

Responses of phytoplankton benthic propagules to macronutrient enrichment and varying light intensities: elucidation from monsoon-influenced Mandovi and Zuari riverine system

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Abstract

The ecological importance of phytoplankton-benthic-propagules (PBP) from coastal sediments, except tropical monsoon-influenced rivers and estuaries, is well documented. PBP in such systems is often transported from upstream (near freshwater) to downstream (estuary) through freshwater discharges during monsoon and thereby experiences higher salinities (>30 PSU) and nutrients with varying light conditions due to reducing discharges during the monsoon-break/withdrawal-phase. However, the PBP responses (germination and subsequent growth) to such changes are unknown and are examined here at ~35 PSU salinity. For the study, macronutrients (nitrate, phosphate, silicate, and nitrate+phosphate+silicate) and light-intensities were considered to assess the response of PBP representing estuarine, brackish, and near freshwater locations of monsoon-influenced Mandovi and Zuari rivers (Goa, India). Diatoms dominated the viable PBP community, but the maximum growth and sustained photosynthetic activity were observed when all macronutrients were supplied than individually. Here, variable fluorescence technique utility in PBP resurrection (detection of viability and responses) was also explored. The PBP growth was similar for macronutrients but increased with light intensity indicating a longer growth response during monsoon. For PBP (germination and photosynthetic activity), light intensities drive the rate of improvement/development, whereas the nutrients are essential for maintaining vegetative growth upon germination in the region. The PBP dominance of common planktonic species (*Skeletonema* and *Thalassiosira*) along the river also signifies the role of seawater intrusion (up to upstream locations) in distribution. *Skeletonema* and *Thalassiosira*, which contribute significantly to the total community, are light-sensitive with a similar response and cause single species blooms during monsoon and non-monsoon, respectively, depending on the species' tolerance to salinity.

Keywords: Diatoms; Germination; Light intensity; Monsoon influenced rivers; Nutrient sources; Phytoplankton benthic propagules; Variable fluorescence

Introduction

Phytoplankton, represented by considerable diversity of algal groups (diatoms, dinoflagellates, coccolithophorids, green algae, and blue-green algae), forms an essential component in the functioning of aquatic ecosystems and biogeochemical cycles [1]. Most phytoplankton groups have known resting stages (e.g., specialized resting spores or resting cells or cysts), formed asexually or sexually, as survival alternatives in an adverse environment to maintain their population [2, 3]. These stages are commonly known to provide short- or long-term survival and contribute to natural dispersal by currents or when transported in ballast water tanks [4]. There is also a hypothesis; (i) resting stages anchor a population at a particular site [5-6], (ii) also have the potential to provide an inoculum that influences present populations through “dispersal from the past” (the storage effect) [7], and (iii) sexual reproduction (and consequently genetic diversity) is influenced by factors such as habitat conditions, life-cycle strategies or population history [8]. These resting stages are dormant and serve as a refuge during adverse conditions, such as a change in temperature, salinity, pH, and especially nutrient depletion [8; 9]. Once resting stages are formed, they sink to the bottom and become a part of the benthos as benthic propagules. Resting stages are reported to play central roles in evolutionary resilience, survival of catastrophic events, and will also aid in managing water bodies used by multiple industries [10-11]. The rich abundance of benthic propagules protects themselves in coastal sediments during periods not suitable for vegetative growth. In most cases, resting stage formation is a survival mechanism for disturbances occurring on various timescales (months to years) and can remain viable for up to about a century in sediments [e.g. 10, 12-14].

Viable ‘banks’ of phytoplankton resting stages have been reported from marine sediments all over the world, encompassing different ecosystems such as bays, coastal waters, estuaries, fjords, and polar regions, including examples from the bays/fjords of Scandinavia [15-17], United Kingdom [18], continental Europe [19], bays and coastal waters of Asia and Indian subcontinent [12, 20-21], Mediterranean sea [22], Bering Sea [23], Arctic sea [24] and even after a century of dormancy from Koljo Fjord [15]. Freshwater studies have also recorded viability in resting stages ‘[25] after decades in the sediments. Viable phytoplankton benthic resting stages or benthic propagules (PBP) repopulated waters if resuspended and switch to active growth following germination. Germination of resting stages and the successful growth of newly germinated vegetative cells also play a significant role in coastal micro-plankton dynamics and bloom formation [30-31]. Therefore, environmental cues that control resting stage germination and growth may determine the seasonal patterns of a species (e.g., when a species appears in the plankton) and the population’s overall

success. In view of this, it is essential to understand how environmental factors regulate germination and growth on a species-by-species basis. Several environmental factors such as light (both quality and quantity), temperature, salinity, and nutrients are known to trigger the germination of PBP [e.g. 10, 26-28, 32]. After the germination, cells growth is further controlled by several factors, such as temperature, light intensity, nutrient concentration, inter-and intraspecific competition, and grazing [29]. However, in monsoon-influenced tropical coastal regions, environmental variables such as light (both quality and quantity), salinity, and nutrients could be the drivers influencing phytoplankton seed formation and germination compared to water temperature due to the prevalent of warm waters [33]

In monsoon-influenced tropical coastal regions, particularly in India, most studies are focused on understanding the role of environmental drivers on the variations in phytoplankton, but the drivers influencing eco-biology and physiology of viable PBP received less attention, including monsoon-influenced rivers and estuaries [21, 34]. Field-based findings from the monsoon-influenced Zuari estuary [21] suggested that in shallow tropical regions, physical processes during monsoon (mostly freshwater discharge) and non-monsoon seasons (currents, waves, and tides) cause resuspension followed by transportation (e.g., from upstream to downstream and vice versa due to river discharge and tides, respectively) of benthic propagules along with sediment and other detritus, which in turn influences water transparency and nutrient concentrations. Although the BP community is dominated by planktonic species (e.g. *Skeletonema*, *Thalassiosira*, *Fragillariopsis*, *Noctiluca*) and mixed (e.g. *Chaetoceros*, *Leptocylindrus*, *Bacteriastrum*, *Skeletonema*), which are known to form seasonal blooms in the region [21], no direct attempts have been made to identify the environmental cues responsible for triggering germination and its subsequent vegetative growth. Therefore light availability and macronutrient availability are considered environmental cues because they are the major growth controlling factors for phytoplankton in this region only [21, 35-36]. Given this, the present study investigates PBP responses (germination and its subsequent growth) to different macronutrients and light intensities at higher salinity from monsoon-influenced Mandovi and Zuari riverine systems. The high annual runoff and a distinctly higher runoff during monsoon season (wet season) compared to the non-monsoon season, i.e., dry season, are the distinguishing feature of the monsoonal estuaries compared to better-studied estuaries in extratropical regions [37]. Therefore, the dominance of freshwater (due to riverine discharge and precipitation) and marine conditions in the estuary is more during monsoon (June to September) and non-monsoon months (October to May), respectively. Given this, higher salinity was selected because the PBP in the downstream of monsoon-influenced rivers systems is often transported from upstream (near freshwater) to

downstream (estuary) through freshwater discharges during monsoon and thereby experiences higher salinity conditions (>30 PSU) and varying levels of nutrient concentrations and light conditions due to reduce discharges during monsoon break or withdrawal phase. Since introduced PBP will experience higher salinity conditions until the return of the next monsoon, i.e., after approximately eight months, delineating PBP responses (germination and subsequent growth) to such variations will be a step ahead in understanding phytoplankton eco-biology and bloom dynamics of the region. As the study was conducted before the onset of the monsoon, the findings will also unravel the distribution and fate of PBP introduced by river discharge from upstream to the estuary region.

Materials and Method

Sampling locations

Mandovi and Zuari estuaries located along the central west coast (Goa coast) of India are dynamic aquatic ecosystems exhibiting a strong seasonal gradient, both in environmental variables and plankton assemblages, because of the tight physicochemical and biological coupling [e.g. 35,36]. The rivers Mandovi and Zuari, together with the Cumbarjua canal, form a major estuarine system in the region (Fig. 1a). Both rivers originate in the Sahyadri hills in the Western Ghats. The Mandovi River traverses ~75 Km, while the Zuari River ~ 70 Km, from their respective places of origin before entering the Arabian Sea along the west coast of India. The narrow Cumbarjua canal about 11Km and 14Km away from Mandovi and Zuari estuaries' mouths interconnects the estuaries, respectively. This area is strongly influenced by the southwest monsoon (June to September). The changes associated with its onset have marked effects on the phytoplankton community, food web, and production [18, 30]. During the monsoon, the surplus influx from the river, and precipitation (~275 cm, i.e., 80% of annual rainfall), are added to the estuary resulting in freshwater dominance. In the Zuari estuary, almost marine conditions prevail with freshwater drainage from the river during post-monsoon months. In contrast, the freshwater discharge continues throughout the year in the Mandovi estuary.

Collection of sediment samples

The sediment samples were collected along a salinity gradient from three stations, each along the Zuari (Z1 – Mormugao; Z2 - Cortalim, and Z3 - Sanvorderm) and Mandovi (M1 – Campal; M2 - Amona, and M3- Usgoa) rivers during January 2011 (Fig. 1a; Suppl Table 1). The stations selected represented upper, middle, and lower regions, covering a wide range of salinities from near freshwater in the upstream location, brackish in midstream, and seawater at downstream (mouth or

estuary) (*Supplementary Table 1*). Samples from Zuari and Mandovi were collected on 10th and 12th January 2011, respectively. Sediment samples from each station were collected using a modified van Veen grab (grabbing area of 0.04 m²), equipped with flaps on the top side, enabling the collection of surface sediments using cores. The uppermost sediments (0-2 cm) using 2-3 cores (PVC cores, 25 cm long, the inner diameter of 2.5 cm) were obtained from the grab. The collected sediment samples were then sealed in airtight bags and kept cool (4 °C) in the dark for more than three weeks to reduce viable vegetative cells. Few studies indicated that dark storage at relatively low temperatures was influential in selecting resting stages while eliminating recently settled vegetative cells [38].

Inocula preparation for the experiment

A stock solution was prepared for the inocula preparation by mixing an aliquot of known weight (3.5 g wet wt) of sediment with 35 ml sterile filtered seawater (SFSW) to achieve a 0.1 g ml⁻¹ concentration. For the 250 ml and 125 ml, experimental bottles 1 ml and 0.5 ml of the stock solution were added as inocula. Inoculum preparation for different sediment samples was prepared separately under sterile conditions, and the details of inoculum preparation are described elsewhere [42]

Experimental setup

All the experiments were conducted in the laboratory. The experiments to evaluate the responses of estuarine phytoplankton benthic propagules (PBP) to different nutrient sources and light intensities were conducted parallel (Fig. 1b). However, these experiments were performed separately for sediments collected from the Zuari and Mandovi rivers. For the experiments, ~20 L of seawater (~34.74 PSU) collected from Dona Paula Bay of Zuari estuary was used. Prior using, the collected water was filtered through 0.22µm cellulose filter paper (Millipore) followed by steam sterilization at 120 °C and cooling at room temperature. This water was used for conducting two experiments. The first and second experiments correspond to the sediments collected from Zuari and Mandovi estuaries. In each experiment, the sediment collected from three stations was used in parallel. Fourteen Nalgene polycarbonate bottles with capacities (250 ml and 125 ml) were used for one station. In each of these bottles, SFSW was poured aseptically, i.e., 250 ml SFSW water in 250 ml bottles and 125 ml SFSW in 125 ml bottles. All these bottles were then enriched with f/2 trace metals and vitamins [39]. These bottles were then incubated as detailed below to elucidate the different macronutrient and light intensity effects on PBB.

Effect of nutrients on phytoplankton benthic propagules

In this experiment effect of types of macronutrients such as nitrate–N, phosphate–P, silicate–Si, and in combination (NPSi) on PBP responses was evaluated. For the experiment, ten 250 ml Nalgene bottles containing trace metal-enriched SFSW were used. Of these, two bottles each were enriched with phosphate ($\text{Na}_2\text{H}_2\text{PO}_4 \cdot \text{H}_2\text{O}$ – 250 μl from 3.62×10^{-5} M stock solution), nitrate (NaNO_3 – 250 μl from 8.82×10^{-4} M stock solution), silicate ($\text{Na}_2\text{SiO}_3 \cdot \text{H}_2\text{O}$ – 250 μl from 1.06×10^{-4} M stock solution) and in a combination NPSi. The preparation of the above nutrients' stock solution is the same as that for the preparation of f/2 stock solutions [39]. The remaining two bottles without nutrient addition were treated as the experimental control. In all these bottles, 1 ml of inocula prepared from the collected sediment was added. After addition, the bottles were gently swirled and incubated for 13 days in the temperature-controlled room (25 ± 1.0 °C) with a 12:12 h light: dark cycle at a light intensity of $70 \mu\text{mol photons m}^{-2} \text{s}^{-1}$. Previously, the growth of a relatively large number of species was observed under this light intensity and was considered as a reference in the light experiments has been regarded as medium light (ML) in the experiment with different light intensity. During the incubation period, phytoplankton's progression in each treatment was monitored through *in vivo* fluorescence measurements (0, 2, 4, 7, 9, and 13 days) using a fluorometer (Turner designs Trilogy (fluorometer) and FIRE fluorometer -Satlantic Inc.), and cell counts by microscope (0, 2, 7, and 13 days). Similarly, the above procedure was repeated for the sediments collected from Zuari and Mandovi. The experiments with Zuari (7th to 20th February 2011) and Mandovi (25th February to 11th March 2011) were conducted separately.

Effect of light intensities on phytoplankton benthic propagules

In this experiment, PBP responses to varying different light intensities on PBP were elucidated. These experiments were performed simultaneously, using four 125 ml bottles containing SFSW enriched with full strength nutrient concentrations (NPSi), with the above experiment. In all these bottles, 0.5 ml of prepared sediment inocula were added. After addition, the bottles were gently swirled and incubated similarly, as mentioned in the above section, at two different light intensities, $25 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ (low light – LL) and $300 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ (high light – HL). The Secchi disc depth (indicative of water transparency) in the monsoon estuary varies between <0.5 m to 3.5 m, and the lowest is observed during peak discharges during monsoon and the maximum during monsoon breaks and non-monsoon months [40]. Therefore, the light intensity selected is an indicator to confirm the role of light intensity on the PBP germination and subsequent growth. For

LL, two bottles were wrapped with neutral density screens and incubated in the same temperature-controlled room as above. In contrast, HL bottles were set in a versatile environment test chamber at $25\pm 1^\circ\text{C}$ (model: SANYO). Since all the nutrient experiments were conducted under ML, the observations carried out from bottles with full strength nutrients (NPSi) were used to compare with the observations done for LL and HL bottles. It should be noted that the criteria of using LL, ML, and HL is just a representation for the three experimental light intensities 25, 70, and $300\ \mu\text{mol photons m}^{-2}\ \text{s}^{-1}$ selected for the study. The light intensities were measured using a light meter (PMA data logger Solar Light). The tested light intensities are by default based on the best possible experimental setup in the growth chamber and were selected mainly to understand the PBP response to varying light intensities. Since underwater light intensity data for the region are not available arbitrary light intensities were selected to examine the differential PBP responses to different light intensities. However, the photocycle and the interval for monitoring the progression of growth are the same as detailed above. The procedure was repeated with sediment samples collected from Zuari and Mandovi rivers.

Parameter analyzed in the experiments

Samples for the analysis of chlorophyll fluorescence, photosynthetic parameters, phytoplankton counts, and taxonomic composition were collected on each sampling occasion during all the experiments. Inorganic nutrients (nitrate, phosphate, and silicate) were analyzed by following standard procedures [41] using a spectrophotometer (Shimadzu Model-2450) during the start (day 0) and end of the experiments, i.e., day 13, and the data are presented in *supplementary table 2*. The nutrient measurements were done manually wherein sample requirement was large. Therefore pooling of the samples was essential and the data presented is from the pooled replicate samples.

Chlorophyll (chl) fluorescence was measured on each sampling occasion through in vivo chlorophyll fluorescence measurements of the samples using Trilogy (Turner designs), equipped with a chlorophyll module. Further, the fluorescence induction and relaxation (FIRE) technique used FIRE fluorometer (Satlantic Inc.) to monitor cells' viability and photosynthetic performance. The FIRE measurements details for sediment samples (including sample preparation) are published elsewhere [42]. Unlike water samples, the background corrections for sediment samples are not straightforward. The only possible way for assessment is first to prepare the inocula of the sediment samples followed by suspension in a known volume of sterilized growth media followed by incubation in proper growth conditions for several days till the signal of the growth is observed. FIRE measurement of the inocula/resuspended sample (day 0) will indicate viable cells presence [42]. The

measurements done during incubation will determine the time taken for growth. The method is rapid and non-destructive. The FIRE fluorometer measures a suite of photosynthetic parameters such as the initial (F_0), maximum (F_m), and variable ($F_v = F_m - F_0$) components of photosystem II (PSII) fluorescence, quantum yield (F_v/F_m), and functional absorption cross-section (σ_{PSII}), which can be used to assess the physiological status of the phytoplankton. The fluorescence transients were measured using a FIRE fluorometer. Before measurements, the samples were dark-adapted for 30 min. The gain was adjusted depending upon the chl concentration of the sample taken for analysis. The curve-fitting procedure was performed by using the FIREPro software (developed by Dr. Maxim Gurbonov). For phytoplankton analysis, 5 ml of the samples were preserved with Lugol's solution in duplicates. The analysis was carried out using a counting chamber having a grid of 20X20 under an inverted microscope (Olympus IX71) equipped with a camera at a magnification of 20x. Phytoplankton was identified based on the standard identification keys [<http://www.algaebase.org/>, 43]. Univariate measures (species count, Shannon–Wiener diversity, and evenness) were calculated using PRIMER ver.5 (PRIMER–E Ltd. Plymouth, UK) software.

Results

Inocula description

Microscopic observations show that diatoms dominated the sediment inoculum prepared from the sediment sample collected from the Zuari and Mandovi rivers. The major taxa in the sediment inoculum of Zuari were *Navicula*, *Skeletonema*, *Coscinodiscus*, *Gyrosigma*, *Ditylum*, *Trigonum*, *Cymbella*, *Grammatophora*, *Pleurosigma*, and *Thalassiosira*. Whereas in the sediment inoculum of the Mandovi river, the major taxa were *Chaetoceros*, *Coscinodiscus*, *Grammatophora*, *Navicula*, *Skeletonema*, *Thalassionema*, and *Thalassiosira*. The prepared inocula's total cell counts ranged from 70 to 180 cells ml⁻¹ in Zuari and 135 to 190 cells ml⁻¹ in Mandovi. The chl fluorescence values of the inocula ranged from 434 to 523 (au) for the Zuari river, and for the Mandovi river, the values ranged from 82 to 250 (au) (Fig. 2). The FIRE measurements on sediment inocula showed a somewhat flat curve instead of the induction curve typically observed for the live organisms curve. The variable chlorophyll fluorescence (F_v ; a proxy for viability) of the inocula was almost the same as that of the initial and final fluorescence (Fig. 3; Suppl Fig. 1), whereas the photochemical efficiency was very low ($F_v/F_m = <0.2$) and σ_{PSII} was unrealistic (Fig. 4; Suppl Fig. 2). These initial values for PSII parameters suggested that the sediment sample used for incubation harbored very low viable chlorophyll (in the undetectable range) with reduced quantum efficiency.

Effect of different nutrient sources on PBP

The nutrient analysis revealed that the macronutrients (nitrate, phosphate, and silicate) in the experiment were not limited in all the treatments at the beginning of the experiments. However, on day 13 (the last day of the experiment), only the nitrate was found low in control, phosphate, and silicate added bottles only (Suppl Table 2). The chl fluorescence values indicated that the increase in fluorescence values was not observed initially for some days in all the treatments (Fig. 2). In the case of Zuari, chl increase was not observed up to seven days, whereas, in the case of Mandovi, chl increase was not observed until day seven (for M1 and M2 stations) and day nine for M3 station. However, significant increases in fluorescence values were observed from day seven onwards, indicating that the phytoplankton growth started after day 4 (Fig. 2; Suppl Fig. 1). Even the variable fluorescence (F_v) responded similarly (Fig. 3). Such a long lag phase was due to the presence of dormant benthic propagules or resting stages. ANOVA showed significant variations between the macronutrient treatments, i.e., maximum and minimum fluorescence values (raw fluorescence, F_0 , and F_v) were observed for NPSi and control treatments, respectively as well as days of incubation ($p \leq 0.001$). However, improvements in photosynthetic efficiency, F_v/F_m values, in most treatments, were observed from day two onwards and reached the maximum on the fourth day and remained the same till the end of the experiment, i.e., Day 13 (Fig. 4). ANOVA showed insignificant variations between the macronutrient treatments and significant variations concerning days of incubation ($p \leq 0.001$). In the case of σ_{PSII} , the initial unrealistic values (>1000 or <200) were found to fall in the normal range upon incubation (Suppl. Fig. 2). These variations in fluorescence parameters were noticed in all the treatments.

The PBP responses from Zuari and Mandovi also showed a similar trend concerning the nutrient treatment and incubation period (Fig. 5). The species count remained almost constant with nutrient treatment and incubation period, which was observed for Zuari and Mandovi (Fig. 5). Initially, the diversity of the benthic propagules was high and evenly distributed. From day seven onwards, the increase in the population of some diatoms resulted in a decrease in diversity and evenness values. This was observed in all the treatments (Fig. 5). Species composition data revealed that 21 diatom species were recorded, of which 19 and 17 species belonged to Zuari and Mandovi, respectively. The abundance of these diatoms in each treatment with time is presented in Figure 5. In Zuari, *Skeletonema* was the most dominant species, followed by *Thalassionema*, *Navicula*, *Grammatophora*, and *Coscinodiscus* (Fig. 6). In Mandovi *Thalassiosira*, followed by *Thalassionema*, *Skeletonema*, *Navicula*, and *Coscinodiscus* were dominant species except for the M1

station (Fig. 6). In station M1, *Thalassionema* was more prevalent than *Thalassiosira*. Irrespective of nutrient sources, the species composition in both Zuari and Mandovi was the same. The maximum abundance was observed when the macronutrients were supplied in combination (nitrate+phosphate+silicate) compared to the individual nutrient source.

Effect of different light intensities on PBP

The effects of different light intensities on chl fluorescence, abundance, species count, diversity, and evenness are presented in figures 2, 7, and 8. The chl fluorescence results indicated that light intensities play a significant role in the PBP germination and subsequent growth. This was observed for both the rivers (Zuari and Mandovi), and in both the cases ANOVA showed significant variations between the light intensities ($p \leq 0.001$), i.e., maximum and minimum fluorescence values were observed for HL and LL treatments, respectively as well as days of incubation ($p \leq 0.001$). The increase in chlorophyll (as evidenced by raw and variable fluorescence) for HL, ML, and LL was observed from four, seven, and thirteen days (Fig. 2, 5; Suppl. Fig. 1), respectively. Even F_v/F_m values were found to be increasing from day two onwards in all the light treatments, but the magnitude was more in HL (Fig. 4). ANOVA revealed significant variations between light intensities ($p \leq 0.001$) and days of incubation ($p \leq 0.001$). σ_{PSII} , unlike that observed in nutrient treatments, the initial unrealistic values (>1000 or <200) were found to fall in the normal range upon incubation and remained in the same range till the end of the experiment (Suppl. Fig. 2). The σ_{PSII} were found to be relatively lower and higher under low and high light conditions between the light treatments, respectively (Suppl. Fig. 2). These results indicate that the high light intensities led to faster growth with a relatively shorter lag period and marked improvements in photosynthetic efficiency compared to ML and LL. Further confirming that the light intensity is one of the critical environmental factors in determining the lag period's duration for the PBP growth and photosynthetic activity.

PBP's response for Zuari and Mandovi also shows a similar pattern concerning different light intensities and incubation periods (Fig. 7). The increase in abundance was observed with an increase in light intensities. However, the species count remained almost constant with the incubation period and light treatment for Zuari and Mandovi (Fig. 7). Initially, irrespective of light intensities, the diversity of the benthic propagules was high and evenly distributed. From day seven, diversity and evenness values decreased with an increase in some diatom populations (Fig. 7) and might be the scenario in the field during the occurrence of single species blooms [18]. Altogether 17 species belonging to only diatoms were recorded from Zuari and Mandovi (Fig. 8). The abundance of these

diatoms for each light treatment concerning days of incubation is presented in Figure 8. The species composition and the dominance pattern seen in this experiment are the same as above for nutrient experiments. In Zuari, *Skeletonema* was the most dominant species, followed by *Thalassionema*, *Navicula*, *Grammatophora*, and *Coscinodiscus* (Fig. 8). In Mandovi, *Thalassiosira* followed by *Thalassionema*, *Skeletonema*, *Navicula*, and *Coscinodiscus* were dominant species except for the M1 station (Fig. 8). In station M1, *Thalassionema* was dominant than *Thalassiosira*. Irrespective of light intensity, the species composition in Zuari and Mandovi was the same, and the maximum abundance was observed under high light compared to the supply of low light levels.

Discussion

Phytoplankton benthic propagule (PBP) viability and community in monsoon influenced riverine systems

The present study demonstrated that the PBP present in the sediments representing the upper, middle, and lower regions of the Zuari and Mandovi rivers are photosynthetically inactive. The variable fluorescence (F_v ; an indicator for cell viability [42]) and unrealistic functional absorption cross-section of PSII (σ_{PSII}) values suggested that the PBP was photosynthetically not viable at the start of the experiment. However, they were capable of reviving viability and photosynthetic activity, i.e., an increase in F_v and photosynthetic efficiency (F_v/F_m) along with restoration in σ_{PSII} values due to vegetative growth after germination under 12h light: dark photocycle and macronutrients (Figs. 2 to 5; Suppl Figs. 1 and 2). Interestingly, the revival of PBP was also observed from those samples representing the middle and upper regions of both the rivers when incubated in the nutrient-enriched high salinity seawater, which is normally found during non-monsoon in the downstream locations. Further, this study also demonstrates the utility of the variable fluorescence technique in resurrection studies (detection of photosynthetic viability and responses to environmental cues). Resurrection ecologists have long recognized sediments as sources of viable propagules (“seed or egg banks”), including diatoms, with which to explore questions of community ecology, ecological response, and evolutionary ecology [44]. Generally, diatom benthic stages and dinoflagellate cysts represent PBP of the region [18, Rodrigues and Patil Unpublished data]. Still, in this study, only the response of diatoms was observed, indicating their dominance in the viable PBP pool present in the stations representing upper, middle, and lower regions of the Zuari and Mandovi rivers. The data from the area suggests dinoflagellate cyst abundance is very low (a few hundred per gram of dry sediment), which could be one reason in addition to specific experimental setup (e.g. different combination of stimulators) for their no response in the incubation (Rodrigues and Patil Unpublished data).

Nevertheless, the focus is on the PBP, which responded to the treatments and contributed significantly to the region's phytoplankton dynamics.

Altogether, 21 diatom species were recorded after incubation (Figs. 6 and 8), and the following species *Skeletonema*, *Thalassionema*, *Thalassiosira*, *Navicula*, *Grammatophora*, and *Coscinodiscus* dominated the BP community. The occurrence pattern of these forms in the estuarine system is as follows: 1. the propagules of *Skeletonema*, a bloom-causing species along the west coast of India, including the present estuarine system, are present from downstream to upstream up to several kilometers (~40 km) in Zuari and Mandovi rivers respectively. In Zuari, the cell abundance of *Skeletonema* after incubation is higher compared to Mandovi; 2. the presence of *Thalassionema* propagules is restricted from downstream to midstream in both Zuari (Z1 and Z2) and Mandovi (M1 and M2) rivers; 3. the propagules of *Thalassiosira* are present only in the stations along Mandovi, and their high cell abundance after incubation was observed in midstream and upstream sediments (M2 and M3) compared to downstream (M1); 4. *Navicula*, *Grammatophora*, and *Coscinodiscus* are present in the upper, middle, and lower Zuari and Mandovi rivers regions. The cell abundance of these species after incubation is low compared to the three dominant species. Nevertheless, the dominant species' presence from the down to upstream stations can be attributed to tides. During non-monsoon, hydrodynamics in Mandovi and Zuari is mainly controlled by tidal activity [45] wherein tidal advection from the sea to the upstream is dominant than river discharge. This is because the tides in the region are semidiurnal type, with the highest height of over 2 m during spring tide. It has been observed that during the spring tide of the non-monsoon season, the salinity of ~1.5 is observed at the upstream location, i.e., ~40 Km from the mouth [46], corroborating the role of tides in the widespread distribution of the plankton, including PBP in the region. For instance, tidal entry of seawater facilitated the transport of phycoerythrin containing *Synechococcus* with salinity preferences up to the upstream end in the Zuari estuary [47]. However, this aspect needs to detailed investigation with respect to PBP.

The resting stages are formed to overcome unfavorable environmental conditions such as nutrient limitation, light, low temperature, salinity fluctuation, and internal regulation [28, 48, 49]. Recent findings also indicated a link between cell density and spore formation in the diatom *Chaetoceros socialis* [49]. Several studies demonstrated that nitrogen depletion is the most important environmental variable for forming resting stages in several microalgae, including diatoms [28,]. A field-based study in the Zuari estuary also revealed that irrespective of season, termination of diatom blooms coincided with nitrate depletion and a corresponding increase in benthic

propagules [21, 36]. However, the onset of favorable conditions such as for non-limiting nutrient concentration, and appropriate combinations of temperature and light conditions (duration, quality, and quantity) seem to favor resting stages to germinate [26, 28, 50-52]. In monsoon-influenced Mandovi and Zuari riverine systems, despite seasonality, the temperature may not be a significant factor due to the prevalence of warmer water [21, 35, 36, 53,54]. Previous field studies in Zuari estuary (including adjacent Dona Paula Bay) and Mandovi river indicated that the peaks in chlorophyll coincided with improved underwater light conditions (except during peak monsoon discharges and periods of the high suspended load during non-monsoon), nutrients influx, and relatively high salinity (except during peak discharges) [21, 35, 36, 53, 54]. In view of such observations, specific experiments were performed and are discussed subsequently.

Phytoplankton benthic propagule (PBP) responses to macro-nutrients

The availability of nutrients (such as nitrate, phosphate, silicate) in addition to carbon dioxide, sunlight, and micronutrient is a known fact affecting the phytoplankton life cycle (including resting stage formation and germination). The nutrient enrichment experiment indicated that the maximum growth was observed when the macronutrients were supplied in conjunction (NPSi) compared to the availability of individual nutrient sources. This was supported by the fluorescence (both raw and variable fluorescence) as well as abundance data. The species composition in each treatment was the same, and this was observed in both Zuari and Mandovi sediments. For Zuari samples, significant PBP growth was observed from day seven onwards. In Mandovi, significant PBP growth was observed from day seven (for downstream and midstream stations) and day thirteen for the upstream station. Previous studies also reported a 1 to 8 days lag period compared to vegetative cells, which can grow almost immediately [30, 34, 50]. Even the photosynthetic efficiency remained low during the lag period but showed a drastic improvement upon the commencement of the growth after the end of the respective lag period (Fig. 3 and 4). Such a delayed lag period is because the benthic propagules first need to germinate, followed by asexual growth, whereas the vegetative cells just need to grow asexually. Another reason for prolonging the lag period could be fewer resting stages in the inoculum [34]. However, this was not the case in this study as the sediment samples harbored sufficient propagules, but there could be other limiting factors (e.g., light) for the long lag period. Further, the prolonged lag period for the PBP growth was almost the same irrespective of nutrient sources, indicating that the nutrients might be playing a minor role in the germination of diatoms in the studied region. However, upon germination, subsequent growth success depends on nutrient availability, which was evident irrespective of the sample's origin. Though main limiting nutrient for

phytoplankton in the region is nitrate [21, 35, 36, 55, and Patil unpublished data) but the experimental data indicated enhanced growth even when supplied with any macronutrients compared to control. Still, the highest increase will be observed when all macronutrients (NPSi) are available above limiting levels.

Phytoplankton benthic propagule (PBP) responses to light intensities

Germination of individual cells occurs whenever sediments are exposed to a well-lit zone. Several studies have indicated that the photoperiod i.e., day length [26], intensity/irradiance [32, 56-57], and quality such as spectral wavelength, e.g., blue, light [52], are known to influence germination. The day length does not vary significantly in the tropical region, unlike the high latitude and temperate areas. Montresor et al. [32] indicated the day length theory holds the key to the germination of high latitude species but does not seem to be affected by changes in the day length for temperate species. Therefore it is presumed that the day length, which varies between 12 to 14 h depending upon the season, should not be a limiting factor [32] in the Indian subcontinent and most probably for tropical species.

Laboratory experiments showed that a threshold irradiance is required to induce germination and subsequent growth [56, 57] but not mandatory. Earlier works have indicated that diatom resting stages can germinate within a few days when exposed to light conditions [28, 48, 56, 59] and can have fully developed plastids after four days [60]. Also, the resting stages of a diatom *Aulacosira* can germinate in dark conditions [58]. Research indicated an increase in the germination rate concerning increasing light intensity from 300 to 4000 lux, also evident in the present study [32]. Fluorescence (both raw and variable fluorescence) and abundance data indicated that the maximum growth of PBP was observed under HL ($300 \mu\text{mol photons m}^{-2}\text{s}^{-1}$) followed by ML ($70 \mu\text{mol photons m}^{-2}\text{s}^{-1}$) and LL ($25 \mu\text{mol photons m}^{-2} \text{s}^{-1}$). Even the photosynthetic efficiency followed a similar trend initially but remained maximum until the end of the experiment. However, the species composition remained the same under all light intensities for the respective samples. The experimental results also indicated that the light intensities play a major role in influencing germination and subsequent growth of PBP, i.e., higher light intensities showed faster growth with a relatively shorter lag period compared with lower light intensities. It should also be noted that germination rates showed species-specific responses to light intensity and could affect species succession patterns when species were exposed to light. In this study, the germination rates of the dominant diatom species were more under HL conditions. *Skeletonema* and *Thalassiosira*, common planktonic species, were more sensitive to light, and both responded similarly to the light levels.

Previous studies revealed that both *Skeletonema* and *Thalassiosira* are dominant in the sediments during all the seasons and were reported to form blooms in the region but on different seasons [21]. *Skeletonema* blooms were observed under lower (during monsoon as single species) and higher (during monsoon break or non-monsoon mixed species) salinities. In contrast, *Thalassiosira* blooms were observed mainly during the non-monsoon season at higher salinity [21, 35]. However, the *Skeletonema* population outbreak's magnitude was more significant than *Thalassiosira* [21]. Although blooms of these species occurred on different occasions, the bloom-forming mechanism for both is the same [18,35,36]. For instance, the physical disturbances such as freshwater discharges, tidal mixing, currents, and strong waves inducing benthic resuspension cause lower water transparency, nutrients influx, propagule transfer to the surface (photic zone) followed by germination when exposed to light, and the vegetative cells that arise from the sediment inoculum proliferate under nutrient-replete conditions. However, the physical forcings inducing benthic resuspension are different during monsoon (e.g. freshwater discharges) and non-monsoon (e.g. tidal mixing, currents) seasons. The physical forcings associated with *Skeletonema* and *Thalassiosira* blooms are the freshwater discharge (during monsoon), and the unnatural loading of nitrate [18, 35 61, 62] by mining effluents as the upstream region is surrounded by mining activities [62] (during non-monsoon), respectively. Although the species' benthic propagules get resuspended, the species' tolerance capabilities to salinity could be the dominant factor that led to causing the population increase on different occasions in the region [18, 35, 36]. Further, both the species lag period depends on the magnitude and duration of the factors such as benthic resuspension and cloud cover affecting the light availability but not to the extent of growth-limiting levels [18]. For instance, light availability is governed by both benthic resuspension and cloud cover (for *Skeletonema* only during monsoon).

Conclusion

We investigated the role of different nutrient sources and light intensities on the germination and subsequent growth of PBP representing downstream, midstream, and upstream locations of the monsoon-influenced riverine systems. This study revealed that the PBP was mostly represented by diatoms, and its distribution is spread along with the monsoon-influenced Mandovi and Zuari riverine systems. It is also evident from the dominance of most common planktonic forms such as *Skeletonema* and *Thalassiosira* in the sediments. Viable diatom benthic propagules having negligible variable fluorescence signals or efficiency was one of the notable observations, and this aided in understanding PBP resurrection (detection of viability and responses) in this study. Among

the tested parameters, light intensities play a major role in germination and the nutrients on the germinated cells' growth, which was not known previously for the study region. Though maximum photosynthetic efficiency was observed in light and nutrient treatments, the rate of improvement was driven by light intensity i.e., higher light intensities resulted in a shorter lag period compared with lower light intensities. *Skeletonema* and *Thalassiosira* (common planktonic species and most dominant PBP) responded similarly to the light levels and are known to form blooms in the region but on different occasions. For instance *Skeletonema* in monsoon and *Thalassiosira* during non-monsoon. However, the mechanism of bloom formation for both is the same. The species tolerance capabilities to salinity are a possible explanation for species seasonality downstream of monsoon-influenced riverine systems. Further research considering changes in monsoon induced physicochemical and biological (e.g., internal clock) variables with better experimental matrix and improved analytical techniques (e.g. flow cytometry, HPLC and molecular approaches) is essential for better understanding benthic-pelagic coupling in bloom-forming species.

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Compliance with Ethical Standards

Conflict of Interest: The authors declare they have no conflict of interest.

Author contributions

Sathish K: performed all the experiments and statistical analysis, data interpretation; JS Patil: Original concept, experiment planning, data interpretation, manuscript elaboration and also supervised all works.

Ethics approval and consent to participate

Not applicable as this study is not related to humans and animals

Consent for publication: Not applicable

Availability of data and material: Please contact the author for data requests

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Figure Legends

Figure 1. (a) Location of sediment collection for germination experiments along Mandovi (M1 – FDownstream; M2 – Midstream; M3 - Upstream) and Zuari rivers (Z1 – Downstream; Z2 – Midstream; Z3 – Upstream). (b) Schematic presentation of experimental design to elucidate responses of phytoplankton benthic propagule (PBP) representing downstream (Z1 and M1), midstream (Z2 and M2), and upstream (Z3 and M3) locations of the Zuari (Z) and Mandovi (M) rivers to macro-nutrient enrichment (nitrate – N, phosphate – P, silicate – Si, and NPSi) and different light intensities (high light – HL, medium light – ML and low light – LL). SFSW – sterile filtered seawater

Figure 2. Effect of nutrient sources (N, P, Si, and NPSi) and different light intensities (high light – HL, medium light – ML, and low light – LL) on chlorophyll *a* fluorescence of phytoplankton benthic propagules collected from downstream to upstream locations along Mandovi and Zuari rivers concerning incubation days. Error bars indicate standard deviation.

Figure 3. Effect of nutrient sources (N, P, Si, and NPSi) and different light intensities (high light – HL, medium light – ML, and low light – LL) on variable fluorescence (F_v) of phytoplankton benthic propagules collected from downstream to upstream locations along Mandovi and Zuari rivers concerning incubation days. Error bars indicate standard deviation.

Figure 4. Effect of nutrient sources (N, P, Si, and NPSi) and different light intensities (high light – HL, medium light – ML and low light – LL) on photosynthetic efficiency (F_v/F_m) of phytoplankton benthic propagules collected from downstream to upstream locations along Mandovi and Zuari rivers concerning incubation days. Error bars indicate standard deviation.

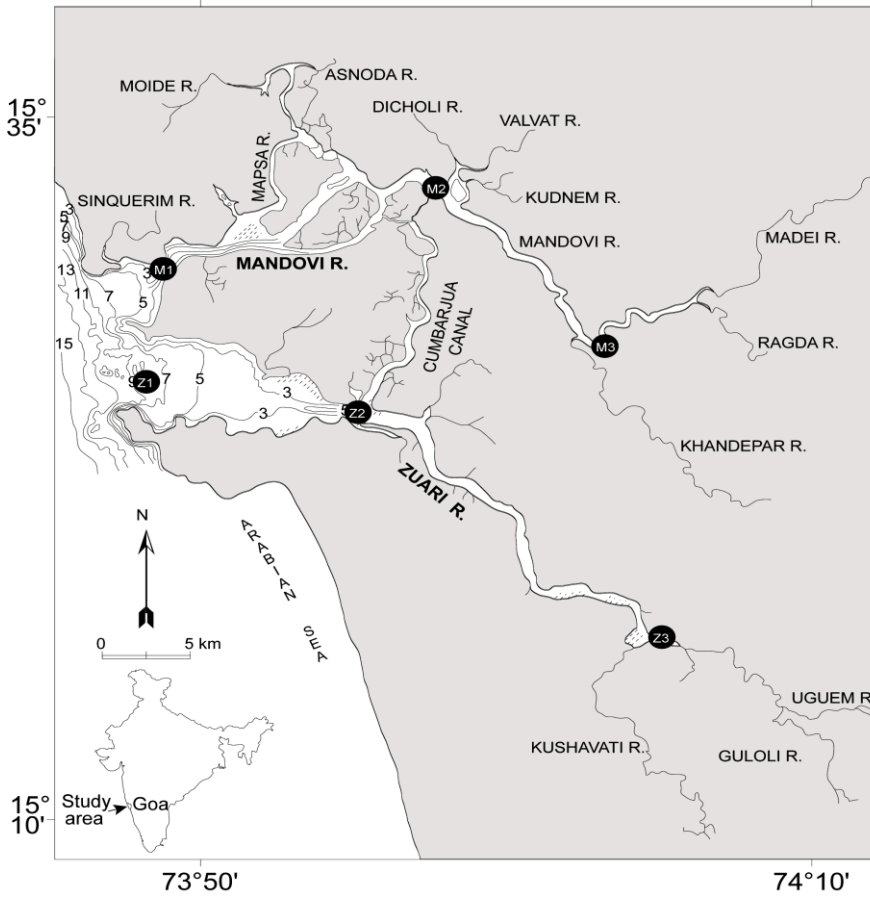
Figure 5. Effect of nutrient sources (N, P, Si, and NPSi) on the abundance (bar), species count (line) diversity (line), and evenness (bar) of phytoplankton benthic propagules collected from downstream to upstream locations along Mandovi and Zuari rivers concerning incubation days. Error bars indicate standard deviation.

Figure 6. Variations in the species composition of germinated phytoplankton benthic propagules under different nutrient sources (N, P, Si, and NPSi) for different locations (downstream to upstream locations) along Mandovi and Zuari rivers concerning incubation days.

Figure 7. Effect of different light intensities (high light – HL, medium light – ML, and low light – LL) on the abundance (bar), species count (line), diversity (line), and evenness (bar) of phytoplankton benthic propagules from downstream to upstream locations along Mandovi and Zuari rivers concerning incubation days. Error bars indicate standard deviation.

Figure 8. Variations in the species composition of germinated phytoplankton benthic propagules under different light intensities (high light – HL, medium light – ML, and low light – LL) for different locations (downstream to upstream locations) along Mandovi and Zuari rivers concerning incubation days.

a



b

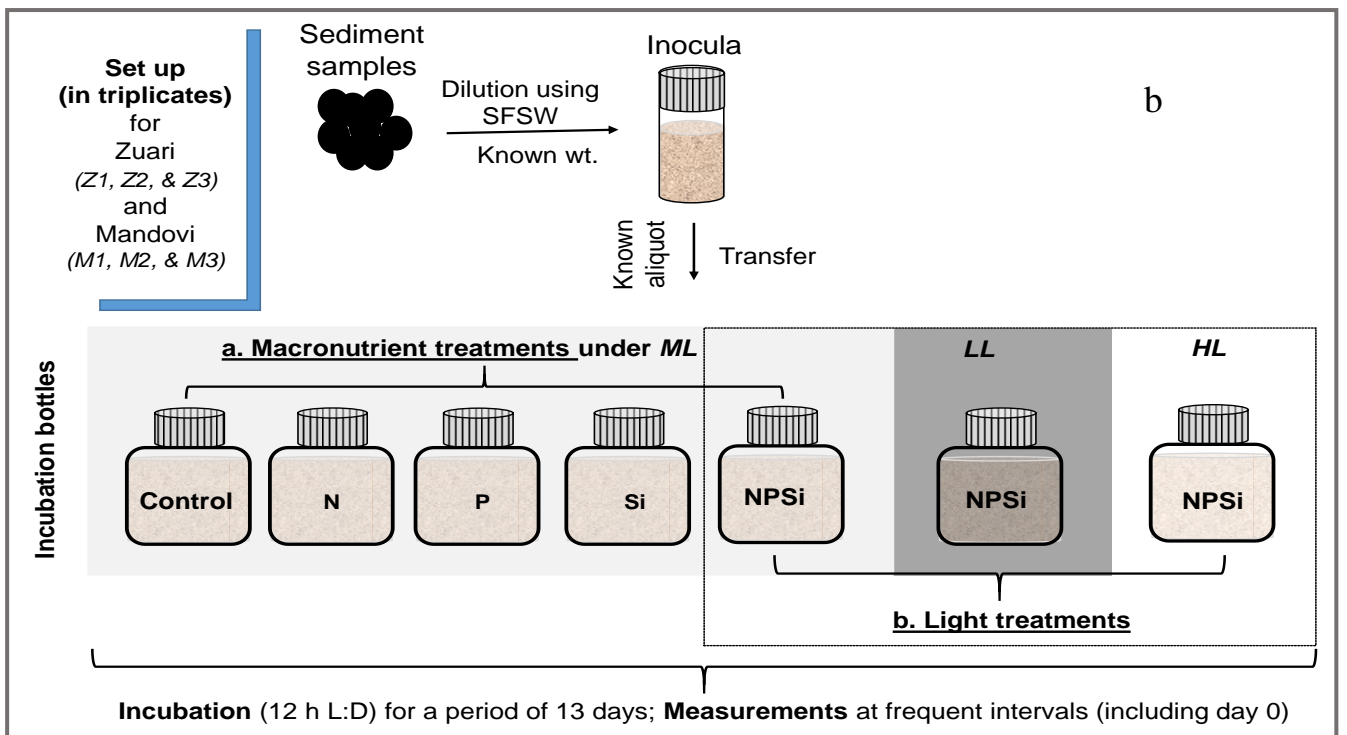


Fig. 1

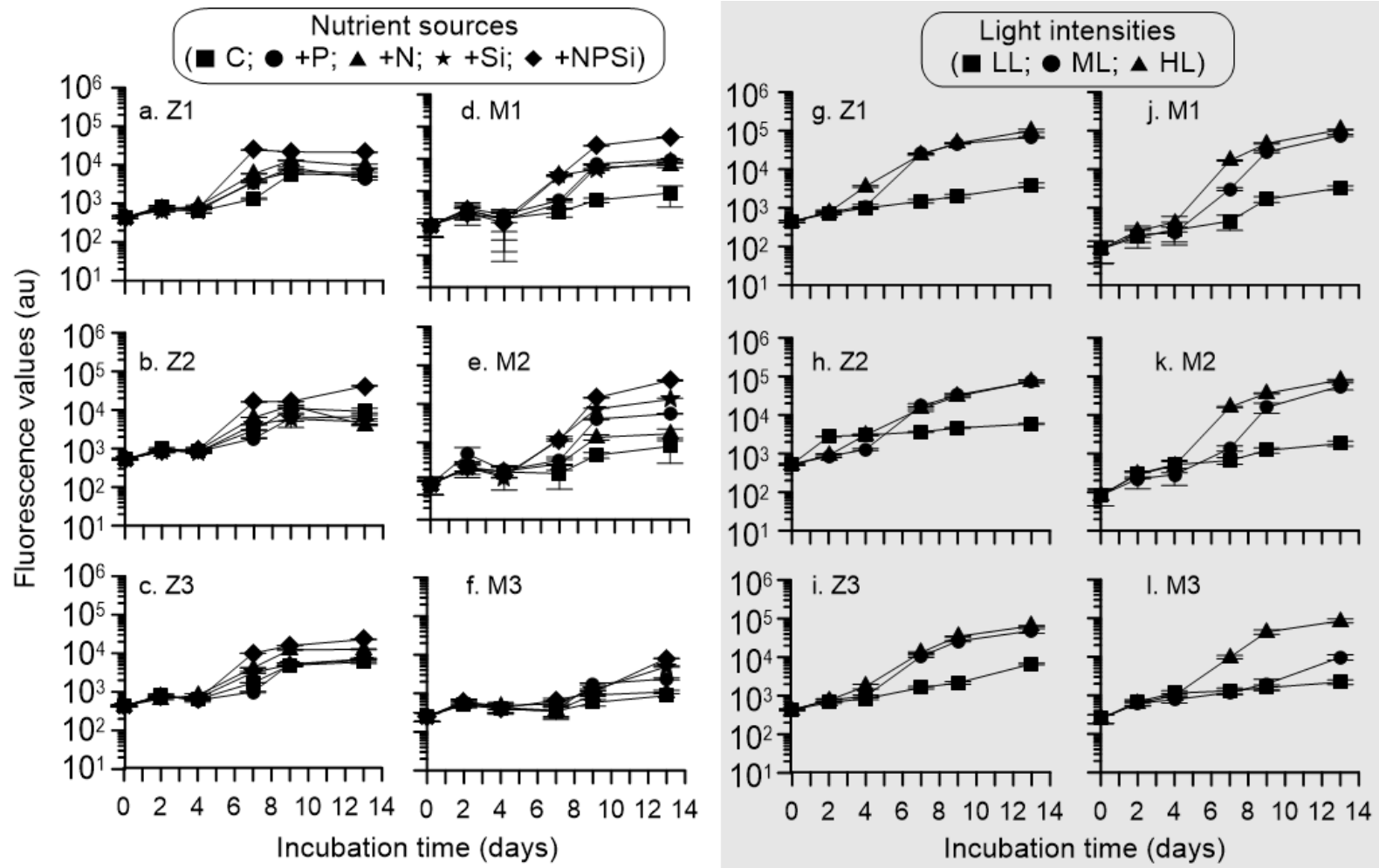


Fig. 2

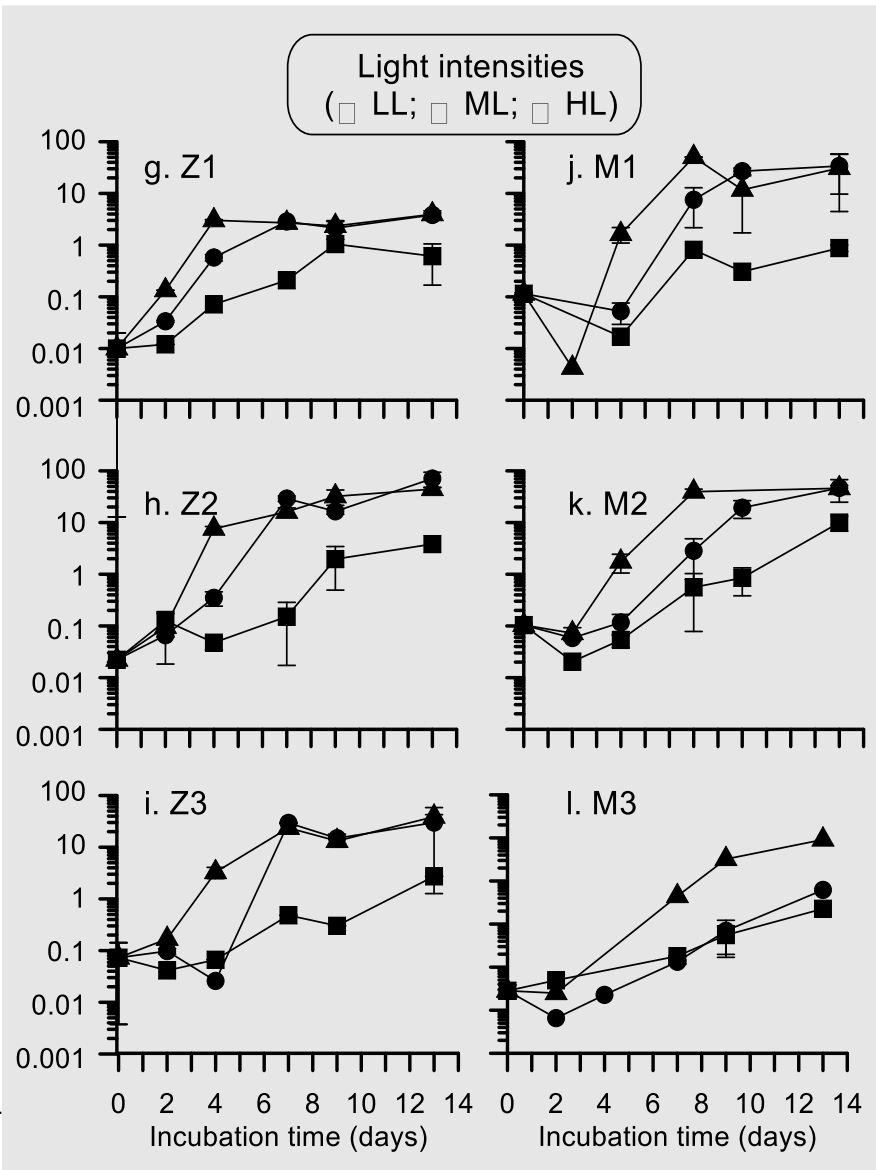
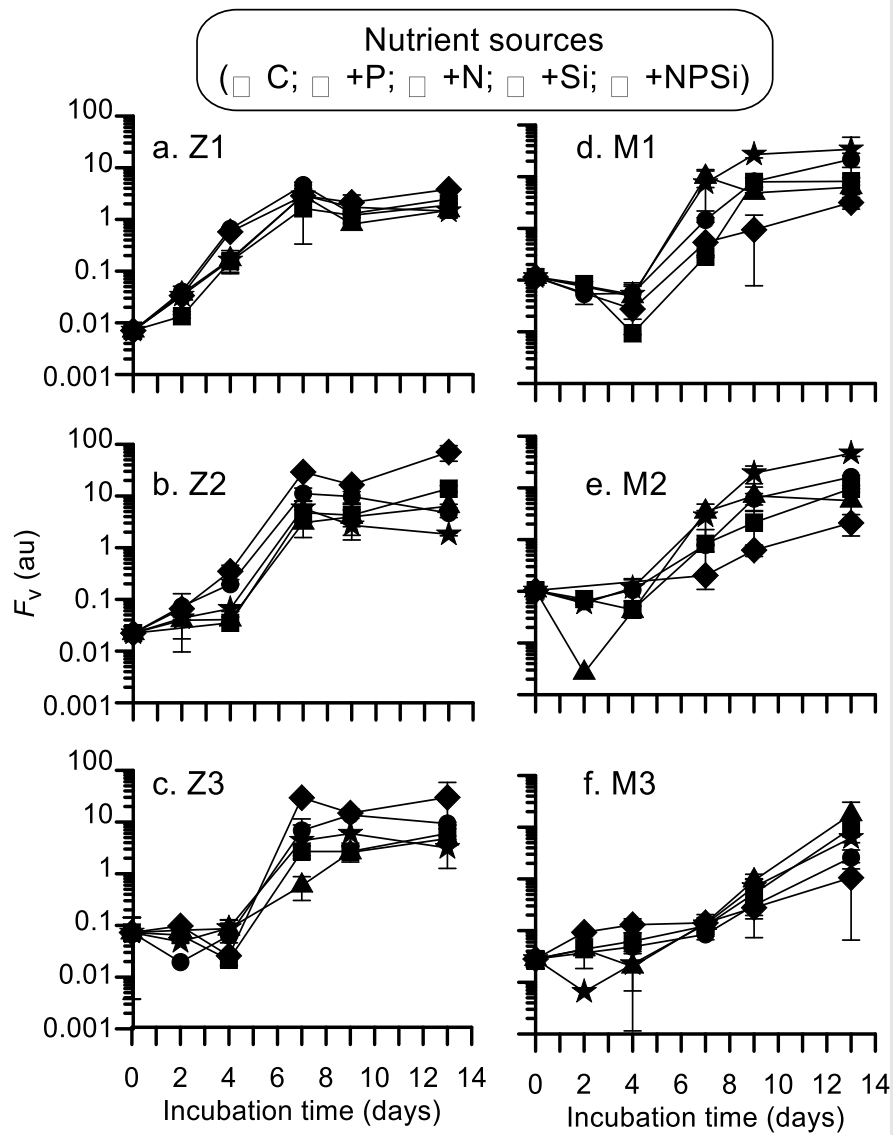


Fig. 3

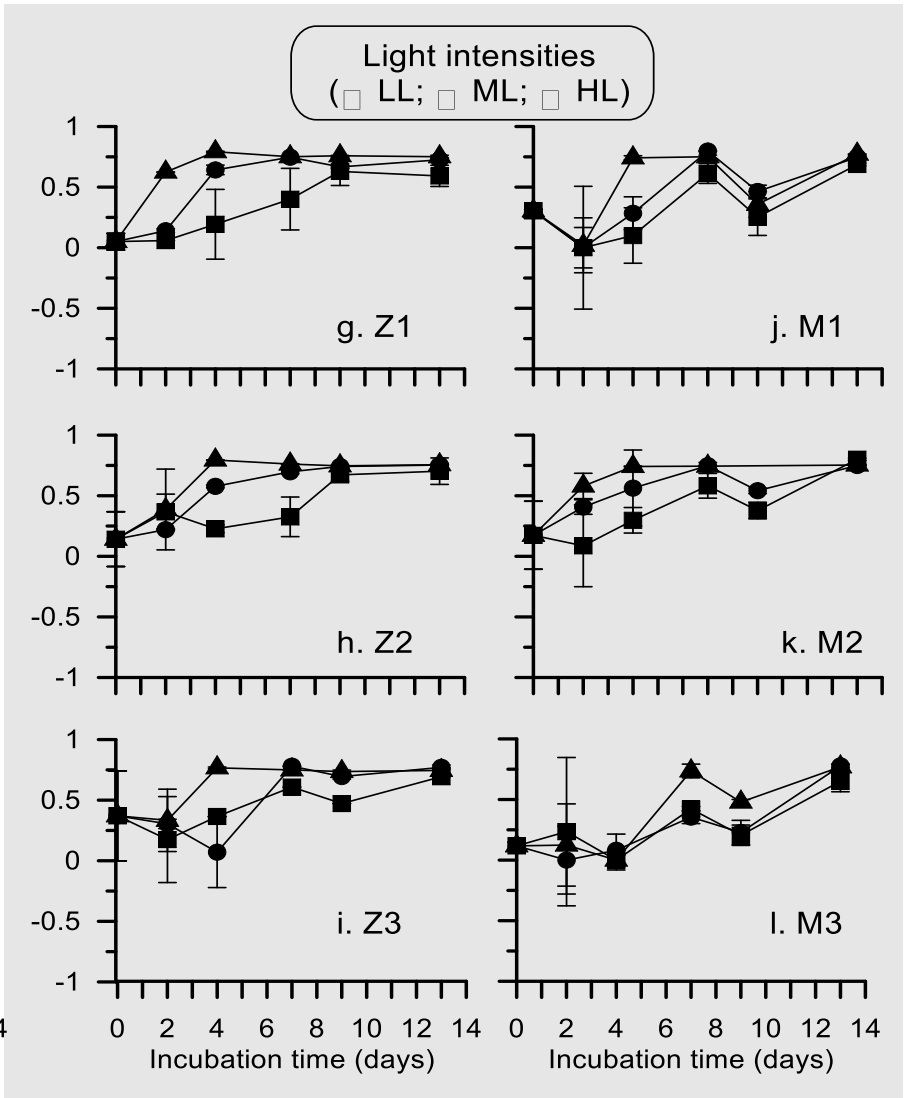
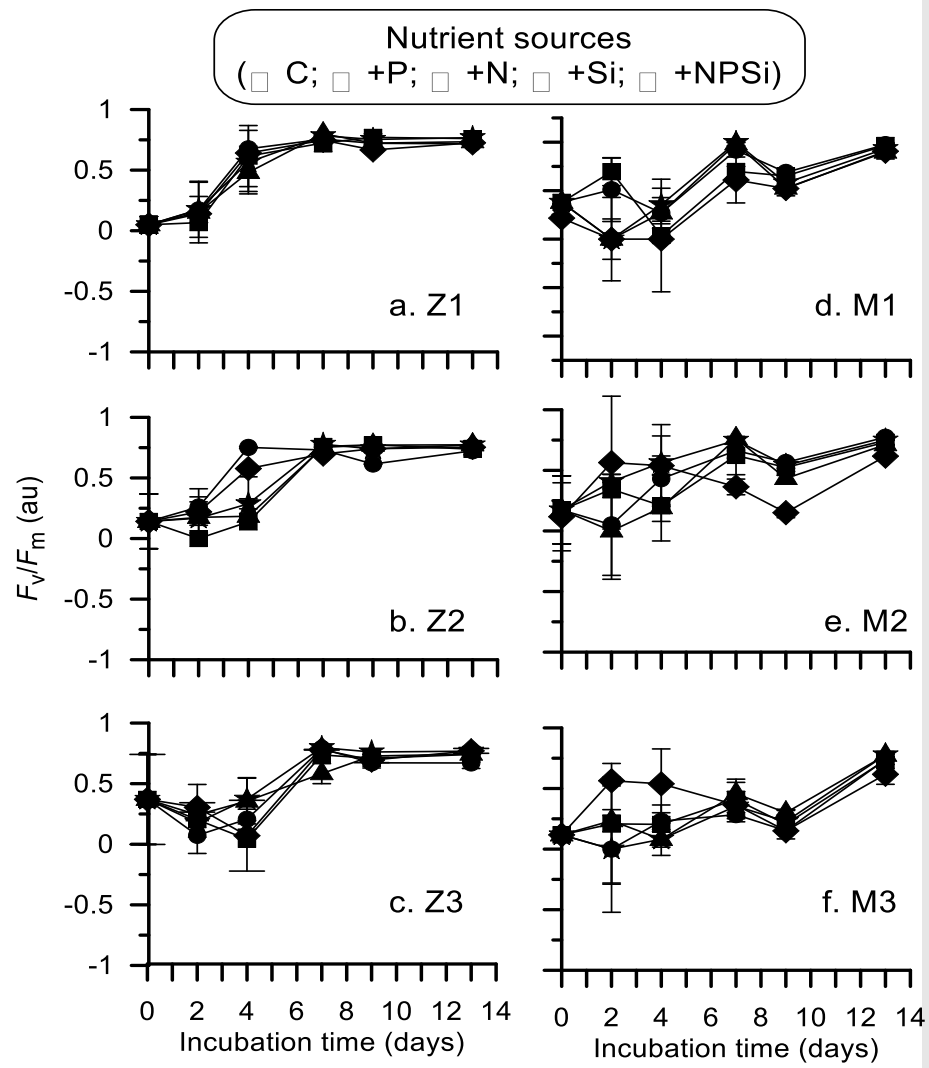


Fig. 4

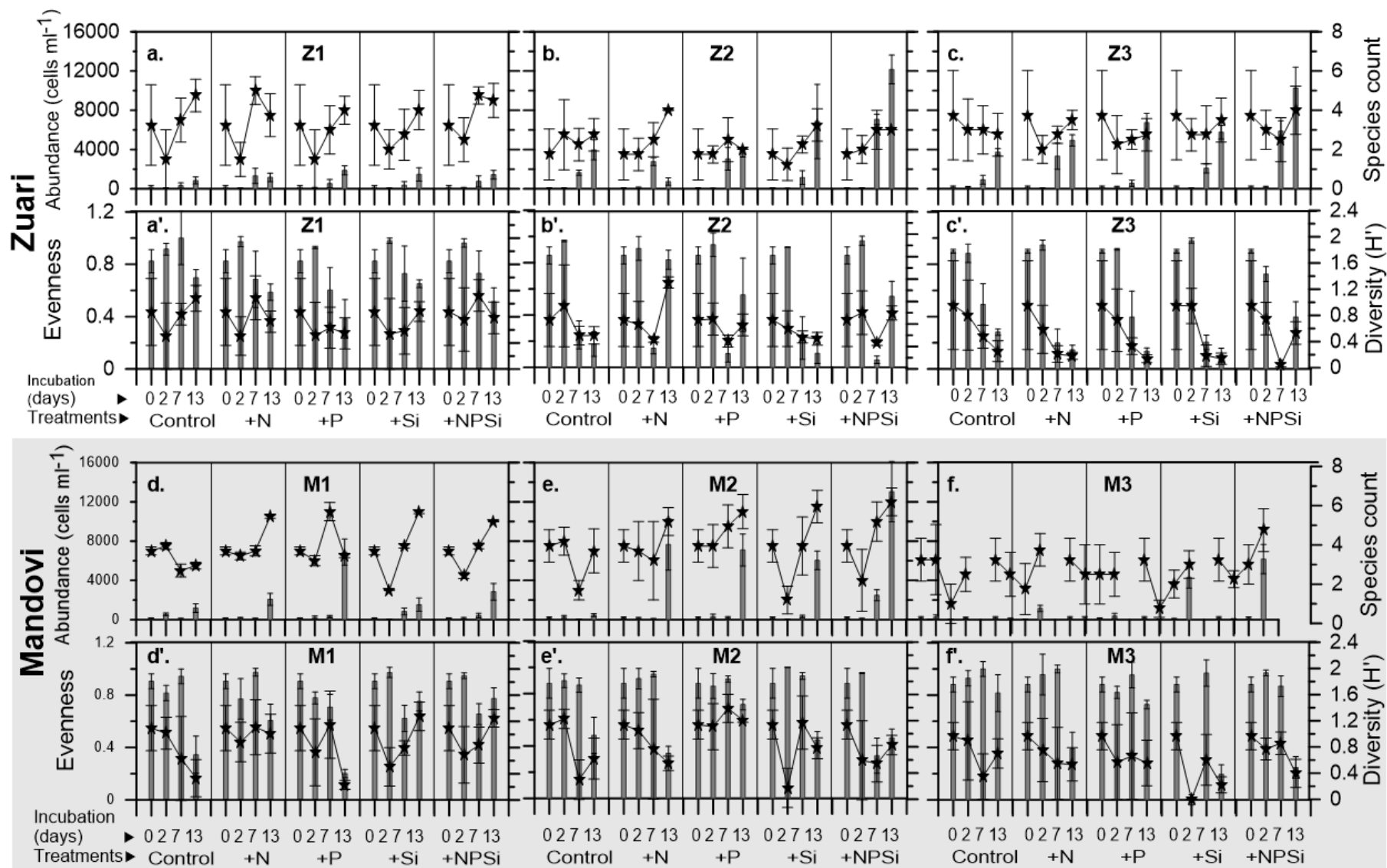


Fig. 5

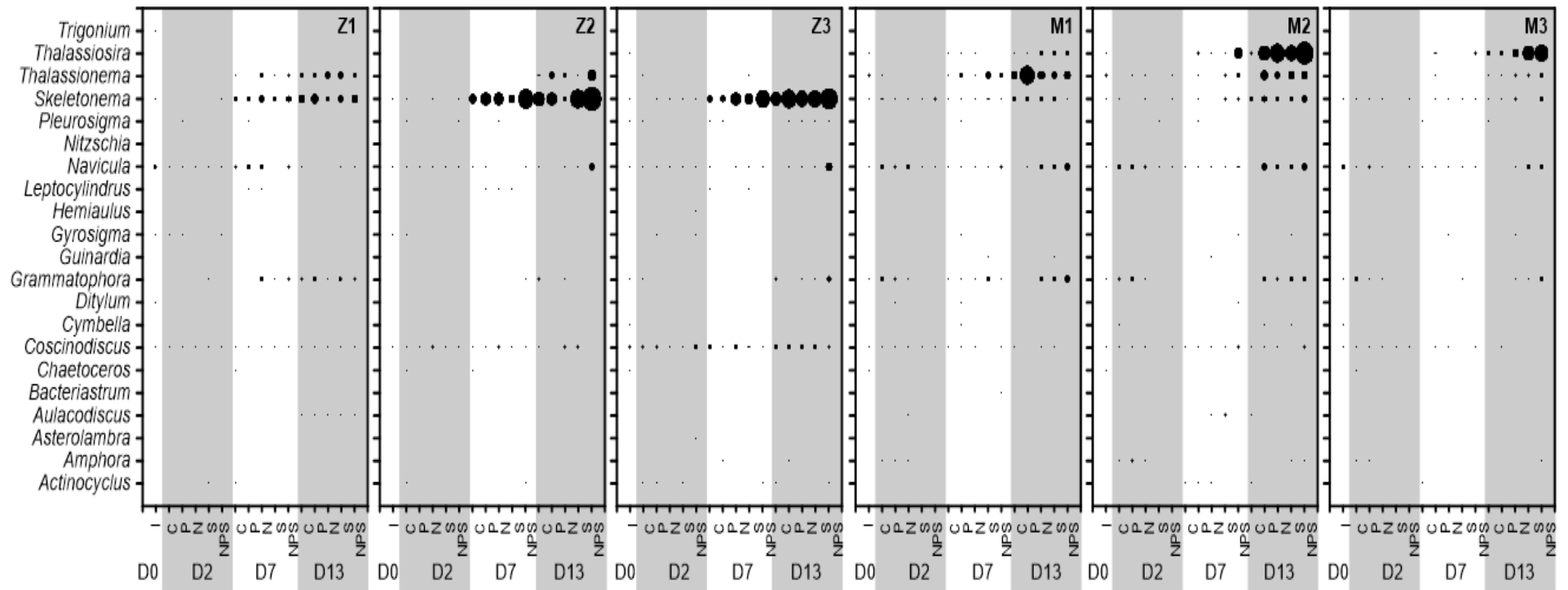


Fig. 6

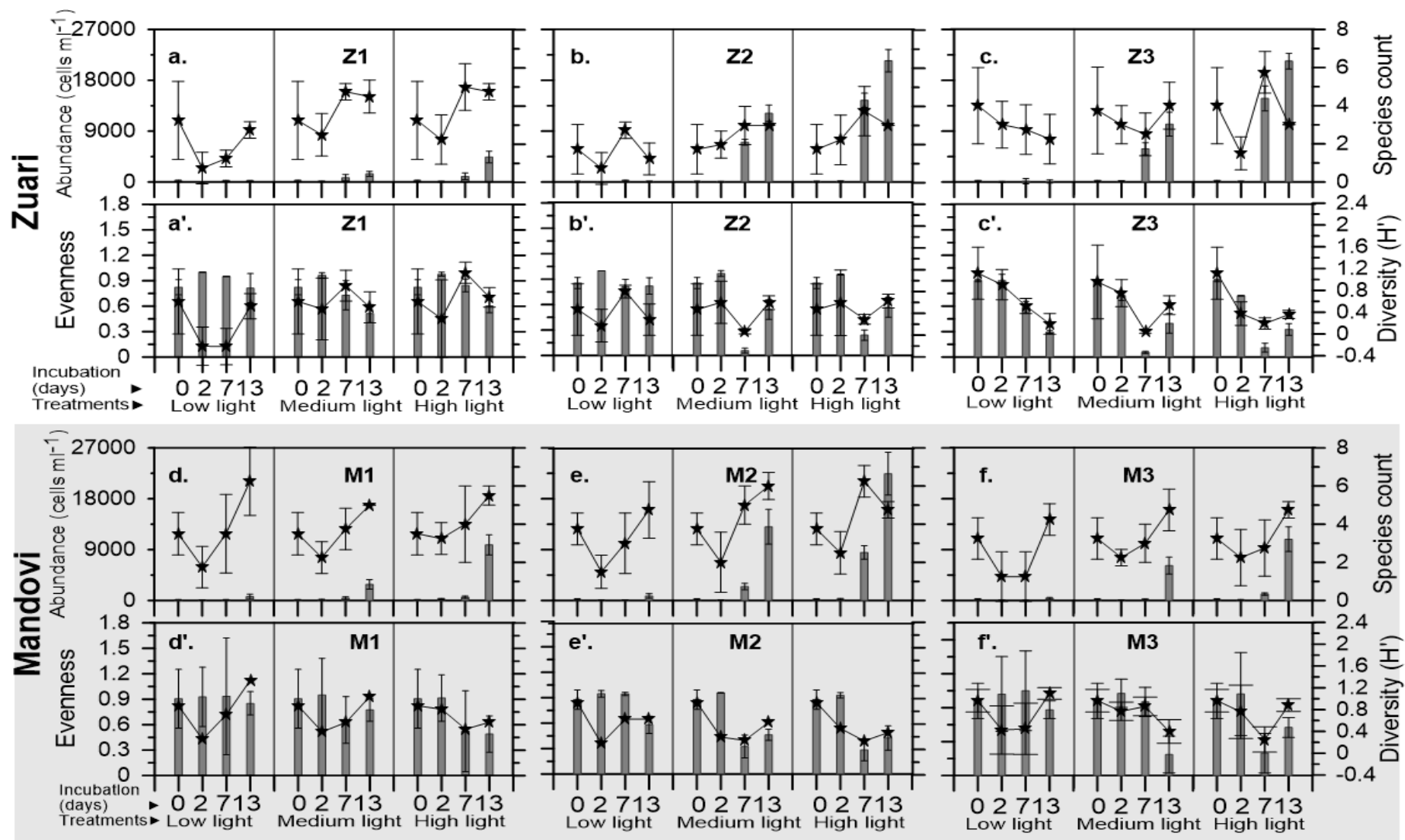


Fig. 7

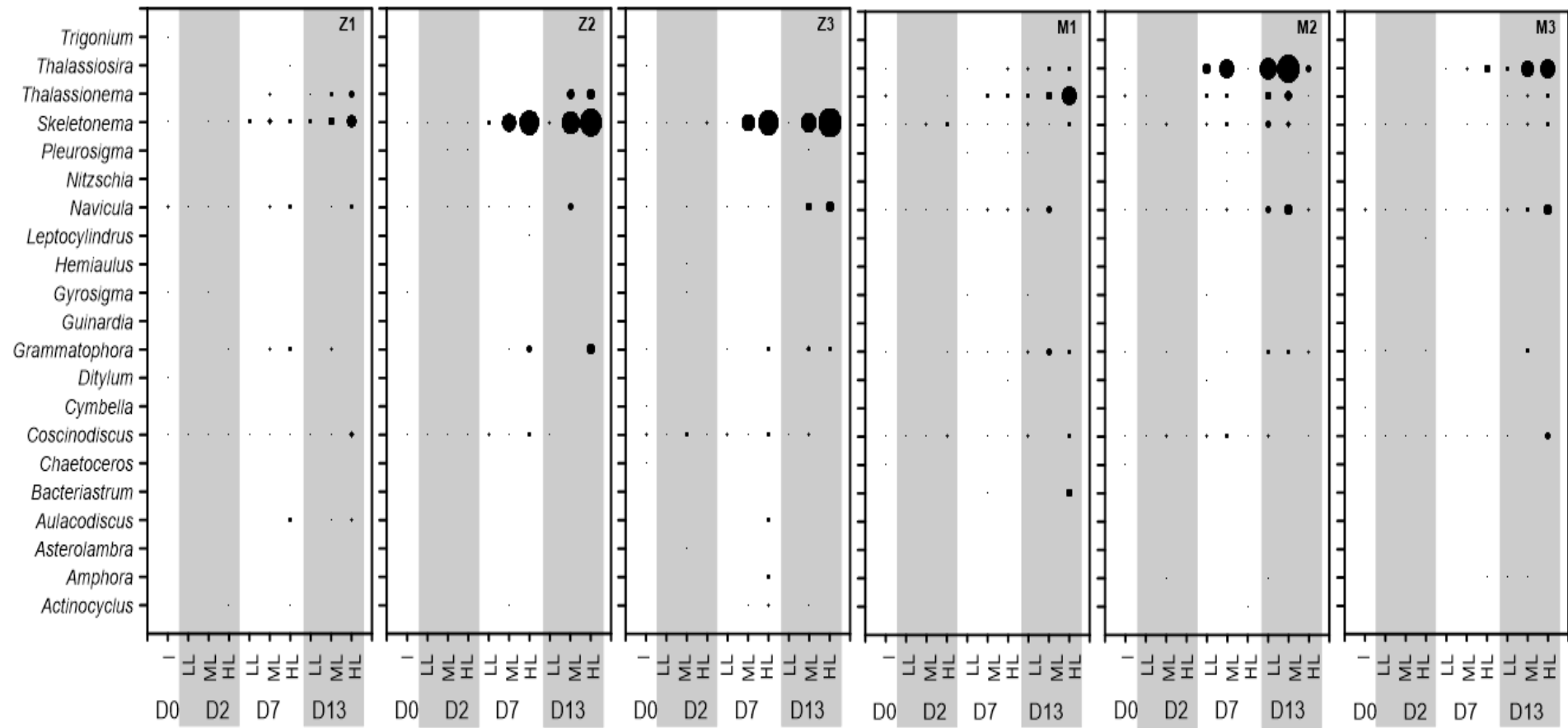


Fig. 8

List of supplementary tables and figures

Supplementary table 1. Details of sampling locations

Station name	Station code	Distance from mouth (km)	Latitude (N)	Longitude (E)	Depth (m)	Temperature (°C)	Salinity (PSU)	Secchidisc depth (cm)
Zuari River								
Mormugao	Z1	0	15° 25' 16.9"	73° 47' 36.9"	16.0	25.9	34.74	255
Cortalim	Z2	13	15° 25' 32.0"	73° 47' 50.2"	9.6	26.3	27.21	122
Sanvorderm	Z3	42.2	15° 25' 01.1"	73° 47' 36.0"	4.9	27.2	0.03	62
Mandovi River								
Campal	M1	0	15° 29' 46.6"	73° 48' 56.4"	8.3	24.7	32.52	217
Cambarjua	M2	14.9	15° 31' 31.4"	73° 55' 34.8"	5.83	25.6	10.36	143
Usgoa	M3	34	15° 29' 09.7"	74° 01' 20.8"	8.07	26.0	6.43	134

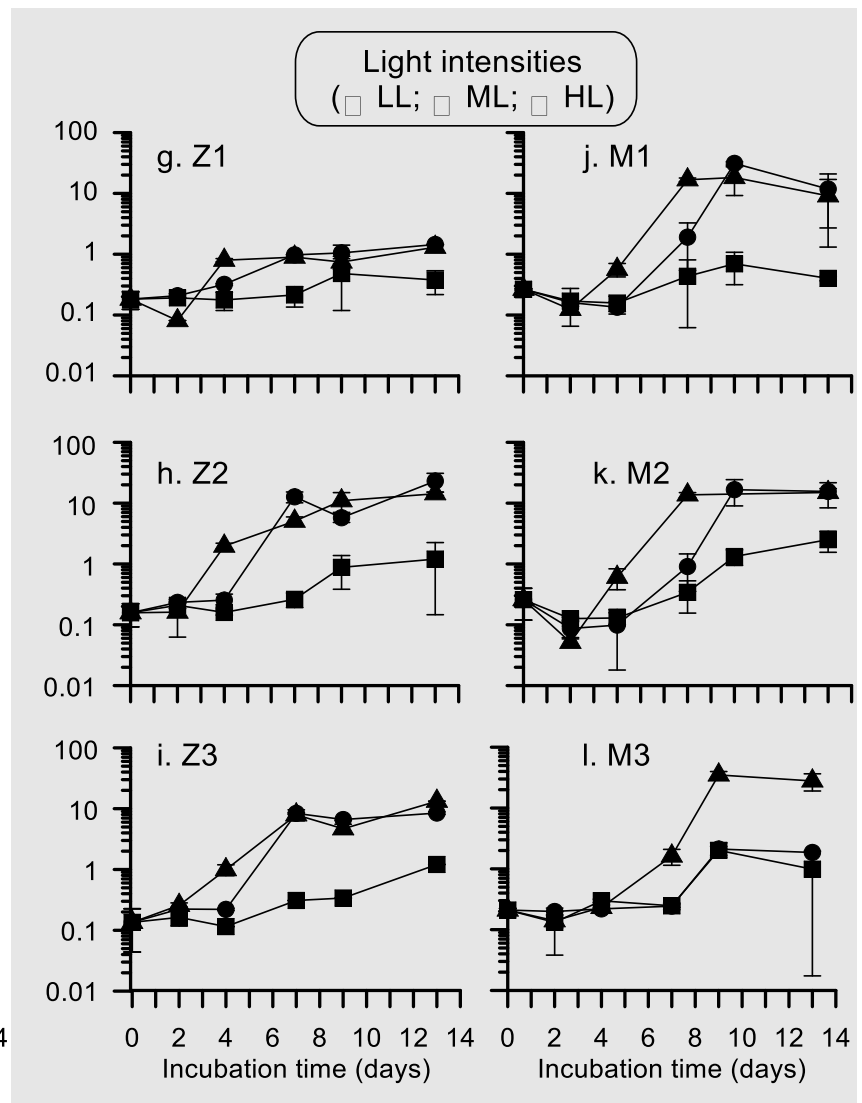
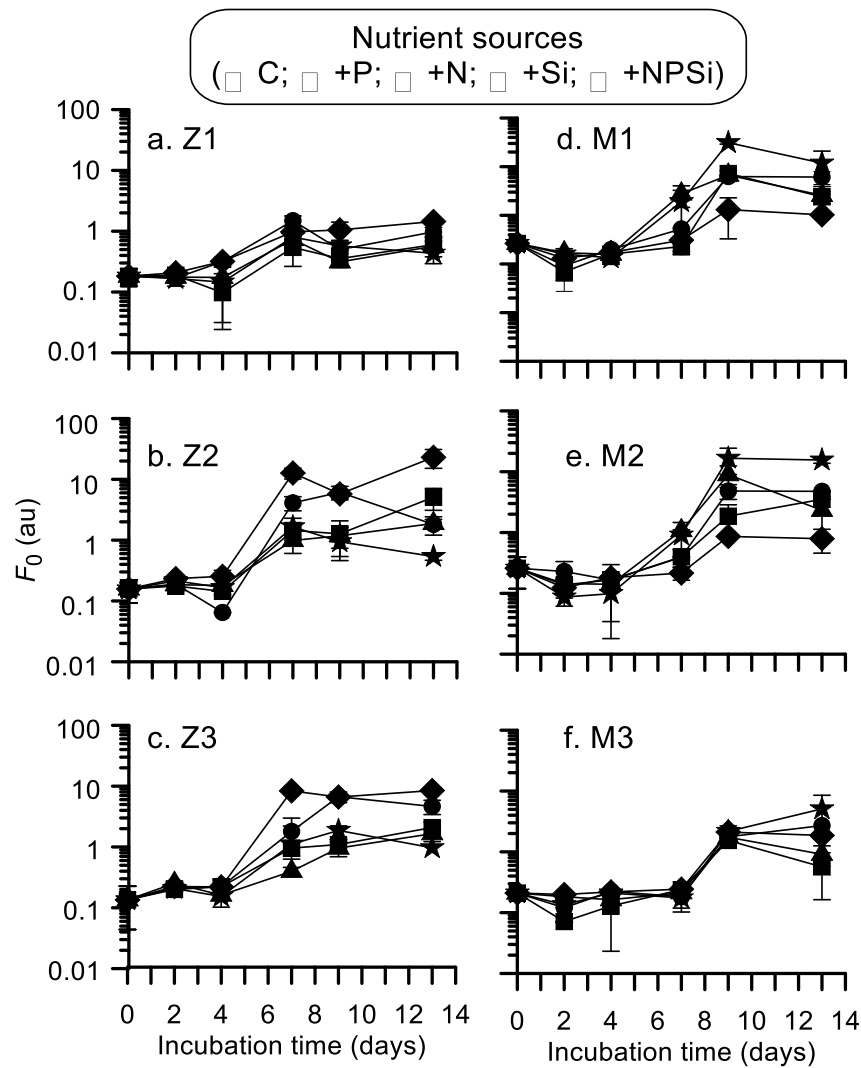
Supplementary table 2. Nutrient concentrations at the start and end of the experiments. The nutrient measurements were done manually wherein sample requirement was large. Therefore pooling of the samples was essential and the data presented is from the pooled replicate samples.

Initial nutrient level for Zuari ($\mu\text{Mol l}^{-1}$)					Initial nutrient level for Mandovi ($\mu\text{Mol l}^{-1}$)				
Sample	PO ₄	NO ₃	SiO ₃	NH ₃	Sample	PO ₄	NO ₃	SiO ₃	NH ₃
Z1 ctrl	10.30	1.92	11.04	10.16	M1ctrl	12.17	1.12	14.78	7.28
Z2 ctrl	10.02	2.07	9.41	18.26	M2 ctrl	12.39	1.18	15.17	10.98
Z3 ctrl	9.99	2.05	16.51	12.91	M3ctrl	16.15	3.21	31.78	15.24
Final nutrient level Zuari ($\mu\text{Mol l}^{-1}$)					Final nutrient level Mandovi ($\mu\text{Mol l}^{-1}$)				
Sample	PO ₄	NO ₃	SiO ₃	NH ₃	Sample	PO ₄	NO ₃	SiO ₃	NH ₃
Z1 ctrl	2.99	0.27	153.98	14.55	M1ctrl	12.51	1.83	136.70	23.20
Z2 ctrl	7.25	0.38	135.74	27.87	M2 ctrl	10.64	1.36	123.46	11.12
Z3 ctrl	6.69	0.29	129.79	30.35	M3ctrl	11.33	1.63	127.68	26.77
Z1PO ₄	20.85	0.20	141.70	11.53	M1PO ₄	65.04	0.87	104.45	36.52
Z2 PO ₄	15.56	0.25	110.69	26.77	M2 PO ₄	68.30	0.83	89.57	53.82
Z3 PO ₄	19.76	0.33	140.93	37.21	M3 PO ₄	64.26	1.76	116.06	58.35
Z1 NO ₃	4.42	233.59	149.47	14.96	M1 NO ₃	13.41	233.70	113.95	29.11
Z2 NO ₃	2.93	237.49	152.35	37.75	M2 NO ₃	13.19	239.60	104.74	21.42
Z3 NO ₃	6.50	239.50	129.02	30.48	M3 NO ₃	18.08	239.50	129.22	44.89
Z1 SiO ₃	4.54	0.36	308.16	15.51	M1 SiO ₃	10.95	1.92	262.08	16.61
Z2 SiO ₃	7.94	0.45	222.24	40.23	M2 SiO ₃	12.82	0.78	220.32	15.79
Z3 SiO ₃	6.97	0.33	204.48	30.34	M3 SiO ₃	15.53	0.94	217.92	20.87
Z1 NPSi/ML	18.05	237.49	351.36	36.79	M1 NPSi/ML	60.22	227.90	124.32	22.39
Z2 NPSi/ML	18.98	237.27	132.48	43.80	M2 NPSi/ML	53.99	233.81	81.12	19.50
Z3 NPSi/ML	21.01	237.49	126.72	49.84	M3 NPSi/ML	53.06	231.69	158.88	45.58
Z1 HL	19.14	170.78	252.96	64.25	M1 HL	48.55	202.46	138.72	35.83
Z2 HL	18.83	128.84	113.28	39.81	M2 HL	46.84	185.84	68.16	18.67
Z3 HL	23.18	155.95	124.8	44.21	M3 HL	36.88	175.91	89.28	29.11
Z1 LL	28.01	230.01	238.56	20.04	M1 LL	57.11	241.62	216.00	59.31
Z2 LL	24.90	231.80	232.8	31.85	M2 LL	61.93	231.91	213.12	42.29
Z3 LL	22.25	231.80	263.52	24.85	M3 LL	58.82	233.81	268.32	58.62

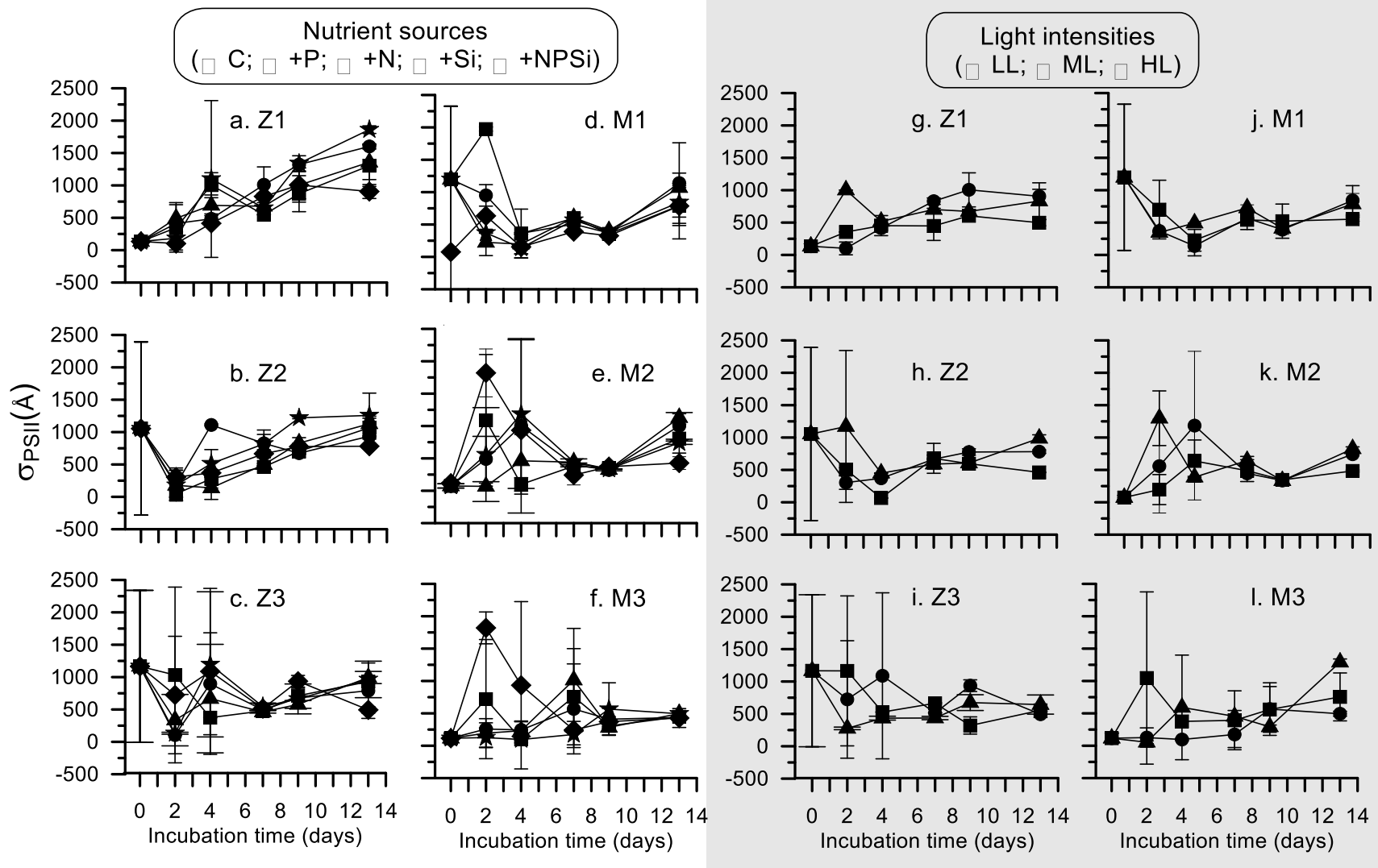
Supplementary figures

Supplementary figure 1. Effect of nutrient sources (N, P, Si, and NPSi) and different light intensities (high light – HL, medium light – ML, and low light – LL) on initial fluorescence (F_0) of phytoplankton benthic propagules collected from downstream to upstream locations along Mandovi and Zuari rivers concerning incubation days. Error bars shown indicate standard deviation.

Supplementary figure 2. Effect of nutrient sources (N, P, Si, and NPSi) and different light intensities high light – HL, medium light – ML and low light – LL) on functional absorption cross-section of photosystem II (σ_{PSII}) of phytoplankton benthic propagules collected from downstream to upstream locations along Mandovi and Zuari rivers concerning incubation days. Error bars shown indicate standard deviation.



Suppl. Fig. 1



Suppl. Fig. 2