the case of +N and P, this occurred more rapidly than the response of F_v/F_m and demonstrated that additional processes preceded the synthesis of the PSII reaction centers (RCIIs) to enable faster electron turnover.

Reduction of σ_{PSII} occurs as a result of less light absorption per unit RCII, i.e. down-regulation of photosynthetic pigment, investment of more photoprotective pigments and/or of more RCIIs (Kolber et al. 1988, Suggett et al. 2004). Since $E_{K(FRR)}$ increased at 24 h relative to the initial value, preferential synthesis of photoprotective (or down-regulation of photosynthetic) pigments is plausible; indeed, pigment synthesis is likely to occur more rapidly than RC synthesis, in particular where cells exhibit a photoacclimative response of preferential changes in photosynthetic unit (PSU) size over number (see Suggett et al. 2007). However, lower values of σ_{PSII} could also represent preferential environmental selection for diatoms or *Synechococcus*. Some strains of *Synechococcus* grown *in vivo* exhibit low values of σ_{PSII} but maintain relatively high values of F_v/F_m (Suggett et al. 2009, I. Berman-Frank unpubl.). From the data available here, we cannot identify the mechanism underlying the changes in σ_{PSII} and τ, but it is clear that the transiently-available N (N + P) resulted in a higher $E_{K(FRR)}$ and faster electron turnover, and drove increased phototrophic production and growth in the short term (24 to 48h).

Changes in PSII characteristics in the long-term bioassay can be explained as a result of both physiological and taxonomic responses. First, the +N and P addition decreased σ_{PSII} at 48 h but increased F_v/F_m at 96 h, a response generally similar to that throughout the short-term bioassay. For these treatments, the 77K spectra also showed a 695 nm peak indicative of an increase in 'healthy and active' PSII units. However, this response may also indicate taxonomic shifts, where the community becomes increasingly dominated by diatoms (Suggett et al. 2009).Second, the +N treatment produced a progressive decrease in both F_v/F_m and σ_{PSII} at 48 h and 96 h. Such a response might be expected as cyanobacterial dominance increased (Raateoja et al. 2004, Suggett et al. 2009) if the PSII fluorescence signal were increasingly 'contaminated' by higher PSI: PSII ratios and/or phycobilin content. 77K spectra for the +N (and N + P) treatment indeed show increased PSI abundance but not phycobilin synthesis (Fig. 5). However, this interpretation is inconsistent with the notion that not all of the chl *a* increases could be explained by the *Synechococcus* (see 'Nutrient (co-) limitation of microbial growth'). Also, phycobilin synthesis in cyanobacteria is unlikely to be the site of nutrient limitation (*sensu* Moore et al. 2008). The increased light intensity of the incubators further confound this notion of preferential pigment (phycobilin) synthesis.

Third, the +DW treatment decreased F_v/F_m and simultaneously increased σ_{PSII} between 48 and 96 h, which is indicative of a general stress response as RCIIs inactivate (Kolber et al. 1988) but also of shifts in the community from dominance by large-celled diatoms to cyanobacteria and/or picoeukaryotes (Suggett et al. 2009). At first observation, the +DW response appeared to conflict with the corresponding 77K spectra, which demonstrated apparent phycobilin synthesis (see Fig. 5). Whilst phycobilin synthesis might be expected from the increasing dominance of the community by cyanobacteria, it is likely that the phycobilin synthesized here was too orange-shifted to 'contaminate' the PSII fluorescence signal and so lower F_v/F_m (cf. Raateoja et al. 2004). Rather, the proportion of the picophytoplankton community comprised of picoeukaryotes (Fig. 1F) increased in the +DW treatment, which is consistent with the observed changes to the FRR fluorescence parameters and which suggests that larger eukaryotes could not have been selected for within this treatment.

Heterotrophic bacteria

Addition of inorganic nutrients to oligotrophic waters induces small increases in heterotrophic bacterial biomass; however, the response is ultimately mediated by the availability of organic substrates (Zohary et al. 2005, Mills et al. 2008, van Wambeke et al. 2008). In our experiments, heterotrophic bacteria responded to the +DW, and to a much lesser extent the +N and P treatment, suggesting that this component of the microbial community was co-limited by organic carbon and N + P. The +DW treatment likely contained enough labile organic material to sustain bacterial growth for at least 48 h (Fig. 5). Although we did not measure DOC in the +DW treatment, previous studies have demonstrated high concentrations and turnover of DOC below the thermocline in these stratified Gulf waters (Grossart & Simon 2002). We cannot discount that microbial cycling of nutrients may have been stimulated as a result of the 'bottle effect' (Ferguson et al. 1984) to increase DOC availability to the heterotrophs; however, the effect is likely relatively small since the bottles exhibiting greatest heterotrophic bacterial stimulation (+DW) did not coincide with the bottles containing greatest phototroph stimulation (+N and P).

Our results are generally consistent with previous studies that have demonstrated N and P co-limitation

of both phytoplankton and bacteria in oligotrophic waters (Zohary et al. 2005, Mills et al. 2008); however, heterotrophic bacterial productivity (BP) responded most positively to the +DW enrichment. In the presence of DOC, heterotrophic bacteria are capable of responding as quickly as autotrophs to inputs of inorganic nutrients (Gasol et al. 2009) and so can become direct competitors for the same resources (Mills et al. 2008). Whilst both BP and phototrophic productivity (i.e. τ) indeed increased rapidly within 24 h of nutrient addition, the simultaneous change of phototrophic and bacterial biomass could not be compared with the data available here. Increased bacterial productivity should result in increased abundance, so that heterotrophs would inevitably compete with the phototrophs for N and P. An argument for potential out-competition by the bacteria assumes that grazing rates upon the different microbial components were the same, which depends on the protist community structure in these waters (Christaki et al. 2005, Vázquez-Domínguez et al. 2005), or that the larger phytoplankton fractions (not observed by flow cytometry) were a significant component of the overall chl *a* biomass. These assumptions seem plausible since, if anything, grazing pressure is typically greater for the smaller components of the Gulf microbial community (e.g. Berninger & Wickham 2005). Furthermore, the increase in phototrophic biomass (chl *a*) in the +DW treatment was negligible over 48 h.

In contrast to the obligate heterotrophs and autotrophs, the facultative photoheterotroph (AAP) bacterio-chl *a* responded positively to +N, but showed some evidence of sequential +N and P limitation in the long-term (48 to 96h) incubations. The increase in bacterio-chl *a* biomass and decrease in τ between the initial sample and 24 h analysis was greater for the AAPs (ca. 4- and 1.25-fold, respectively) than for obligate autotrophs (ca. 1.25- and 1.20-fold, respectively) indicating that the AAPs were potentially more efficient at utilising the available inorganic resources for photosynthesis. AAP biomass (as chl *a*) was only ca. 1% that of the obligate autotroph chl *a*. After 24 h, pigment synthesis exhibited only a small further increase in bacterio-chl *a* but a gradual sustained increase in τ.

Up-regulation of phototrophy in response to N is perhaps not surprising since AAP bacterio-chl *a* concentrations are typically lower in oligotrophic waters than nutrient rich waters (e.g. Kolber et al. 2001), and these organisms can synthesize pigments rapidly upon relief of nutrient limitation (Biebl & Wagner-Döbler 2006). Also, N is likely to be the primary limiting inorganic nutrient for pigment synthesis. The gradual increase in τ with length of incubation implies a cumulative exhaustion of nutrients once bacterio-chl *a* biomass had accumulated.

CONCLUSIONS

We have shown that there were physiological and taxonomic changes in response to nutrient addition in the oligotrophic microbial community sampled from the Gulf of Aqaba. Our observations are consistent with previous bioassays that identified N-P co-limitation of phytoplankton biomass and productivity from oceanic waters (e.g. Zohary et al. 2005, Mackey et al. 2007, 2009, Davey et al. 2008, Moore et al. 2008), although co-limitation was not found for all parameters. We also observed N limitation of the biomass and productivity of AAP over 48 to 96 h duration. The increases in biomass (chl *a*) in response to N and N + P additions did not appear to be driven entirely by the picoplanktonic fraction (<10 µm, as measured by flow cytometry). Thus, our bioassays emphasize the importance of accounting for larger eukaryotic phytoplankton during incubations of oceanic waters. Given the lower nutrient requirements of smaller cells, it is perhaps not surprising that larger cells that persisted following deep winter mixing were likely nutrient-starved and responded to nutrient additions. Relief from nutrient limitation of larger cells would explain many of the observed bulk photo- physiological responses.

For both of the phototrophic fractions (phytoplankton and AAPs), changes in photosynthetic physiology and productivity in response to N and N + P additions preceded an increase in biomass (as chl *a* or cell number). Increases in photochemical efficiency (F_v/F_m) and decreases in electron turnover (τ) were observed within 24 to 48 h, signifying rapid up-regulation of PSII activity, in contrast to slower (48 to 96 h) changes in biomass. Low temperature (77K) fluorescence spectroscopy confirmed that N and N + P additions resulted in active synthesis of new PSII reaction centers. Our approach demonstrates that the physiological 'kick-start' as observed by FRR fluorescence is rapid and short-lived (24 to 48 h); consequently, detection of nutrient stress in nature will be determined by the time-scale at which transient nutrient perturbations occur.

Our bioassays provided evidence for minor N and P co-limitation of the heterotrophic bacteria; however, this fraction responded most to the +DW addition, demonstrating the importance for growth of additional resources in the deeper water. The contrasting responses of the bacteria and phototrophs are consistent with previous bioassay experiments and highlight the critical role of transient nutrient enrichment in regulating the structure and function of oceanic microbes.

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