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Impact of salinity and pH on phytoplankton community in a tropical freshwater system: An investigation with pigment analysis by HPLC

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Abstract:

An in vitro study was carried out to understand the effects of salinity shock and variation in pH on phytoplankton communities in a tropical freshwater system of Godavari River (a major peninsular river of India). The effects were assessed by pigment analysis using HPLC technique. Subtle changes in the salinity of freshwater by one practical salinity unit (PSU) completely removed green algae from the system and allowed cyanobacteria to come into dominance. The cyanobacteria were found to tolerate higher osmotic stress until salinity reached a PSU of 16. The higher salinity tolerance range of the cyanobacteria was attributed to enhanced synthesis of zeaxanthin as a protective xanthophyll against the osmotic stress. However, the effect of pH was not as dramatic as salinity where green algae and cyanobacteria from the same freshwater system showed a considerable acclimation towards fluctuating pH. These findings are environmentally relevant to understand the likely impact of salt water intrusion and pH variation on phytoplankton communities in a tropical freshwater system.

Key words: Salinity shock; sea water intrusion, phytoplankton community; tropical freshwater, pH shock

Introduction:

Two potentially important factors that can regulate estuarine phytoplankton community and biomass are salinity and pH. Salinity in an estuary is a dynamic entity that is chiefly regulated by the river discharge, local rainfall and tidal amplitude. In an unperturbed estuary, different groups of phytoplankton communities are adapted to withstand a certain range of salinity and therefore show complex pattern of distribution along the salinity gradient of the estuary from the head to the mouth [1, 2].

It was reported [3, 4] that the distribution of phytoplankton along estuary gradients tends to favour cyanobacteria and chlorophytes in brackish waters. However, Kies [1] reported that mid-to-high salinities in an estuary favour dinoflagellates and diatoms. Species diversity usually becomes very low at high salinities. Rijstenbil [2] reported that high salinities can be a lethal limit for many phytoplanktons in estuaries.

Guillard [5] reported that the salinity change can result in osmotic stress on cells, uptake or loss of ions and effects on the cellular ionic ratio in phytoplankton. To maintain osmotic balance due to frequent alterations in salinity level result in an increased respiratory activity in phytoplankton. Inhibitory effects on physiological processes of phytoplankton can follow changes in salinity.

Alterations in salinity level frequently result in increased respiratory activity to maintain osmotic balance [6]. A rise in NaCl levels in the medium has been shown to increase respiration rate and decrease photosynthetic O_2-evolution for two species of Scenedesmus [7-9]. It has also been showed that an increase in salinity immediately reduced rates of net carbon fixation by Nitzschia americana.

Much of our current knowledge of salinity effects is based on laboratory studies of cultured algae [10-12] and on observations from field studies. Surprisingly only few studies have experimentally determined the effects of changes in salinity on phytoplankton community structure for naturally occurring phytoplankton assemblages.

Effect of salinity shock on species diversity of natural assemblages of largely freshwater phytoplankton was studied by Floder and Burns [13]. In their study, the transition to brackish water conditions resulted in reduced phytoplankton diversity, especially during the first few days of incubation. They attributed this effect to osmotic stress.

The salinity tolerance of phytoplankton differ and based on their tolerance extent they are grouped as euryhaline (able to tolerate wide range of salinity) and stenohaline (having very narrow salinity tolerance range) species. Any unnatural change in the salinity is strong enough to affect stenohaline

phytoplankton sp. and could irreversibly change local phytoplankton community structure and establish a new stable climax community.

Any drastic change in phytoplankton community (which is at or near the bottom of the food chain in the aquatic system) can have serious ecological impact [14-16]. Godavari estuary is one of the major estuaries of the Indian east coast and recently a huge natural gas reserve has been identified in its basin [17]. Thus, it is expected that heavy dredging activity will be performed in the near future that may facilitate tidal salt wedge to intrude further which may have serious ecological impact.

Rivers and estuaries are also prone to pH fluctuation due to poor buffering capacity of riverbed clay mineral and fresh water and periodic tidal mixing with alkaline sea water. Variation in pH also can affect phytoplankton growth in a number of ways [18-20]. It can change the clay buffering system thus influencing the availability of trace metals and essential nutrients [21,] and inflict direct physiological effects at extreme levels.

The main stimulus in this investigation was to find out how freshwater phytoplankton communities respond when they encounter different levels of salinity shock and pH change. The distribution of, and variations in, the phytoplankton community was assessed by quantitative determination of their class specific marker pigments. Highly significant linear regressions were reported for *chl a*-total biomass, fucoxanthin-diatoms, lutein-green algae and zeaxanthin-cyanophytes [22]. High Performance Liquid Chromatography (HPLC) [23, 24] was used to identify and quantify these phytoplankton communities by their pigment markers.

This study is environmentally relevant in global scale (a) to understand the likely impact of salt water intrusion by dredging and predicted sea level rise on local phytoplankton community in estuary (b) recognizing the effect of pH changes in freshwater.

Experimental Section

Study Area

Godavari is the third largest river in India. It has formed two major distributaries; Goutami and Vasishta before debouching into the Bay of Bengal in the east coast of India. Freshwater sample was collected at Gautami Godavari as it carries the majority of the discharge.

Sampling was done further upstream at Alamuru (40 km upstream from Yanam as shown in Figure 1) that represents typical freshwater which is not affected by tidal intrusion at any time throughout the year. As high discharge in the river overshadows all other potentially important factors [25] that may regulate phytoplankton production and succession, the sampling was carried out in September,

when river discharge virtually stops and tidal effect plays a major role in the salinity values of the estuary in Yanam but not enough to impart its effects as far upstream as Alamuru

Sampling and analysis

Surface water samples from Godavari River were collected at Alamuru station by using 5-L Niskin bottles and finally transferring it to 20 L Nalgene bottles. Before starting the incubation experiment, the exact salinity of the water samples were measured by Autosal (Guild line Autosal 8400B salinometer) in 'practical salinity units' (PSU) which defines salinity in terms of the conductivity ratio of a sample to that of a solution of 32.4356 g of KCl at 15°C in a 1 kg solution according to the definition of the International Association for the Physical Sciences of the Ocean (IAPSO). pH was measured by a Metrohm auto titration unit.

99.5% pure sodium chloride (NaCl) from Merck India Ltd was used for salinity manipulation. The salt treatment will be indicated by means of the term 'NaCl', which is the main component of the sea salt.

Water samples were distributed into fourteen 1L bottles (NALGENE), five bottles in one set was manipulated by adding NaCl in such a way that the salinity of freshwater of Godavari changed to 1, 2, 4, 8 and 16. To manipulate the salinity of the river water, NaCl was dissolved in a glass beaker with the same river water and added all at once to the original water sample. Salinity change was allowed to take place suddenly instead of gradual dissolution of crystal based on the hypothesis that salinity change in the natural system might takes place by sudden intrusion of tidal front having high salinity gradient.

Another set of five bottles were pH manipulated by adding ultrapure HCl (Fluka) and NaOH which resulted in pH values for the water solutions of 6.0, 6.5, 7.5, 8.0, 8.5. The remaining 2 pairs of bottles were kept as controls.

All the experimental solutions (after adjusting the salinity and pH) were incubated for 5 days (120 hours). Incubation experiments were carried out (in triplicates) in transparent bottles under the natural light source. Temperature was found to be ~25±2°C during the incubation experiment under the natural condition.

Filtration and Extraction:

After five days of incubation, 0.5L of samples was filtered through Whatman GF/F filter paper (0.7µm nominal pore size and 47mm diameter) under reduced vacuum. Filters were dabbed in tissue paper to remove water. Each filter was cut into small slivers and was placed in heavy-walled 10 ml

amber coloured culture tubes (Shimadaju: P/N 638-41462). 4mL of 90% Acetone was added to the tubes using a dispensette. Each tube was covered and placed in an ultrasonic bath filled with ice slurry to prevent heat accumulation. Ultrasonification (Cole Parmer, Model: 08893-26) of samples enables disruption of cells and facilitates the extraction of the pigments. All the tubes were then placed in a freezer (-20^{o}C) overnight. Sample slurry was then vortexed (GeNei India Pvt. Ltd.) and clarified by pushing the contents through a nylon HPLC syringe catridge filter (PAL, P/N 4118) with 0.45um pore size.

HPLC Analysis

Analysis was performed by using Agilent 1200 HPLC system equipped with quarternary pump, autoinjector, Peltier column thermostat, temperature controlled autosampler and chemstation software. Pigments were detected with the diode array detector using 450 and 665 nm wavelength (20 nm bandwidth was used in both cases). 665 nm was used to quantify chlorophyll-a, divinyl chlorophyll-a, chlorophyllide-a, phaeophorbide-a and phaeophytin-a as they respond similarly and strongly in this wavelength. All other carotenoids and xanthophylls were detected and quantified at 445nm.

An injector program was optimized to deliver sample extract and buffer composed of 28 mM aqueous tetrabutyl ammonium acetate (TBAA) (AR Grade, Fluka) at pH 6.5 and methanol (GC Assay 99.7% pure,Merck) in 90: 10 ratio. The sample extract and buffer was mixed automatically within the sample loop which enabled effective retention of early eluting chlorophylls and lessened peak distortion. The method proposed by Heukelem [26] was adapted for pigment analysis during the study. The method outlined below.

Column: ZORBAX eclipse XDB-C8, 4.6 × 150 mm (diameter by length); PN: 963967-906

Gradient: Binary gradient elution

A: 70/30 methanol/TBAA pH 6.5

B: methanol

Linear gradient from 5-95% solvent B in 22 min, isocratic hold of 95% solvent B

From 22-29 min, return to 5% solvent B at 31 minutes, equilibration for further

5 minutes (31-36 minutes)

Injection volume: 200 µl

Oven temperature: 60 degree celcius

Solvent flow rate: 1.1 ml/min

In this study, zeaxanthin was used as a marker of cyanobacteria, fucoxanthin for Diatoms, lutein for green algae and *Chl a* as overall total phytoplankton biomass. Presence of Merismopedia sp. (colonial and coccoid cyanobacteria) in Godavari estuary (our unpublished data) gave us additional confidence of using zeaxanthin as their marker pigment and to trace their biomass change.

Results and Discussion

The pH of the sample was measured immediately after collecting the sample by a Metrohm auto titration unit and found to be 8.15. The pH of the sample was measured prior to the incubation experiment and after the incubation of 60 hrs. No variation in the pH value was observed. The total organic carbon (TOC) concentration was found to be 12.0 ± 0.6 mg. L^{-1}. The major ion concentrations such as NO_2^{-1}, PO_4^{3-}, NH_4^+ ions were found to be 4.10 μM, 0.60 μM, and 3.40 μM respectively. The total concentrations of Na, K, Ca and Mg obtained from the literature were (0.70 ± 0.17) mM, (0.18 ± 0.05 mM, (0.63 ± 0.13) mM, and (0.41 ± 0.12) mM respectively in Godavari River. However, it is important to note that the concentrations of major cations presented here are the average values obtained from the literature [27-29]. Total trace metal concentrations in the natural sample were analysed by ICP-MS (X-SERIES II, Thermo Scientific) and all the biologically important metals were present in the range of nM concentrations. This indicates that the surface water collected in Godavari River at Alamuru station (with no discharge from Dawleshawaram Dam) was not contaminated with trace metals.

Effect of salinity on total biomass and phytoplankton communities in freshwater system.

Figure 2a shows that the maximum phytoplankton biomass in terms of *Chl-a* was in the control and was equally contributed by green algae and cyanobacteria (as revealed from the zea/*Chl-a* and lut/ *Chl-a* ratio as shown in Table 1.

Increasing concentration of NaCl in the salt manipulated bottles caused consecutive decrease in the total biomass and the effect became most pronounced at the salinity of 16.

Pigment derived phytoplankton community structure revealed some interesting features. No signal was detected by HPLC for lutein which is a representative marker pigment for green algae in all the salt treated samples at salinity of 1. This indicates that green algae which was present initially in freshwater (concentration of lutein in control was 2.7 ± 0.1 $mg.m^{-3}$) were very sensitive to subtle changes of salinity. However, the concentration of zeaxanthin enormously increased with the increasing salinity and maximum concentration of 25.6 ± 1.3 $mg.m^{-3}$ was recorded at the salinity of 4 (Fig 2a, Table 1).Further treatment with higher concentration of NaCl (at the salinity of 8) reduced the concentration of zeaxanthin compared to the salinity at 4. Phytoplankton biomass was severely

reduced at a salinity of 16 PSU (Fig. 2a) and indicating their salinity tolerance maxima. Importantly, in all the salt treated samples, zeaxanthin was higher than the control. A striking shift in phytoplankton community took place where green algae sp. were completely replaced by more salt tolerant cyanobacteria (Merismopedia sp.) whenever there was an increase of salinity in the freshwater. In a similar laboratory based salinity enhancement experiment in Myall lakes system, Australia [30], the reduction in the abundance of green algae at the salinity of 4-8 and Merismopedia as the most abundant taxa at the salinity of 16 was reported. In our investigation, the tolerance maxima of green algae and cyanobacteria was found to be 1 and 16 respectively which shows that the fresh water phytoplankton community in Godavari river are quite sensitive towards salinity hikes.

Scatter plot of zeaxanthyn: *Chl-a* ratio against respective salinity manipulations gave a linear fit with R^2 value of 0.97 (Fig 2b). Table 1 reveals that increase in the zeaxanthin concentration was always at the cost of *Chl-a,* which is clearly the most plausible explanation for the linear fit. Another plausible reason for the reduction in over all *Chl-a* could be explained by the complete removal of green algae which was initially contributing to *Chl-a* in the control but not at all in the salt manipulated samples. It is known that intracellular reactive oxygen species (ROS) formation is triggered in hyper osmotic condition [31]. As a part of the antioxidative mechanism algae can increase the ratio of xanthophylls to light harvesting pigments [32, 33]. Zeaxanthin and ß carotene are such non-photosynthetic carotenoids which protect the photosynthetic centre against the destructive singlet oxygen.

It is evident from Table 1 that with the increasing salinity level cyanobacteria combat the salinity stress by synthesizing more zeaxanthin. Energy cost associated with the zeaxanthin synthesis is probably balanced by the adaptability of cyanobacteria to tolerate wider range of salinity fluctuation that helps them to occupy the niche left by the green algae (green algae disappeared from the system at the salinity of 1). It is apparent that increase in zeaxanthin concentration by cyanobacteria to combat the progressively higher salinity stress is not indefinite and after a certain salinity maxima (16, in this study) the population can not tolerate the stress. The highest zeaxanthyn: *Chl-a* value (Table 1) was observed at the salinity of 16. This observation suggests that thriving population of cyanobacteria could only survive by synthesizing higher than average concentration of zeaxanthin per cell.

ß-carotene is reported as a minor (1-10 %) marker pigment of cyanobacteria. In HPLC chromatogram ß-carotene also found to follow similar trend like zeaxanthin however the former was not quantified as it is nonspecific and widely distributed among the phytoplankton taxa [11]. Apparently ß-carotene also similarly responds with salinity stress and is synthesized by cyanobacteria as a protective carotenoid.

A second set of laboratory based incubation experiment was simultaneously conducted to understand the fate of the fresh water phytoplankton in brackish (intermediate salinities of 4 and 6) water by mixing virtually fresh (with salinity value of 0.02) upstream river water from Alamuru with the aged coastal water from the Bay of Bengal (with salinity of 35) in appropriate proportion. Another aim of this experiment was to compare the phytoplankton community and biomass in salt treated samples with that of the manually mixed water parcels of different salinities. Table 1 shows that the mixed water samples at the salinity of 4 accumulated phytoplankton biomass (*Chl-a*, 4.8 ±0.2 $mg.m^{-3}$) was comparable to salt added sample at salinity 4 (*Chl-a*, 5.0± 0.3 $mg.m^{-3}$). *Chl-a* concentration decreased to 3.5 ± 0.2 $mg.m^{-3}$ at the salinity of 6. Pigment analysis revealed that the major phytoplankton community is cyanobacteria followed by diatom as presented in Table 1 (identified from the marker pigment zeaxanthin and fucoxanthin, respectively). No signal of lutein was observed in the chromatogram indicating the disappearance of green algae once again at elevated salinity. Presence of diatom in the salt manipulated mixed samples could only be explained by the proliferation of diatom propagules in optimal salinity and nutrient as intact diatom cell neither could come from [34-36] fresh water side (no signal of fucoxanthin was observed in fresh water) nor from the aged coastal water. Higher phytoplankton biomass accumulation in the second experimental set could be explained by the fact that diatom also contributed to *Chl a* along with cyanobacteria. In contrast, in case of the salt treated samples (with salinity of 4, 8, 16) only lower numbers of cyanobacteria could proliferate by synthesizing higher concentration of zeaxanthin at the expense of *Chl- a*.

Daily time series observation study (2007-2008) at the head of this estuary revealed that phytoplankton community is dominated by cyanobacteria followed by diatoms whenever the surface salinity goes beyond > 1 (unpublished data).Such community scenario is in accordance with our laboratory experiment and strengthens the hypothesis that intrusion of saline water can change the phytoplankton community structure which may have serious ecological impact.

Effect of pH on total biomass and phytoplankton communities in freshwater system.

Figure 3 and table 2 shows the effect of pH on *Chl-a* concentration in the freshwater system. Concentration of *Chl-a* gradually increased from acidic to neutral and reached at its maxima 9.35 $mg.m^{-3}$ at the pH of 8.15. Carotenoid signature revealed that in all the pH manipulated samples lutein concentration was much higher than zeaxanthin (average concentration of zeaxanthine was found to be 2.16 ± 0.57 $mg.m^{-3}$) indicating that green algae was dominating phytoplankton compared to cyanobacteria. In the control having pH 8.15 lutein: zeaxanthin ratio was 1.08. At more alkaline pH of 8.5, the ratio was not significantly different while in the pH range from 6 to 7.5, the average ratio

of lutein: zeaxanthin was 7.57 ± 0.18 (Average ± 2 SD). This result is completely contrasting from that of salinity manipulation where cyanobacteria always dominated the community. It appears that green algae inhabiting in the fresh river water system have evolved through the ages to tolerate wide fluctuation of pH ranges that is more common due to the poor buffering capacity of river bed clay and fresh water. However the same green algae could not tolerate even subtle changes in salinity as Alamuru station (from where the sampling was done) is still free of influence from tidal salt wedge.

There is some obvious enhancement in zeaxanthin concentration in the alkaline pH range which prompts us to consider the possible adaptability of estuarine cyanobacteria with the periodic tidal mixing with the alkaline seawater although their response was not as strong as observed in case of salt treated samples.

It is quite evident from this study that phytoplankton community is affected by sudden salinity changes. Complete exclusion of green algae in the salinity enhanced samples indicates that they are stenohaline sp having very narrow salt tolerance range. This is probably because of the fact that the green algal sp. present in the Godavari river estuary does not have potent osmoregulatory mechanism thus vulnerable to local extinction for subtle salinity increment. Cyanobacteria seem to be well adapted to withstand such salinity fluctuation as they are capable of enhancing the production of zeaxanthin to combat ROS. Consequently, cyanobacteria can completely replace green algae in freshwater because of intrusion of saline water in estuary. This shift in phytoplankton community (which is at or near the bottom of the food chain in the aquatic system) can have serious ecological impact. Cyanobacteria are well known for their nuisance bloom formation. There is no documentation of toxic bloom formation by cyanobacteria in Godavari river estuary. However, it is not possible to rule out possible consequence in the higher tropic level. Ultimate outcome could also vary in different estuaries of the world depending on the salinity tolerance level of the local phytoplankton and the extent of salt water intrusion.

On the other hand green algae seemed quite adaptable to pH fluctuation and are potentially more tolerant to any natural or anthropogenic pH change. Further studies are being carried out to understand the complex interaction of other biogeochemical factors with salinity and pH to shape the phytoplankton community structure.

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Captions and Legends for Figures

Figure 1 Geographical location of the study area

Figure 2. (a) Variation in *chl-a*, zeaxanthin and lutein concentrations obtained from phytoplankton in Godavari River at different salinity shock ; (b) Variation in zeaxanthin /*chl-a at* different salinity shock

Figure 3. (a) Variation in *chl-a*, zeaxanthin and lutein concentrations obtained from phytoplankton in Godavari River at different pH; (b) Variation in zeaxanthin /*chl-a* and lutein /*chl-a* ratios *at* different pH

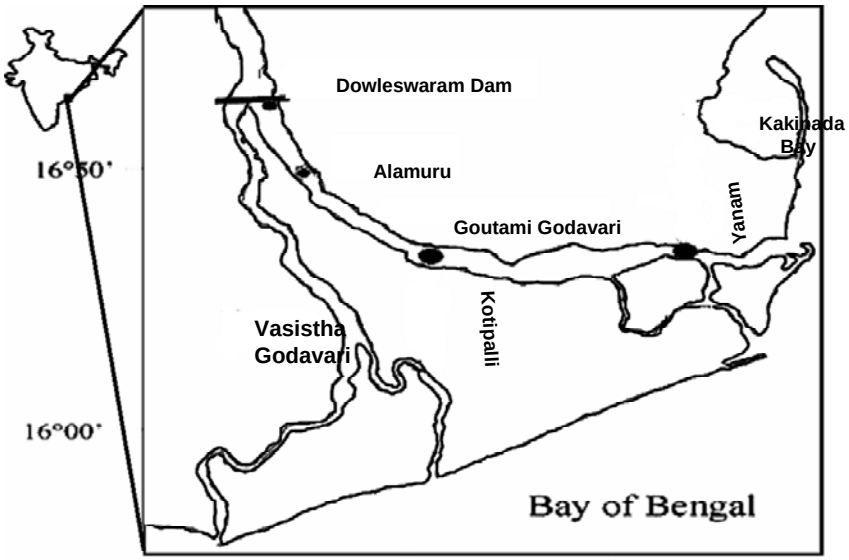
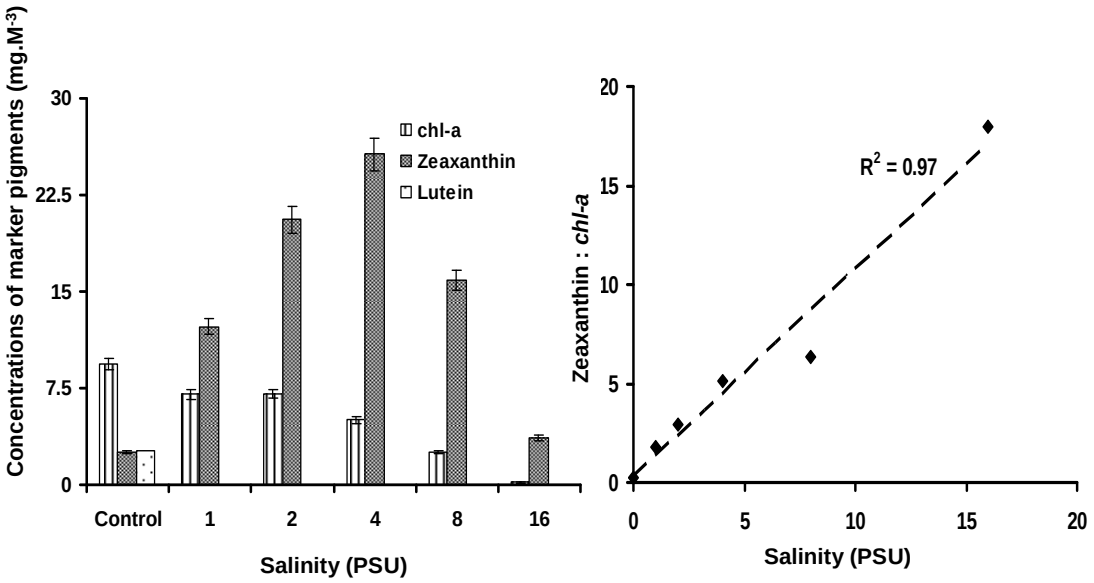

Dowleswaram Dam
Kakinada Bay
16°30'
Alamuru
Yanam
Goutami Godavari
Kotipalli
Vasistha Godavari
16°00'
Bay of Bengal

Figure 1


Concentrations of marker pigments (mg.M-3)
30
22.5
15
7.5
0
chl-a
Zeaxanthin
Lutein
Control
1
2
4
8
16
Salinity (PSU)
Zeaxanthin : chl-a
20
15
10
5
0
R2 = 0.97
0
5
10
15
20
Salinity (PSU)

(a) (b)

Figure 2

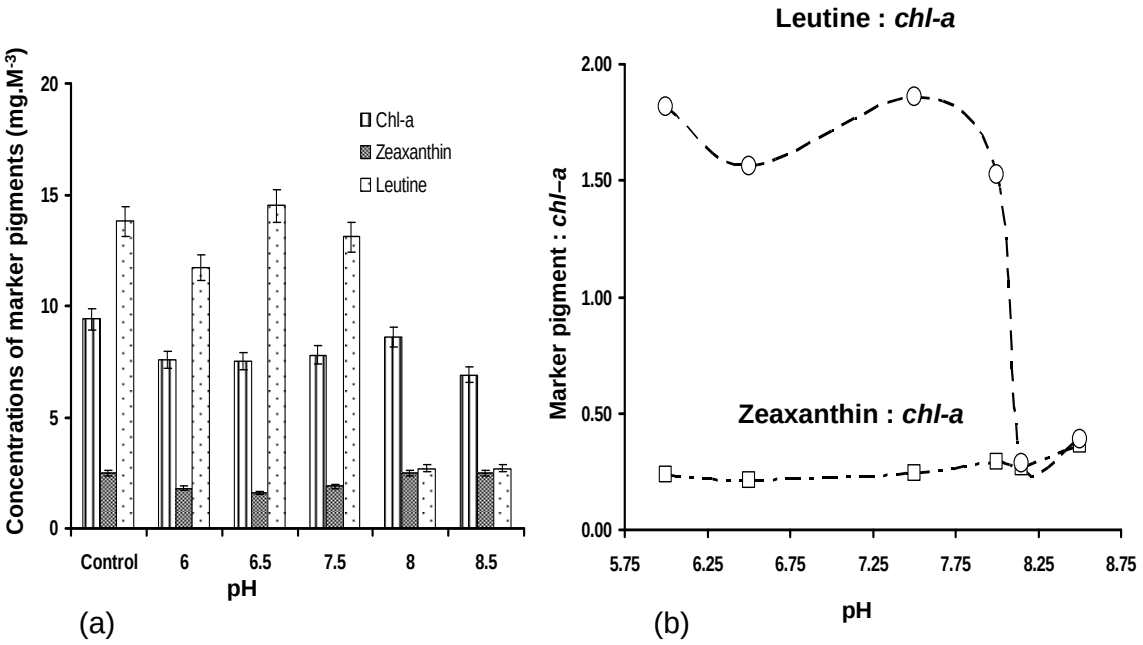

Concentrations of marker pigments (mg.M-3)
20
15
10
5
0
Chl-a
Zeaxanthin
Leutine
Control
6
6.5
7.5
8
8.5
pH
(a)
Leutine : chl-a
Marker pigment : chl–a
2.00
1.50
1.00
0.50
0.00
Zeaxanthin : chl-a
5.75
6.25
6.75
7.25
7.75
8.25
8.75
pH
(b)

Figure 3

Table 1. Variation in concentrations of *chl-a*, zeaxanthin, lutein and fucoxanthin obtained from phytoplankton in Godavari River with respect to the different salinity shock

Salinity (PSU) (manipulated by the adding NaCl)	*Chl-a* (mg. M^{-3})	Zeaxanthin (mg. M^{-3})	Zeaxanthin / *Chl-a*	Leutine (mg. M^{-3})	Leutine / *Chl-a*
Control	9.4 ± 0.3	2.5 ± 0.1	0.3	2.7±0.1	0.3
1	7.0 ± 0.2	12.3 ± 0.6	1.8	0.0	-
2	7.0 ± 0.2	20.6 ± 1.0	2.9	0.0	-
4	5.0 ± 0.3	25.6 ± 1.3	5.1	0.0	-
8	2.5 ± 0.1	15.9 ± 0.8	6.3	0.0	-
16	0.2 ± 0.0	3.6 ± 0.2	18.0	0.0	-
Salinity (PSU) (manipulated by the mixing freshwater and aged sea water)	***Chl-a*** **(mg. M^{-3})**	**Zeaxanthin (mg. M^{-3})**	**Zeaxanthin / *Chl-a***	**Leutin (mg. M^{-3})**	**Fucoxanthin (mg. M^{-3})**
4	4.8 ± 0.2	23.4 ± 1.2	4.9	0.0	0.5 ± 0.1
6	3.5 ± 0.2	10.2 ± 0.5	2.9	0.0	2.5 ± 0.1

Values presented as the mean ± 2 × standard deviation, n = 3
All the data are presented with 95.5% confidence interval.

Table 2. Variation in concentrations of *chl-a*, zeaxanthin, lutein and fucoxanthin obtained from phytoplankton in Godavari River with respect to the varying pH

pH	Chl-a (mg/M^3)	Zeaxanthin ($mg.M^{-3}$)	Zeaxanthin / *Chl-a*	Leutine ($mg.M^{-3}$)	Leutine/Chl-a
6.0	7.6 ± 0.4	1.8 ± 0.2	0.24	13.8 ± 0.7	1.82
6.5	7.5 ± 0.4	1.6 ± 0.1	0.21	11.7 ± 0.6	1.55
7.5	7.8 ± 0.4	1.9 ± 0.1	0.24	14.5 ± 0.7	1.86
8.0	8.6 ± 0.4	2.5 ± 0.2	0.29	13.1 ± 0.8	1.51
8.15 (Control)	9.4 ± 0.5	2.5 ± 0.2	0.27	2.7 ± 0.1	0.82
8.5	6.9 ± 0.3	2.5 ± 0.1	0.36	2.7 ± 0.2	0.38

Values presented as the mean ± 2 × standard deviation, n = 3

All the data are presented with 95.5% confidence interval.