

Short term response of plankton community to nutrient enrichment in Central Eastern Arabian Sea: elucidation through mesocosm experiments

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Abstract

Oligotrophic waters (OW), generally favour longer food chain facilitated by the microbial loop. In such ecosystems, physical mixing (e.g. upwelling, and winter convection) inject nutrients and propagules from subsurface to the photic zone. Such events are expected to alter the food chain through shifts in the plankton community. Mesocosm experiments were carried out to evaluate the influence of nutrient enrichment from the deep (100-150 m) on the surface plankton community for the first time in the Arabian Sea, through custom-designed enclosures in OW of the central-eastern Arabian Sea (CEAS). Surface water was characterized by low nutrients and phytoplankton biomass (chlorophyll-a of $<0.2 \mu\text{g m}^{-3}$) and upon nutrient enrichment yielded differing response. Higher abundance of picophytoplankton, bacteria and protists was noticed at a depth of ~ 100 m than at surface. The inoculation of such a population to the surface, resulted in a significant enhancement of autotrophic (picophytoplankton) and heterotrophic (bacteria and protists) populations. However, significant changes in the abundance of larger plankton was not evident till three days of incubation. Even though autotrophic picophytoplankton responded positively, a distinct increase in chlorophyll-a was not evident. This study points out that the lack of sufficient viable microphytoplankton propagules, neither at the surface nor at the depth (inoculum) are the possible reasons for the lack of their distinct positive response. These experiments suggest the dominance of microbial community response to physical mixing in the OW regions of the Arabian Sea and the importance of propagule diversity. The insights from this experiment will serve as a precursor for appropriate modifications in ocean modelling and forecasting studies and help in building global environmental management tools.

Key words: Oligotrophic waters; Mesocosm; Central Eastern Arabian Sea; Nutrient Enrichment; Bacteria; Protist; Phytoplankton; Zooplankton

Highlights

- First free floating mesocosm experiments in the Arabian Sea
- In situ evaluation of nutrient enrichment (NE) on oceanic plankton community
- Nutrient enrichment triggered microbial (picoplankton & protist) response
- Micro phytoplankton & zooplankton response not evident within 3 days of NE
- Biological response to NE depends on viable propagule density & incubation period

1. Introduction

Seasonal reversal in winds due to atmospheric pressure difference bring significant modifications in the circulation patterns in oceanic waters leading to occurrence of upwelling and convective mixing (Madhupratap et al., 1996; Shankar et al., 2016). The changes in the physical forcing over space and time in the Arabian Sea is shown to influence the dynamics of summer, and winter blooms as evidenced through satellite derived chlorophyll-a (Levy et al., 2007; Shankar et al., 2018). In addition to the influence of large scale features, temperature fronts are shown to influence, smaller domain changes in the phytoplankton population (Roy et al., 2015). The physical forcing injects nutrient-rich waters from subsurface to surface leading to increase in plankton abundance in the surface waters. Nutrient concentrations at the surface are close to detection levels during non-monsoon period (March to May and October) and increase in nitrate concentrations to $>2 \mu\text{M}$ are noticed during winter and summer associated with convective mixing and upwelling respectively (de Sousa et al., 1996; Morrison et al., 1998; Sarma and Dalabehera, 2020). The depth of nitracline, where $>1 \mu\text{M}$ of nitrate occurs, exists between 25 and 50 m depth associated with deep chlorophyll-a maximum in the Arabian Sea (Sarma and Dalabehera, 2019; Dalabehera and Sarma, 2020). Higher abundance of total and viable bacteria and picoplankton are also reported in the subsurface waters in the Arabian Sea (Khandeparker et al., 2018; Rajaneesh et al., 2017).

Efforts under the Ocean Finder project of CSIR-NIO in the north eastern Arabian Sea (NEAS; Roy et al., 2015; Vipin et al., 2015; Sarma et al., 2015; 2018; 2019) revealed that vertical mixing occurs in the temperature fronts from water below 50-100 m resulting in enhanced phytoplankton biomass. Roy et al. (2015) noticed the dominance of coccolithophores (prymnesiophytes) and green algae (prasinophytes) in the frontal zone, whereas cyanophyceae was abundant in the non-frontal zone. Recently Sarma et al. (2018) sampled 5 temperature fronts in the NEAS during winter and noticed elevated nutrient concentrations in all the fronts but a significant response of bacteria (Khandeparker et al., 2018) was observed in the open ocean fronts while phytoplankton and zooplankton responded in the shelf fronts. Higher bacterial response in the open ocean fronts was attributed to deeper mixed layers than shelf fronts leading to the input of bacteria-rich subsurface waters to the surface.

On the other hand, higher phytoplankton biomass in the shelf region was promoted due to the addition of nutrients through mixing in the shelf front. Based on these observations, Sarma et al. (2018) suggested that the ecosystem response in the frontal zone is dependent not only on the amount of nutrients injected to the photic zone but also the initial conditions under which the front is

developed. Further, the observations have shown that that the central-eastern AS (CEAS) over the continental margin is subjected to short term hydrographic variations resulting in transient variations in picophytoplankton composition during the transition phase from stratified to vertical mixing (Rajaneesh et al., 2017). In the coastal waters of AS, phytoplankton diversity is the highest during the winter and is coupled with the availability of high concentration of nutrients (Sawant et al., 1996). In the open ocean region, the limitation of nutrients under stratified condition is expected to influence phytoplankton biomass and diversity. Generally, stratified systems transform into an oligotrophic conditions wherein the food chain is longer due to activation of the microbial loop (Ducklow, 1993). The predominance of the microbial loop is considered as one of the explanations for zooplankton paradox (i.e., zooplankton presence during all the seasons) in AS (Madhupratap et al., 1996). Thus, it is hypothesized that pulsed inputs of nutrients through physical processes in the oligotrophic open ocean waters may trigger phytoplankton growth, and the classical food web may be operational. Here, we elucidate the impact of nutrient enrichment of the surface waters from subsurface waters on planktonic community through *in situ* mesocosm experiments using custom-designed enclosures in CEAS during winter. *In situ* mesocosm experiments with large volumes allow to carry out studies at the community level. So far community-level studies have mostly focused on the impact of carbonate chemistry in eutrophic environments (Orr et al., 2005; Yamamoto-Kawai et al., 2009), and also the regions of low inorganic nutrient concentrations (Paul et al., 2015; Sala et al., 2015; Bach et al., 2016; Hornick et al., 2016). On the other hand, studies from the oligotrophic environment are limited (Taucher et al. 2017, Hernández-Hernández et al., 2018). In this study, we examined the response of plankton community to natural nutrients fertilization (from a depth of 100-150 m) with variable initial conditions of propagules mixed from the deep to the surface waters.

2. Material and methods

2.1. Mesocosm design

Since the mesocosm experiments were planned to be conducted in open ocean water, free-drifting enclosures with two different designs were adopted (Figs. 1 a,b). The material used for the mesocosm enclosure was a transparent polypropylene sheet to make a cylindrical bag by fusing the edges with a hot air gun. In one design, the cylindrical bag was sandwiched between the two stainless steel (marine grade) cages to provide reinforcement and protection from the wave action (referred as Cage design; Fig. 1a). The mesocosm enclosure was kept afloat with fiber floats and was used in experiments III and IV.

The second design comprised of a cylindrical transparent polypropylene bag mounted on to a circular ring at the open end, which acted as a float made out of the same material as of the bag and inflated with air (referred as Float design; Fig. 1b). This design was used for Experiments I and II. The difference between the cage and float designs being that the former had the weight due to steel reinforcement and fibre floats and had to be deployed with on board winch, whereas the later was without any such weight. The advantage of the float design was that being lighter in nature, it was floating over waves and drifting with currents, resulting in natural stirring of water inside the bag. It was deployed manually by gently placing on the sea surface and filled the surface water. Prior to the experiments the polypropylene sheet was soaked in seawater for 24 hrs to remove dissolved substances, if any.

2.2. Initial condition and mixing procedure

Altogether, four mesocosm experiments (I, II, III, and IV) from 4 different locations within 25 km radius in the offshore of Goa located in central, eastern Arabian Sea (CEAS; Fig. 2, Table 1) were performed during early winter months (25th November to 1st December 2014). On each occasion, two mesocosms bags were filled with ~2 m³ of seawater from the surface. Of which, one was used as control and the other as an experiment. Each of the experimental bag was amended with different proportions of water from below the mixed layer (~100m) to enrich the surface water nutrients (Table 1). The volume of the water in control and experiment was maintained the same i.e., ~2 m³. Later both the mesocosm bags were deployed in the sea with one end tied to the side of the ship with a long rope so that the enclosures are free-floating. Appropriate care was also taken to ensure that both the enclosures donot cross each other when the ship is drifting. This set up is referred to as experiment I, and similar setups and deployment procedures were repeated thrice on subsequent days, and they are referred to as experiments II, III and IV respectively. The time taken for the preparation of a pair of mesocosms and its deployment was almost an entire day, and during this period, the ship was drifting. Hence, the mesocosms deployed at different locations are filled with different surface and bottom waters of the oligotrophic zone. The vertical profiles of temperature, salinity, chlorophyll-a and dissolved oxygen are plotted at the four locations, where surface water was collected, suggested that water column is uniform, however, both concentration and depth of chlorophyll-a maximum was different (Fig. 3). In order to avoid input of phytoplankton biomass from chlorophyll maxima, the depth of deep water was chosen from below the depth of chlorophyll maxima (100-150 m).

Surface waters were oligotrophic with low nitrate ($<0.5 \mu\text{M}$), phosphate ($<0.5 \mu\text{M}$) and silicate ($<2.0 \mu\text{M}$) when sampled (Table 2). The deep waters (100 m) were several times enriched in terms of nutrients compared to surface ($<5 \mu\text{M}$ of nitrate, $>0.8 \mu\text{M}$ of phosphate and $>10 \mu\text{M}$ of silicate). After the addition of deep water (inoculation), the nutrient concentration, and chlorophyll-a concentrations were of the same order in all the experimental bags (Table 2&4). However, when compared to control, nutrient concentrations doubled in the experimental bags, but the chlorophyll-a changes were negligible. The increase in nutrient addition was maintained close to natural levels as the increase in concentrations of nitrate, for instance, was observed up to 1 to 2 μM during convective mixing in the northern Arabian Sea, and upwelling driven by Findlater Jet (de Sousa et al., 1996; Morrison et al., 1998; Naqvi et al., 2002), and frontal zone (Sarma et al., 2018) in the open ocean of the Arabian Sea.

2.3. *Water sampling in the mesocosm bags*

Soon after enrichment with the deep water, water samples were taken from the bag for several biogeochemical parameters for up to 3-7 days (Table 1). However, we have considered the experiments carried out for three days only in this study to maintain uniformity in all experiments. Every day, between 10:00 and 12:00 h, each bag was sampled (both control and experiment) for different chemical and biological variables using 5 L Niskin bottle. Bag was pulled close to the ship and 5L Niskin bottle tied to the hydrographic wire, coated with Teflon, was sent to the mesocosm bag and closed the bottle using messenger. Subsampling for different parameters were done from the Niskin bottle using glass tube.

2.4. *Hydrography and chemical analysis in the water column*

Vertical profile of temperature, salinity, chlorophyll, and dissolved oxygen was measured using conductivity, temperature and depth sensors (Sea-Bird Electronics, *19 Plus* USA) along with fluorescence (Wet Labs) and dissolved oxygen (Sea-Bird Electronics) sensors mounted on the Rosette. Water samples to enrich surface water nutrients were obtained from 100 m depth using 20 L Niskin bottles fitted to the rosette. The concentrations of nutrients (nitrate, phosphate, and silicate) were measured using Skalar nutrient autoanalyzer (Skalar SAN++ System, Netherlands). About 2 to 3 litres of water samples were filtered through $0.7 \mu\text{m}$ glass fibre (GF/F; 25mm diameter, Whatman) filters and pigments retained on the filters were extracted to 90% acetone and measured using High Performance Liquid Chromatography (HPLC) (Agilent Technologies; 1200 series,) following the detailed methodology given by Roy et al. (2015). About 5 liters of water samples from mesocosm

bags were filtered through pre-combusted GF/F filter (0.7 μm pore size 25 mm diameter; Whatman) at 300 °C for 6 h, at low vacuum and dried at 60 °C for at least 24 h. An aliquot of the filter was acid fumigated for 12 h in a desiccator to remove inorganic carbon. The particulate organic carbon (POC) and particulate nitrogen (PN) were measured using elemental analyzer (M/S Thermo Electron, Germany) using internal reference materials of Glutamic Acid, Alanine, Marine sediments, and IAEA standards. The long term precision of the instrument was 0.02% for both carbon and nitrogen (Sarma et al., 2012; 2014). Total organic carbon (TOC) was measured using total organic carbon analyser (TOC CPH, Shimadzu Corporation, Japan) and DOC was estimated as the difference between TOC and POC.

2.5. Microbiological analyses

2.5.1. Enumeration of total viable bacterial count (TVC)

The seawater samples were diluted as required and spread plated (0.1 ml) on Zobell Marine Agar 2216 (ZMA; Hi-media), which has been extensively used to grow marine bacteria. Subsequently, the samples inoculated on the agar plates were incubated for 24 h at room temperature. After incubation, the colony-forming units (CFU) were counted and expressed as CFU L^{-1} .

2.5.2. Enumeration of total bacterial count (TBC)

Aliquots of 4.5 ml of sample were taken (n=3) for the analysis of the TBC and fixed with 0.22 μm filtered paraformaldehyde (1% final concentration), stored at -80°C until samples could be processed. The samples were thawed at room temperature, subsequently stained with SYBR Green I (1:10000 final concentration Molecular probes, USA) and incubated in the dark for 15 min before measurement. Absolute cell counting was measured using BD FACS Aria II Flow cytometer equipped with 488 nm blue laser. The sheath fluid was BD FACS flow. The emitted light was collected through 488/10 band pass (BP) for right-angle light scatter/side scatter (RALS/SSC) and 530/30 band pass (BP) for green fluorescence. Fluorescent beads (1 μm , Polysciences) were used as internal standards. The TBC is expressed as cells L^{-1} . The flow cytometric data were processed using BD FACS Diva (version 6.2) software (Khandeparker et al., 2017).

2.5.3. Enumeration of protists

Aliquots of 4.5 mL of seawater samples were taken in triplicates (n = 3). Subsequently, the seawater samples were filtered through 20 μm mesh, preserved with 0.22 μm filtered paraformaldehyde (1% final concentration), quickly frozen in liquid nitrogen and stored at -80°C until analysis (not more

than a month). Protists were analysed using a modified protocol by Christaki et al. (2011). Briefly, frozen samples were thawed and stained with SYBR Green I (1:10000 final concentration Molecular probes, USA) and incubated in the dark for 15 min before measurement. Emitted light and fluorescent signals were collected through 488/10 bandpass filter for SSC, 530/30 bandpass filter for green (FL-I) and 695/40 for red (FL-II) fluorescence. 1µm yellow-green fluorescent size beads (Polysciences, USA) were used as an internal standard. Gating was done against green versus red fluorescence. The protists abundance was expressed as cells L⁻¹. Data obtained were processed with the BD FACS Diva (Version 6.2) software.

2.5.4. Estimation of bacterial production (BP)

5-Bromo-2-Deoxyuridine (BrdU) was used for estimating bacterial production (BP). Briefly, the samples were treated with 5-Bromo-2-Deoxyuridine (BrdU; 1µM final concentration; BD-Biosciences), incubated for 3 hrs at in situ temperature. Subsequently, the samples were fixed with 0.22 µm filtered paraformaldehyde (1% final concentration) and stored at -80° C. During analysis, the samples were stained with FITC labelled anti – BrdU with DNase I (BD – Biosciences), kept for 30 min in the dark and analysed using a BD FACSAria™ II flow cytometer equipped with 488nm nuclear blue laser (Khandeparker et al., 2018). For calculating the bacterial carbon production, 11 fg C per bacterium as a cell-to-carbon conversion factor (Garrison et al., 2000) was used, and expressed as µg C l⁻¹ h⁻¹.

2.6. Picophytoplankton analysis

Water samples (2 ml) for pico-phytoplankton analysis were collected in cryovials and preserved with paraformaldehyde (final concentration of 0.2%). After 15 min dark incubation, the samples were flash-frozen in liquid nitrogen and later stored at -80°C in the laboratory until analysis. Prior to analysis preserved samples were thawed to room temperature and analyzed through FacsVerse flow cytometer configured with 20 mW and 40 mW air-cooled lasers exciting at 488 nm (blue) and 664 nm (red), respectively. Sample acquisition was set for 10000 events. Flow rates were standardized before analysis of samples (Marie et al., 2005). Forward and right-angle light scatters, orange (564– 606 nm) and red (>650 nm) fluorescence were collected from each particle and analyzed with Facs Suit™ software. Cell fluorescence emission and light scatter signals were normalized to that of the yellow-green latex beads of 2 µm (polysciences co., USA) which were added to samples as an internal standard. Three major groups of pico-phytoplankton were determined based on size and fluorescence properties. *Synechococcus* (SYN) was identified based on the orange fluorescence of the pigment phycoerythrin. Based on the pigment fluorescence intensities, two sub-

groups of *SYN* were identified, *SYN-PEI* and *SYN-PEII*, with lower and higher intensity, respectively. Picoeukaryotes and *Prochlorococcus* were identified based on their larger, and smaller right-angle light scatters along with higher and lower red fluorescence characteristics, respectively.

2.7. *Micro-phytoplankton analysis*

A known volume of the water sample (1 L) was preserved with 4% Lugol's iodine solution at the site for phytoplankton analysis using an inverted microscope (IX-73 Olympus Make). Before analysis, the preserved material was concentrated by subjecting to sedimentation in a settling chamber. The identification of phytoplankton species was carried out based on the standard identification keys.

2.8. *Nutrients utilization rates*

Incubations were initiated by adding trace quantities of ^{15}N labelled (99 at%) nitrate and ammonium. Based on the nutrients concentrations of the samples, trace concentrations, adjusted to 10% of an ambient level, of tracer was added to the sample. The samples were then immediately placed in deck-board incubator supplied with flowing surface seawater to prevent solar heating and neutral density screen to simulate *in situ* light levels. Incubations were terminated by filtration on combusted GF/F glass fiber filters. The particulate organic nitrogen concentrations and ^{15}N enrichment from our tracer experiments were determined using mass spectrometer (Delta V Plus; Thermo Electron, Germany) attached to the elemental analyzer. All water samples were processed in duplicate, and the maximum difference in the measured particulate organic nitrogen of duplicate samples was found to be <10%. The coefficient of variation in ^{15}N atom% measurement was less than 1% of samples. Uptake rates were calculated using the equation of Dugdale and Wilkerson (1986).

2.9. *Zooplankton analysis*

The mesozooplankton were collected by filtering 80 l of waters from different sets of experiment and filtering through 200 μm mesh, and this filtered seawater was then passed through 50 μm mesh for the collection of microzooplankton. The samples were preserved in 5% buffered formaldehyde for microscopic analysis. The entire sample was analyzed for taxonomic identification up to group level using a stereo microscope (Olympus SZX16) (Kasturirangan 1963; Postel et al., 2000; Conway et al., 2003), and the abundance is expressed as individuals (No.) m^{-3} .

3. Results

3.1. Initial conditions of the mesocosm experiments

Before the addition of deep water, the concentrations of nitrate in the surface waters ranged between 0.1 and 0.4 μM in the experiments, except in experiment III where it was $\sim 1.0 \mu\text{M}$, whereas phosphate varied within a narrow range of 0.4 to 0.5 μM (Table 2). The concentration of dissolved silicate was mostly above limiting levels ($>2 \mu\text{M}$) in the experiments II and IV, and it was $< 2 \mu\text{M}$ in the experiments I and III (Table. 2, Fig. 4a). The low N:P ratios (<1) were observed in all 4 the experiments. Ammonium concentrations were observed to be $\sim 2 \mu\text{M}$ in all experiments (Fig. 4a). The concentration of particulate organic carbon (POC) varied between 5.1 and 14.0 μM . The concentrations of DOC varied from 63.3 to 105.8 μM and it is consistent with the abundance of bacteria and pico-phytoplankton. The phytoplankton biomass occurred in concentrations (0.02 to 0.18 mg m^{-3}) that are typical for oligotrophic conditions (Fig. 4b).

Total viable bacteria (TVC) varied from 1.1 to 25.3 $\times 10^6 \text{ CFU L}^{-1}$. Similarly abundance of Protists and total bacteria ranged from 8.6 to 424 $\times 10^6 \text{ cells L}^{-1}$ and 0.6 to 7.6 $\times 10^9 \text{ cells L}^{-1}$ respectively in the all experiment. The bacterial production varied from 0.97 to 7.98 $\mu\text{g C l}^{-1} \text{ h}^{-1}$ in all the experiments (Table 3).

Cyanophyceae dominated the phytoplankton community in all four experiments, and the contribution of other groups was $<5\%$. *Synechococcus* was the dominant group among Cyanophyceae, and it was 3 to 16 times higher than *Prochlorococcus* in all the experiments (Table 3, Fig. 4b). Picoeukaryotes abundance varied from 0 to 685 $\times 10^3 \text{ cells L}^{-1}$ in all the experiments. *Prochlorococcus* were present more or less uniformly in all the experiments, but their abundance was ten times less than that of picoeukaryotes. Among the microphytoplankton diatoms and dinoflagellates dominated the community. In the case of diatoms, centric followed by pennates dominated the community whereas, in the case of dinoflagellates, mixotrophic and autotrophic forms dominated the community. The initial abundance of diatoms (64 to 184 cells L^{-1}) and dinoflagellates (144 to 280 cells L^{-1}) was variable between the experiments (Table 4, Fig. 4b). The contribution of diatoms was higher only in the experiment II whereas the dinoflagellates contribution was higher in the remaining three experiments.

The initial microzooplankton abundance in the surface waters varied from 138 to 425 No. m^{-3} in all the experiments (Table 4, Fig. 4b). The initial mesozooplankton abundance ranged from 575 to 788 No. m^{-3} in all the experiments (Table 4, Fig. 4b). A large number of invertebrate larvae

including nauplii of copepods were reported in all the experiments. The zooplankton abundance (without considering the larval count) ranged from 450 to 1487 No. m⁻³ in experiments I and II respectively, indicating the contribution of larval forms to the total zooplankton abundance was considerable in experiment II. Adult poecilostomatids followed by calanoid and cyclopoid copepods dominated the zooplankton community in all the experiments. Among the larval forms, calanoid copepod nauplii were the most dominant, followed by poecilostomatid nauplii in all the experiments. Pontellid larvae were observed in considerable numbers in experiments III and IV.

3.2. Biogeochemical characteristics of inoculum (subsurface waters)

Since the sub-surface waters are mixed with the surface waters in the mesocosm tank to fertilize the nutrients, it is also possible that some components of the sub-surface plankton might have also got inoculated into the experimental bags. It has been observed that TVC, TBC and protists were higher in the inoculum water than the surface except in experiment III, wherein TVC and protists were lower in the inoculum, while *Prochlorococcus* were 2 to 55 times higher in the former than latter respectively (Table 3). In contrast, the inoculum water mixed in the experiment had no significant difference with reference to the surface waters for *Synechococcus*. This suggests that a significant number of plankton (TVC, TBC, Protists, and *Prochlorococcus*) were injected to the incubated waters along with the nutrients which might have contributed to the response of the ecosystem in the mesocosm bags (Table 3).

3.3. Immediate changes in biogeochemical properties after fertilization

After fertilization of surface water with subsurface waters in the experimental bags, the concentrations of nutrients increased to 1.1 to 2.2 µM of nitrate, 0.5 to 0.6 µM of phosphate and 3.8 to 5.5 µM of silicate in all the experiments, which are well above the limiting levels (Table 2). A significant increase in Chl-a was observed in experiment II (0.03 to 0.14 mg m⁻³) and III (0.02 to 0.1 mg m⁻³) on the other hand decrease in Chl-a was observed in experiment I (0.18 to 0.12 mg m⁻³) and no change was observed at experiment IV. The micro-phytoplankton abundance decreased in the experiments (I, II and IV), whereas, it increased moderately in experiments III. A relative increase in TVC was observed in experiments I, II and IV but decreased in experiment III, whereas, an increase in the TBC was noticed in experiment I and IV but decreased in experiments II and III after mixing with subsurface waters. An increase in the abundance of protists was noticed in all the experiments. In contrast, an increase in *Prochlorococcus* was observed only in experiment III (Table 3). Picoeukaryotes increased in all the experiments (except in experiment I), whereas, in *Synechococcus* increase was observed in experiments III and IV and decreased in experiment I and II). A slight

increase in diatoms was observed in experiments I and III, whereas, it decreased in the other experiments. On the other hand, the abundance of dinoflagellates decreased in all the experiments after addition of subsurface waters. A decrease in micro and mesozooplankton was noticed in all the experiments, except experiment IV and III respectively. The changes observed after the fertilization were mainly due to the higher abundance in the sub-surface waters, as discussed in the above section.

3.4. Variations in response of biology to fertilization

After three days of incubation, variable response of plankton was observed with reference to initial concentrations in both control and fertilized bags. After incubation for three days, the concentration of nitrate decreased to limiting levels in the control (0.05 to 0.25 μM) in all the experiments, whereas, phosphate (0.24 to 0.39 μM) and silicate (0.9 to 2.11 μM) were above the limiting levels. Though significant decrease in nitrate concentrations were observed, variations in Chl-a were insignificant in the control. A significant increase (16-60%) in DOC was observed in the control during three days of incubation. An increase in POC was also observed compared to initial conditions, however, this increase was not prominent in the control (Table 2; Fig. 4a). The TVC increased by 2 to 4 times during incubation in the control, whereas, TBC and Protists decreased by 10-30%. Similarly, a significant decrease in *Synochoccus*, *picoeukaryotes*, *Prochlorococcus*, phytoplankton abundance, micro and mesozooplankton abundance was observed in the control after three days of incubation.

In the experimental bag, the concentration of nitrate decreased to non-detectable to 0.2 μM , while phosphate (0.3 to 0.5 μM) and silicate (2.8 to 4.4 μM) were still available (Table 2). A significant increase in DOC was observed by 14-37% in the experiments (Fig. 4a). Similarly, an increase in POC was also observed compared to initial in the experimental bags (Table 2; Fig. 4a). The phytoplankton biomass (Chl-a) showed a variable response. Though rapid removal of nitrate was noticed in all the experiments, compared to initial levels, the distinct chlorophyll accumulation was observed only in experiment II. In all the experiments no chlorophyll-a (Chl-a) accumulation was noticed. Uniformly significant increase in TVC was observed in all the experiments compared to control (Table 3; Figs. 5; t-test: $p < 0.03$). A slight increase in TBC was noticed in experiments II and IV while decreased in experiment I and III. Despite a significant amount of TBC input from the subsurface waters in experiment III and IV, it decreased during incubation in the former, and increased in the latter experiment. Interestingly, an increase in *Synechococcus* was noticed in all the experiments, whereas, *picoeukaryotes* population increased in fertilized bags of experiments I and II and *Prochlorococcus* population increased in experiment I and II (Table 3, Figs. 4 and 5). An

increase in protist numbers was noticed in all the experiments while it decreased in experiment III (Table 3, Figs. 4 and 5). On the contrary total micro-phytoplankton abundance showed a marginal increase in all the experiments (Table 3, Figs. 4 and 5). Among the microphytoplankton, diatoms and dinoflagellates dominated the community. Among these, diatoms increased by more than one order in experiments I and IV, whereas it was dinoflagellates in experiments I and II. Among the grazers, microzooplankton showed a variable response. The mesozooplankton abundance decreased (Table 4, Figs. 4 and 5). T-test (n=4) for individual treatment indicated that the response of plankton (except for TVC) after incubation was not statistically significant even though there were positive responses in picoplankton (both autotrophs and heterotrophs).

4. Discussion

4.1. Variable response of microphytoplankton community to the nutrient inputs

After fertilization with the subsurface waters, it was observed that concentration and uptake rates of nutrient, both nitrate and ammonium, increased significantly by an order of magnitude resulting in limitation of dissolved inorganic nitrogen within three days (Table 2). Though nutrients in the experimental bags were above the limiting levels after fertilization, a poor phytoplankton response was observed, which can be due to their physiological status. Due to the existence of oligotrophic conditions in the mixed layer, the phytoplankton might have been adapted to low nutrients levels, and this could take some time for them to respond to the enriched nutrients due to fertilization. Further, the addition of deep water not only increased the nutrients but also introduced a relatively higher population of zooplankton and micro-phytoplankton bottom propagules (MBP), which are mostly the dormant cells and it takes some time upon transfer to well-lit zones to develop into a growing cell. As a result, in the experiments, the contribution of surface growing/viable cells decreased, and the zooplankton population increased when compared to the control. Such biological changes in the nutrient-enrichment experiments using bottom waters are the main reason for the observed differences with the control.

An increase in the nutrients was observed in control during incubation, suggesting release of nutrients through organic matter decomposition. Due to less phytoplankton abundance in control, the increased nutrients through organic matter decomposition was accumulated than consumed. Microphytoplankton in the surface waters (initial) responded only in the Experiment I but not in other experiments to nutrients addition (Table 4). Though concentration of silicate was above the limiting levels, both in the initial (1.4-2.2 μM) and after addition of inoculum (3.8-5.5 μM) in all experiments, but the positive response in the Experiment I may be due to the presence of more

number of microphytoplankton cells initially in the treatment. Increase in diatom cells was noticed after addition of inoculum in the experiments I and III, whereas, it decreased in the Experiment II and IV. A decrease in the dinoflagellate abundance was observed in all the experiments after addition of the inoculum suggesting that lower abundance of dinoflagellates in the subsurface waters and in inoculum did not enhance their abundance. Increase in diatoms abundance was noticed after incubation of 3 days in the experiment I and IV, whereas, increase in the dinoflagellates was evident only in experiment I. Therefore it is possible that the response of dinoflagellates, owing to their complex nutritional mode and slow growth rate compared to the diatoms, may not be captured (Table 4). The abundance of mesozooplankton was the least in the experiment I (400 No. m⁻³) after addition of inoculum compared to other experiments (425 to 1413 No. m⁻³) indicating less grazing pressure in the former experiment. It is a well-known fact that copepod species belonging to the order Calanoida and genus *Calanus* are omnivorous (Turner, 1984) and studies have shown that they graze upon diatoms, dinoflagellates, silicoflagellates, *Phaeocystis*, coccolithophorids, radiolarians, tintinnids, molluscan larvae, and other crustaceans (Arashkevich 1969; Turner 1984). The grazing by zooplankton on phytoplankton is often related to their body size as well as their taxonomic composition (Cyr and Curtis 1999). Copepods have an ability to consume large particles and also use a combination of active and passive strategies to collect particles of different size (Vanderploeg, 1981). The difference in the abundance of larger zooplankton (mesozooplankton) in the experimental bags may lead to differential grazing pressure on the phytoplankton, and alter the overall chlorophyll biomass. The present experiments clearly indicate that abundance of phyto and zooplankton in the inoculum and their overall abundance in the incubation bag controls their response to addition of nutrients to surface waters. The variability in response in different bags is also caused by heterogeneity in the plankton abundance in both surface and inoculum waters. On the other hand, initial nutrient concentrations (mainly nitrate) were the highest in experiment III, only viable bacteria (TVC) responded significantly compared to other plankton, but their abundance was also higher in the initial waters itself. Though protists were also dominant in the initial waters of experiment III (424 x 10⁶ cells L⁻¹), they did not respond significantly after fertilization (316 x 10⁶ cells L⁻¹). A rapid increase in the TVC in all the experiments probably resulted in the utilization of all the nutrients (Table 1). Despite initial higher silicate concentration (above limiting levels of 2 µM) in experiments II and IV, diatoms responded only in experiment IV, and it is consistent with the response of mesozooplankton. The mesozooplankton did increase in any experiments despite variable initial abundances. bags. Probably variable mesozooplankton grazing might have regulated the growth of diatoms. Though a high amount of nutrients were added from subsurface waters (see

inoculum nutrients) in experiment III, no response of microphytoplankton was observed due to the increased abundance of micro and mesozooplankton. Therefore this study suggests that irrespective of amount of nutrients in the initial waters and fertilized waters, plankton addition through inoculum is important for the response of micro-phytoplankton in the open ocean regions.

4.2. *The response of picophytoplankton to nutrient addition*

Unlike micro-phytoplankton, a significant response was observed with pico-phytoplankton to nutrients increase (Figs. 4b and 5). Following nutrient enrichment, *Synechococcus* responded in all the experiments. Earlier observations have also shown an increase in *Synechococcus* abundance within the frontal regions compared to non-frontal regions in response to the relatively higher nutrient concentrations in the NEAS (Mitbavkar and Anil 2018). The response of *Synechococcus* to nutrient enrichment varied between 57 and 404% and did not correlate with initial (after addition of inoculum) abundance suggesting that grazing might have significantly influenced their response. A significant increase in picoeukaryotes was also observed in experiments and variable rate of increase may be driven by either slower growth response to nutrient enrichment or higher grazing pressure, as evidenced by the relatively higher abundance of protists, micro, and mesozooplankton. Bach et al. (2017), based on mesocosm experiments conducted in temperate and cold waters, noticed that the picoeukaryotes are winners among other planktons in the future ocean. However, the mesocosm experiment in the sub-tropical region revealed contrasting results with dominant microphytoplankton than picoplankton (Hernández-Hernández et al., 2018). An earlier study involving high-resolution observations on the picophytoplankton community responses to nutrients in the eastern Arabian Sea showed initial picoeukaryotes response to nutrients fertilization (Rajaneesh et al., 2017). Similarly, *Prochlorococcus* also responded positively in the experiments to nutrient enrichment (Fig. 4). The Arabian Sea harbours both high and low light (below mixed layer depth) adapted *Prochlorococcus* (Roy and Anil 2015) and therefore its decrease in the mesocosm could be due to the cell death driven by photosystem II inactivation (Six et al. 2007).

4.3. *The response of bacteria to nutrients addition*

An increase in the DOC was observed in all experiments associated with a significant increase in the TVC and protists. The response of protist was low in the experiment III associating with higher response of TVC suggesting a time-lag for protist to respond to increase in TVC.. A decrease in protists in experiment III associated with high microzooplankton abundance (800 No. m⁻³) than other experiments (300-375 No m⁻³) suggests that grazing played a significant role in controlling Protists. (Fig. 5).

Predaceous protists are ubiquitous in pelagic ecosystems, range in size from 2 μm flagellates to $>100 \mu\text{m}$ ciliates and dinoflagellates (Sherr and Sherr, 2002). Predation by protists can be a significant pathway of mortality for suspended bacteria in both freshwater as well as marine ecosystems. Heterotrophic protists can also be a significant food source for metazooplankton (Sherr and Sherr, 2002). Heterotrophic marine flagellates graze upon bacteria and release a large amount of dissolved free and combined amino acids during exponential growth (Nagata and Kirchman, 1991). In experiment III and IV, the DOC concentration was considerably higher than the other experiments which can be attributed to higher abundance of bacteria (TVC and TBC) and protists.

Flow cytometry analysis revealed two types of protist populations, small ($\sim 2 \mu\text{m}$, P1) and big ($\sim 4 \mu\text{m}$, P2). In the control as well as the experiments, both the populations were present, and the count was initially low, however, increased upon incubation. Except in experiment II, an increase in P2 type protists was observed. Protists graze selectively on bacteria and may strongly influence the taxonomic composition, morphology, physiology, and metabolism of bacterial communities (Jurgens and Matz, 2002; Kinner et al., 1998). Since the factors that influence grazing also include the feeding capacity of the protists, studies focusing on their size and taxonomic identity would be significant in elucidating their role in prey-predator interaction and relevance.

In response to an increase in bacteria and cyanobacteria, a significant increase in protists abundance was also noticed in all experiments. Despite an increase in the bacterial population, an increase in DOC can be attributed to organic carbon production through picoplankton. In addition to this, since the system is closed, the contribution of dead organisms to increase in organic carbon content in the experimental bags cannot be ruled out. It was further observed that POC decreased with an increase in DOC suggesting, their hydrolysis by bacteria, which could have resulted in the release of DOC.

4.4. The response of zooplankton to nutrients addition

It is observed that the abundance of micro and mesozooplankton varied with the experiments. In experiment II the abundance of microzooplankton increased, whereas in other experiments it decreased. However, in the case of mesozooplankton the abundance decreased in all the four experiments after the incubation. Pontellid larvae were observed in considerable numbers in experiments III and IV. This study suggests that either the dominance of microbial loop or our experimental duration was short for the zooplankton to respond and may be a potential reason for their weak response.

5. Summary and Conclusions

In summary, the responses of surface plankton of the oligotrophic region of the central-eastern Arabian Sea to natural nutrient enrichment from subsurface water showed distinct inter and intraspecific variation with respect to groups or taxa. The surface water used for the experiments was characterized by very low chlorophyll ($<0.2 \text{ mg. m}^{-3}$) and dissolved inorganic macro-nutrients. Surface plankton, upon nutrient enrichment, compared to control, showed a range of responses depending upon groups/taxa. For instance, positive (autotrophic *Synechococcus*, and heterotrophic bacteria and protists), negative (both micro-and meso-zooplankton), variable (picoeukaryotes and microphytoplankton), and no (*Prochlorococcus*) responses to nutrient enrichment were observed (Fig. 6). Since surface waters in the open ocean region during the study period was oligotrophic with very low phytoplankton biomass, both autotrophic (picophytoplankton) and heterotrophic microbial (bacteria and protists) populations responded to the natural fertilization than hitherto hypothesized as micro-phytoplankton, particularly diatoms. Response of zooplankton was not detected and that might be due to the short experimental duration. These findings are consistent with our observations in the frontal and non-frontal zones in the eastern Arabian Sea (Sarma et al., 2018). In addition to this, decrease in both microphytoplankton and zooplankton may also have been caused by their sedimentation to the bottom of the mesocosm bag. We could not collect the samples from the bottom of the mesocosm bags, at the end of the experiment, due to rupturing of the bags while retrieving. Nevertheless, this study suggests that bacteria and pico-phytoplankton do respond initially to the nutrients input to the surface waters in the Arabian Sea than hitherto hypothesized as diatoms. In the numerical models, only nutrients brought by physical mixing is considered, and based on the nutrients utilization rates, chlorophyll-a (phytoplankton biomass) is developed. In contrast, our experiments suggest that ecosystem components brought from the depth of mixing govern the plankton response to mixing in the photic zone. Unfortunately the climatology of the ecosystem (bacteria, and plankton) is not available in most parts of the global ocean to know what kind of plankton are brought to the surface due to physical mixing. This could be one of the potential reasons for the inaccurate performance of ecosystem models in many regions in the tropical oceans (Sarma et al., 2013). Considering the existence of large spatial and temporal variability in the plankton community in the surface and subsurface, further investigations at different regions and times are essential for better understanding of the plankton response to physical mixing in the Arabian Sea.

Declaration of Interests

None

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Table 1. Details of the mesocosm experiments conducted in the Arabian Sea

Expt	Latitude	Longitude	Depth of deep water	Volume mixed (L)	Surface to bottom water ratio	Experiment period	Expt. Days	Expt. Design
I	15.89° N	72.69° E	150	100	20:1	25 th Nov-1 st Dec 2014	7	Float
II	15.86° N	72.72° E	100	250	8:1	26 th Nov-1 st Dec 2014	6	Float
III	15.84° N	72.68° E	100	230	8.7:1	27 th Nov-30 th Nov 2014	4	Cage
IV	15.72° N	72.62° E	100	200	10:1	28 th Nov-30 th Nov 2014	3	Cage

Table 2. Chemical characteristics of water of sea surface water (initial), inoculum, after addition of inoculum to surface waters and after incubation of 3 days in Experiments I to IV. NO₃ – nitrate, PO₄ phosphate, SiO₄ - silicate, POC – particulate organic matter, DOC – dissolved organic matter, - nitrate uptake, - ammonia uptake.

Expt.	Experiment status	NO ₃ (μM)	PO ₄ (μM)	SiO ₄ (μM)	POC (μM)	DOC (μM)	NO ₃ up (μM/l/d)	NH ₄ up (μM/l/d)
Expt. I	Initial	0.4	0.5	1.4	5.1	69.7	0.01	0.13
	Inoculum	9.4	0.9	15.5				
	After addition of Inoculum	1.3	0.6	4.2	5.8	63.8	0.13	0.33
	After incubation	0.1	0.4	3.1	14.3	84.3	0.07	1.09
Expt. II	Initial	0.1	0.4	2.1	12.3	63.3	0.06	0.42
	Inoculum	4.8	0.8	10.8				
	After addition of Inoculum	1.1	0.5	3.8	9.2	64.7	0.03	0.51
	After incubation	0.2	0.4	2.8	10.3	103.2	0.23	0.19
Expt. III	Initial	1.0	0.4	1.7	9.6	105.8	0.01	0.06
	Inoculum	6.5	0.9	12.8				
	After addition of Inoculum	2.2	0.5	3.9	18.2	124.7	0.11	0.60
	After incubation	0.0	0.5	4.4	40.2	149.9	0.02	0.27
Expt. IV	Initial	0.3	0.4	2.2	14.0	93.5	0.05	0.11
	Inoculum	5.5	0.8	16.8				
	After addition of Inoculum	1.3	0.5	5.5	10.3	107.8	0.17	0.36
	After incubation	0.0	0.3	2.8	11.2	123.5	0.40	0.78

Table 3. Microbiological characteristics of water of sea surface water (initial), inoculum, after addition of inoculum to surface waters and after incubation of 3 days in Experiments I to IV. TVC – total viable count, TBC – total bacterial count, Syn – *Synechococcus*, Pro- *Prochlorococcus*, Pico – *picoeukaryotes*, BP – bacterial production in $\mu\text{g C l}^{-1} \text{h}^{-1}$.

Expt.	Experiment status	TVC $\times 10^6$ CFU L ⁻¹	TBC $\times 10^9$ cells L ⁻¹	Protist $\times 10^6$ cells L ⁻¹	Syn $\times 10^3$ cells L ⁻¹	Pro $\times 10^3$ cells L ⁻¹	Pico $\times 10^3$ cells L ⁻¹	BP $\mu\text{g C l}^{-1} \text{h}^{-1}$
Expt. I	Initial	1.1	1.0	9.7	570	50	125	2.93
	Inoculum	3.3	2.2	9.5	616	103	41	
	After addition of Inoculum	2.5	1.8	24.0	453	0	20	
	After incubation	9.4	1.4	58.4	744	51	215	4.89
Expt. II	Initial	2.7	0.6	8.6	909	80	0	7.98
	Inoculum	10.1	25.2	24.9	59.5	213	2205	
	After addition of Inoculum	5.9	0.2	16.2	268	16	88	
	After incubation	151	1.1	37.5	1352	127	215	21.4
Expt. III	Initial	25.3	7.6	424	141	52	316	0.97
	Inoculum	2.2	22.5	7.5	180	4384	322	
	After addition of Inoculum	2.9	3.1	628	587	623	959	
	After incubation	189	2.8	316	2080	47	92	3.35
Expt. IV	Initial	2.5	1.0	18.2	959	60	685	0.12
	Inoculum	3.8	46.9	19.5	782	170	393	
	After addition of Inoculum	3.9	1.2	22.3	1571	51	888	
	After incubation	174	2.7	53	2477	47	29	9.30

Table 4. Microphytoplankton, and zooplankton characteristics of water of sea surface water (initial), inoculum, after addition of inoculum to surface waters and after incubation of 3 days in experiments I to IV.

Expt	Experiment status	Diatoms (Cells L ⁻¹)	Dinoflagellates (Cells L ⁻¹)	Micro-phytoplankton (Cells L ⁻¹)	Chl a (mg m ⁻³)	Micro - Zooplankton (No. m ⁻³)	Meso - Zooplankton (No. m ⁻³)
Expt. I	Initial	80	160	240	0.18	383	600
	Inoculum				0.12		
	After addition of Inoculum	160	40	200	0.12	317	400
	After incubation	352	293	645	0.10	375	263
Expt. II	Initial	184	160	344	0.03	425	788
	Inoculum				0.1		538
	After addition of Inoculum	64	48	112	0.14	300	425
	After incubation	61	60	120	0.50	813	375
Expt. III	Initial	120	280	400	0.02	300	575
	Inoculum				0.14		313
	After addition of Inoculum	200	224	424	0.10	800	1413
	After incubation	187	72	259	0.05	238	463
Expt. IV	Initial	64	144	208	0.20	138	663
	Inoculum				0.15		133
	After addition of Inoculum	24	80	104	0.20	375	625
	After incubation	99	72	171	0.05	150	375

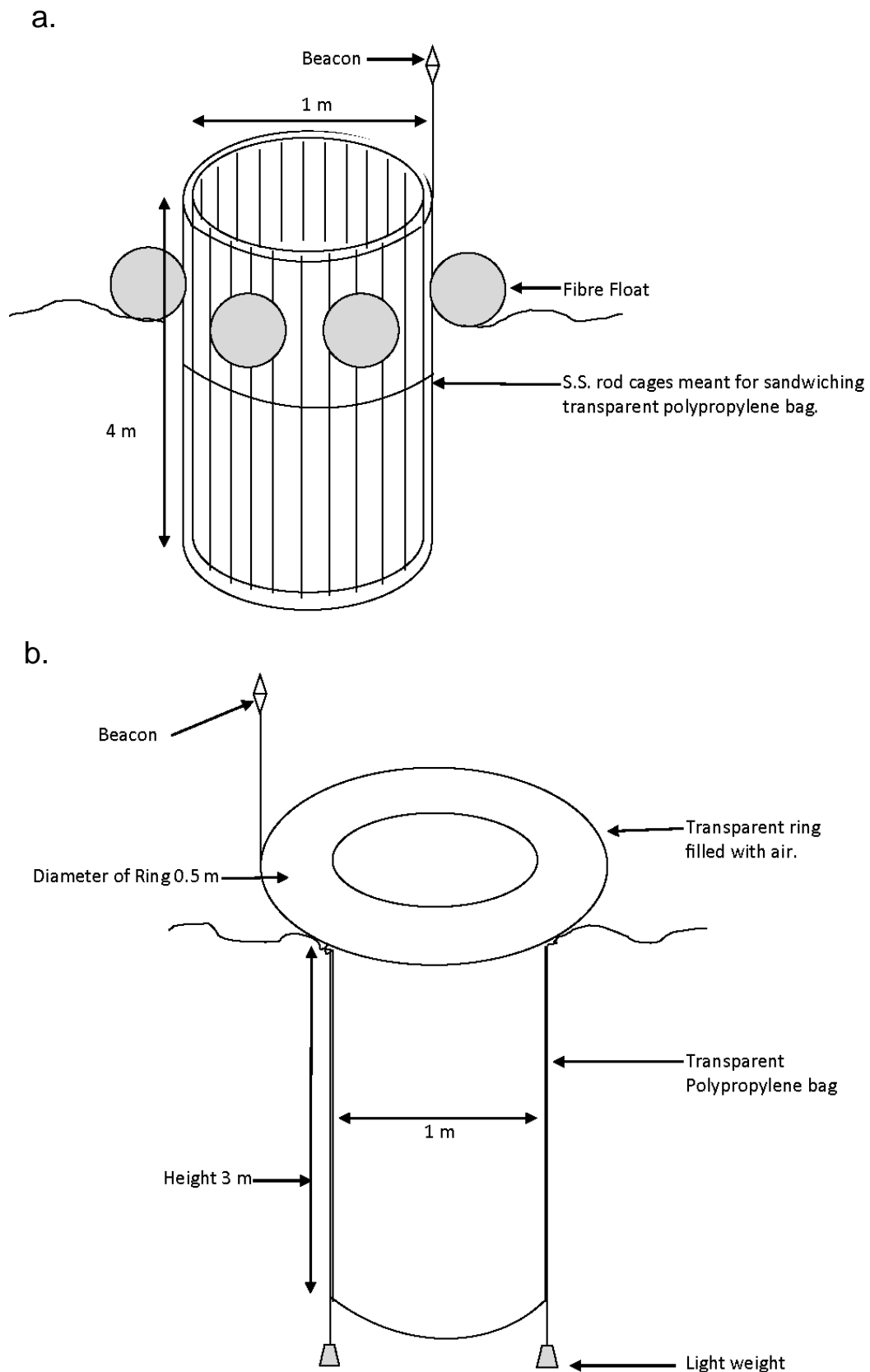


Fig. 1. Different design mesocosm enclosures (a) Cage design enclosures reinforced with stainless steel cages and this design was used in experiments 3 and 4 and (b) Float design enclosure with a transparent ring filled with air to act as a floatation system and this design was used in experiments 1 and 2.

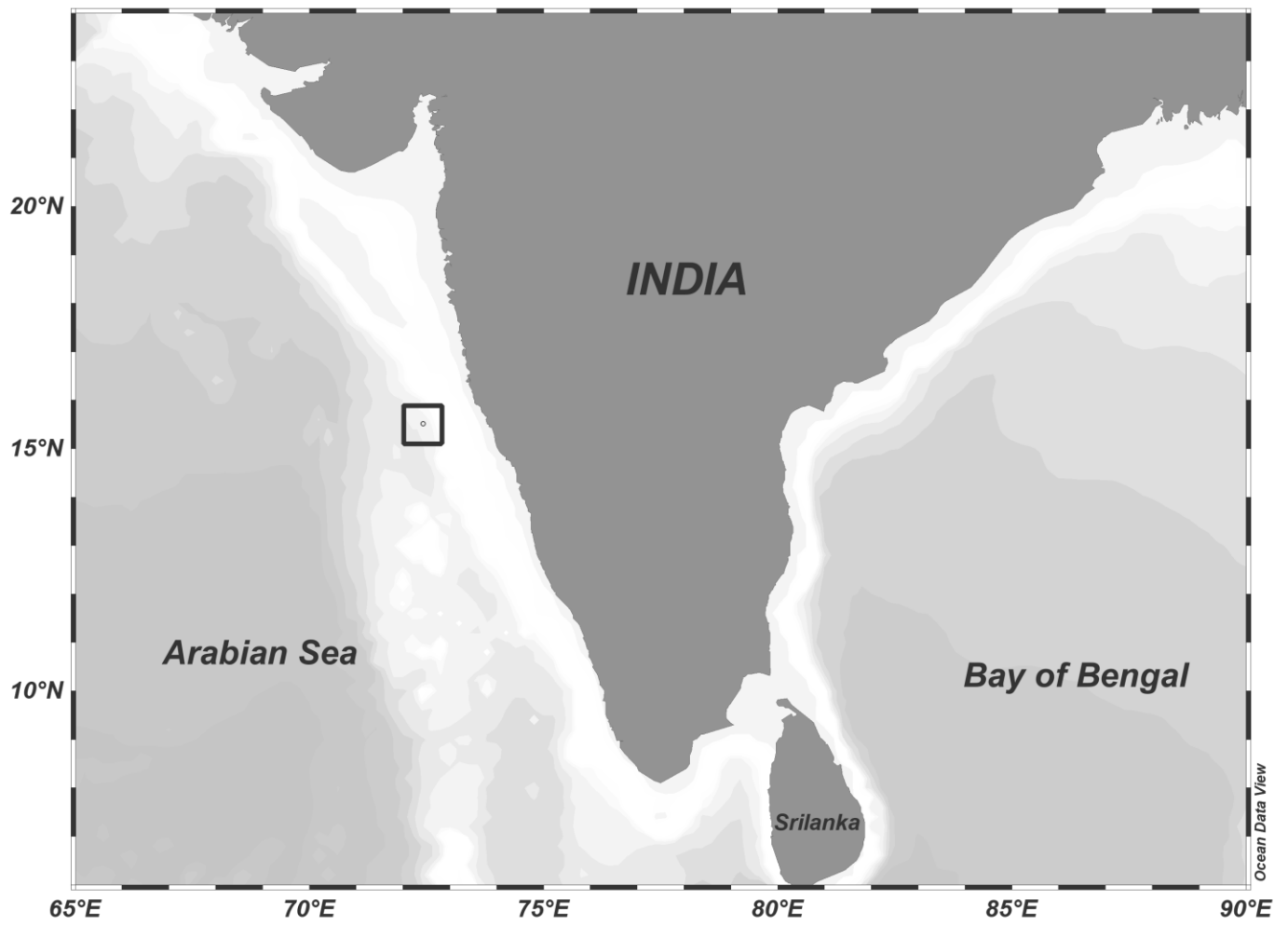


Fig. 2. Location of the experimental site in central eastern Arabian Sea

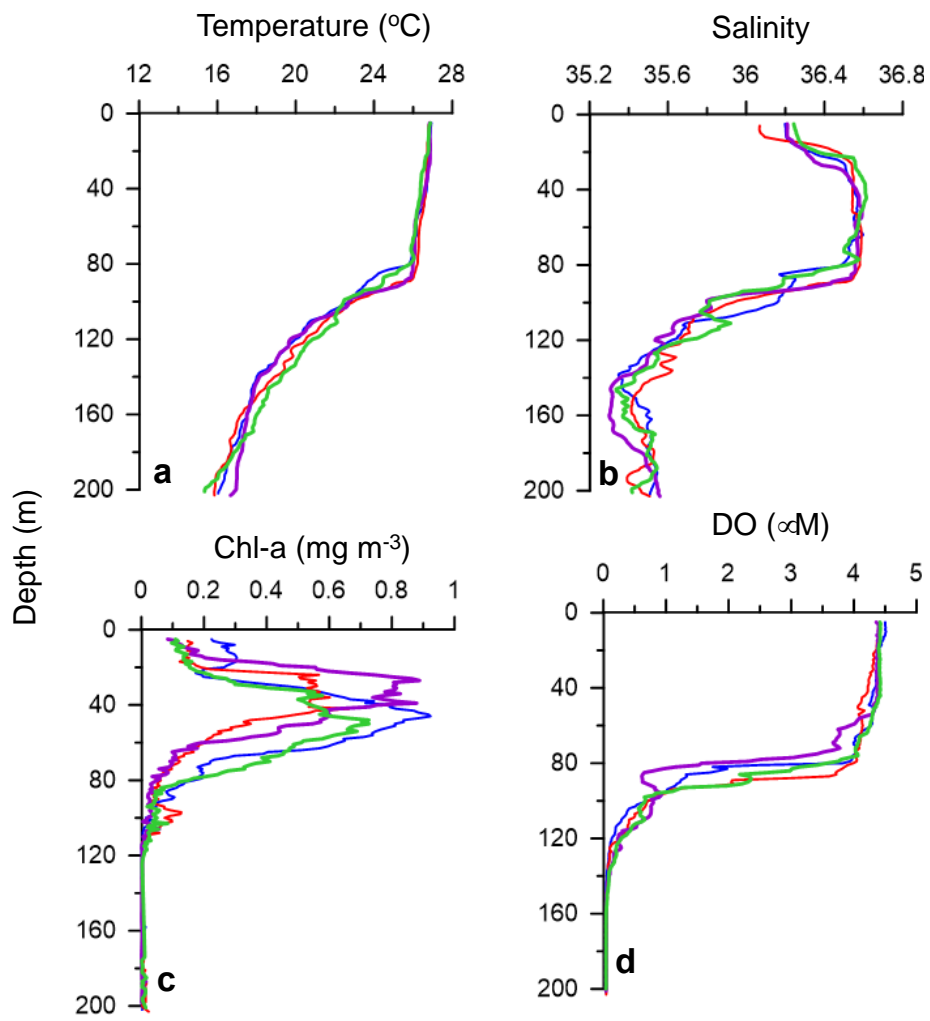


Fig. 3: Vertical distribution of a) temperature (°C), b) salinity, c) chlorophyll-a (mg m⁻³) and d) dissolved oxygen (DO) (μM) at the experimental sites of I, II, III and IV).

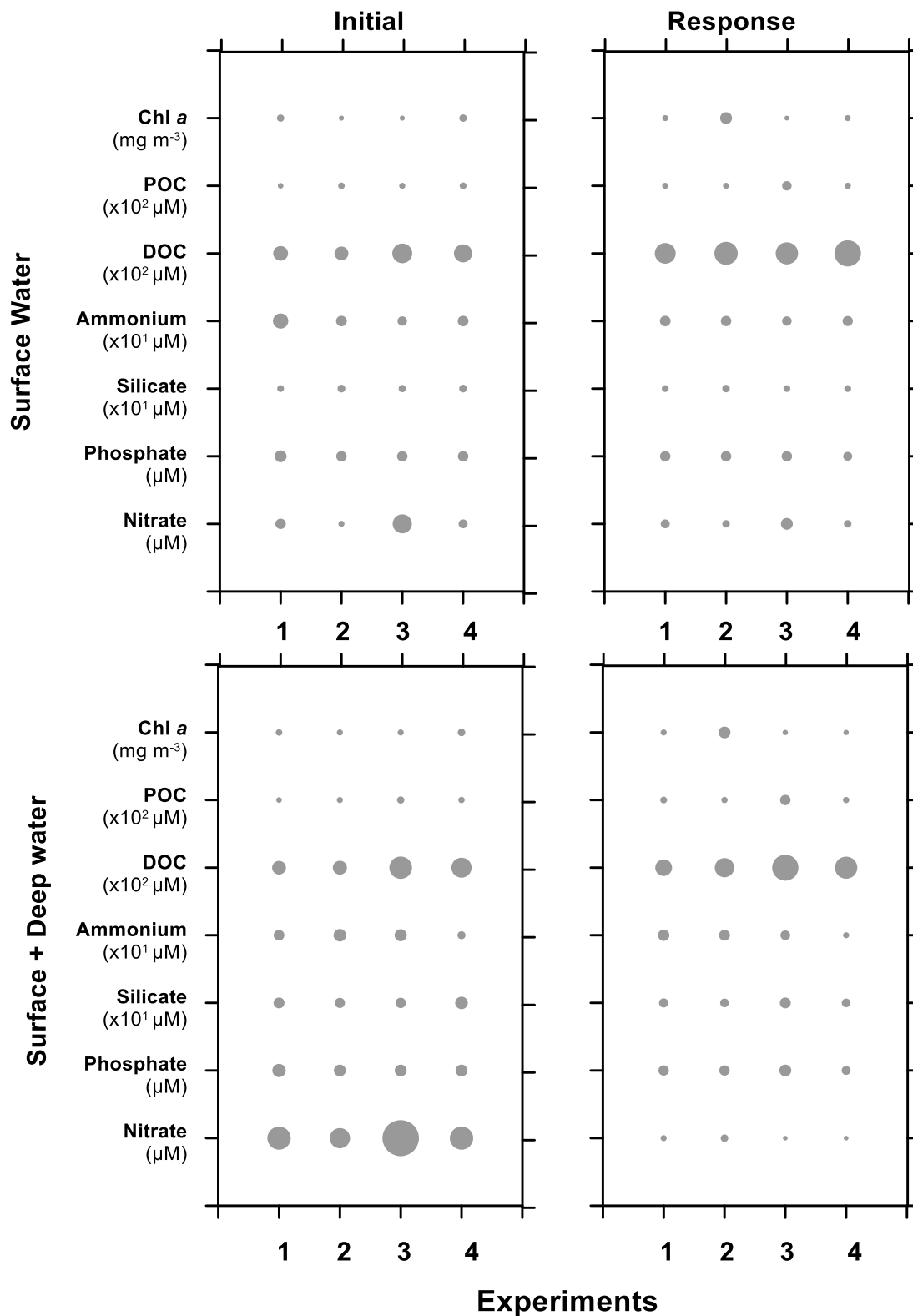


Fig. 4a. Bubble plot showing different chemical characteristics (dissolved inorganic macronutrients, dissolve (DOC) and particulate (POC) organic carbon) when incubated with and without nutrient rich bottom waters in 4 experiments. The size of the bubble (minimum and maximum radius is 0.02 and 0.150 inch) is a relative measure of the increase or decrease during incubation and 2.2 is the maximum data value.

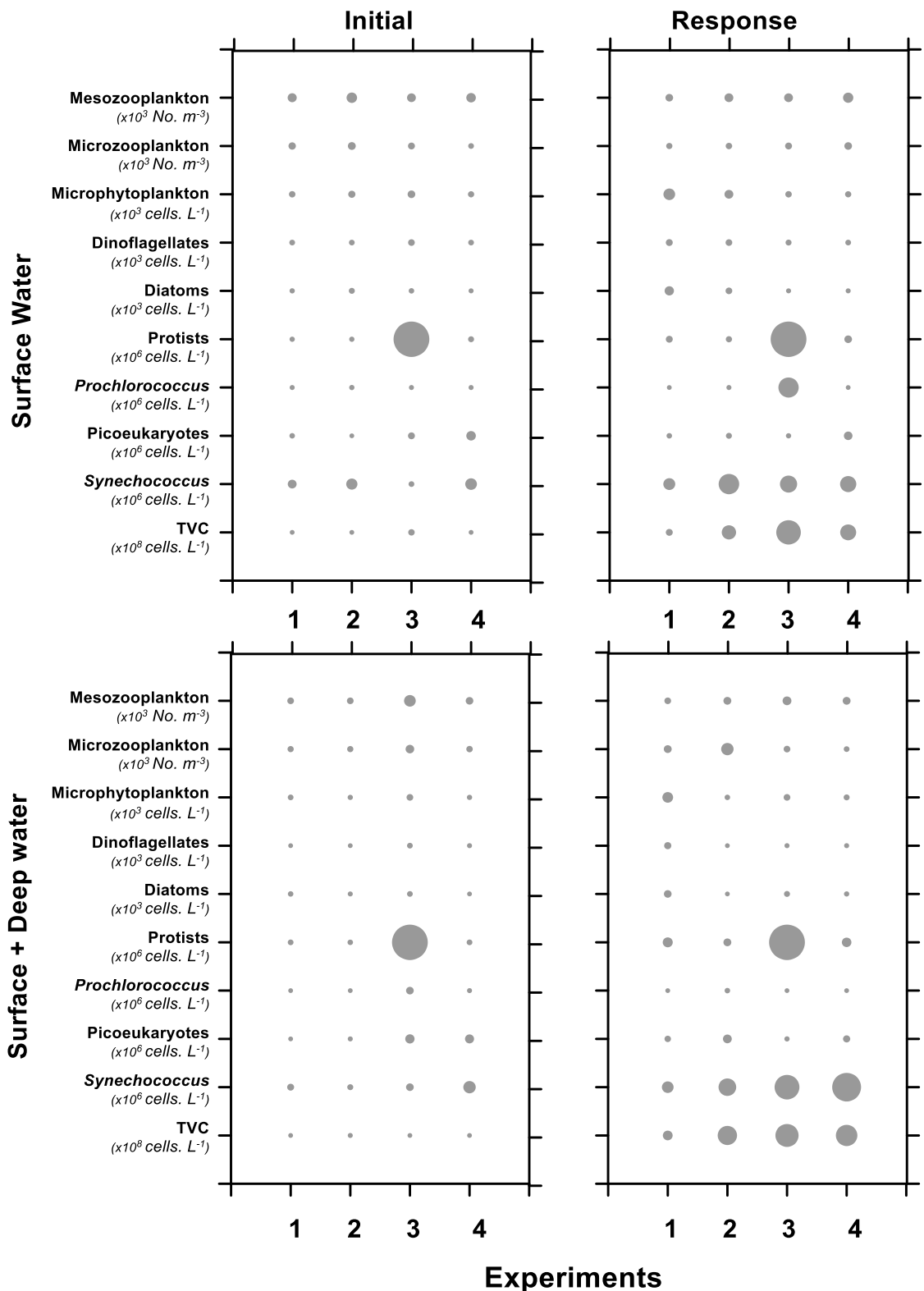


Fig. 4b. Bubble plot showing the response of surface planktons (bacteria, phytoplankton, and zooplankton) when incubated with and without nutrient-rich bottom waters in 4 experiments. The size of the bubble (minimum and maximum radius is 0.02 and 0.150 inch) is a relative measure of the increase or decrease during incubation and 2.5 is the maximum data value.

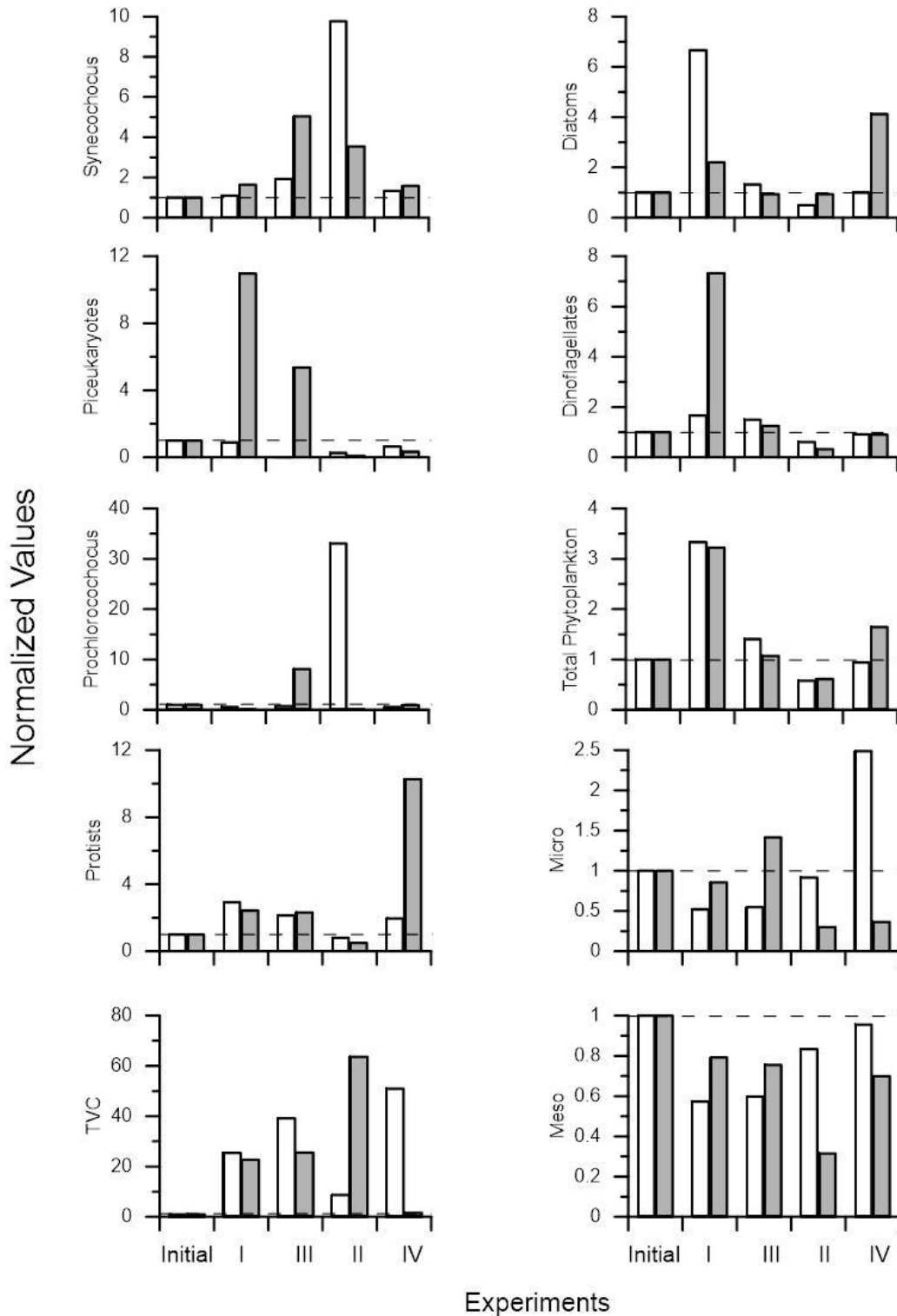


Fig. 5. Response of surface water biology (bacteria, pico- and micro-phytoplankton, micro- and meso-zooplankton) to nutrient enriched deep water in oligotrophic condition of central eastern Arabian Sea. White and grey filled bars corresponds to control mesocosm and the mesocosm enriched with deep water, respectively.

Variable	Control	Experiment	<i>Response</i>
Chlorophyll	↓↑	↓↑	<i>Positive</i>
DOC	↑	↑	
Bacteria	↑	↑	
Protists	↑	↑	
Synechococcus	↑	↑	
Picophytoplankton	Prochlorococcus	—	<i>Variable</i>
	Picoeukaryotes	—	
		↓↑	
Microphytoplankton	↓↑	↓↑	<i>Negative</i>
Microzooplankton	↓	↓	
Mesozooplankton	↓	↓	

↓↑ Variable; ↑ Increase; ↓ Decrease; — No

Fig. 6. Short term biological response in of oligotrophic water to nutrient enrichment after three days of incubation in the control (surface water) and experiments (surface water +deep water). The variable response (↓↑) indicate differed response with reference to experiment I to IV.