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Phytoplankton assemblage and UV-protective compounds in the river Ganges

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Interactions between physico-chemical parameters such as pH, temperature, nitrate, phosphate, alkalinity, acidity, biological oxygen demand (BOD), chemical oxygen demand (COD) and solar ultraviolet radiation (UVR) strongly affect aquatic ecosystems. Due to fluctuations in several environmental factors including river water pollution and UVR, organisms are under constant threat. However, phytoplankton protects themselves from environmental extremes by adopting several defense strategies including synthesis of photoprotective compounds such as scytonemin and mycosporine-like amino acids (MAAs). We investigated the presence of scytonemin and MAAs in phytoplankton of some polluted sites of one of the holy rivers of the world, the Ganges at Varanasi, India. We observed phytoplankton assemblages and studied certain environmental parameters which could possibly affect phytoplankton diversity in the river. Phytoplankton consisted mainly of 49 taxa of 34 genera belonging to Bacillariophyceae, Chlorophyceae, Cyanophyceae, and Chrysophyceae. The members belonging to Bacillariophyceae and Chlorophyceae were the two dominant classes, which comprised up to 75% of the total phytoplankton. Photoprotective compounds were isolated and characterized from phytoplankton. Electrospray ionization-mass spectrometry (ESI-MS) analysis of MAAs showed the presence of shinorine, palythinol, mycosporine-glycine and palythine. A high concentration of scytonemin was also observed with an absorption maximum at 386 nm in the studied phytoplankton.

Keywords: Mycosporine-like amino acids, Phytoplankton, River Ganges, Scytonemin, Ultraviolet radiation

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The river Ganges drains one of the world's largest, continuously and densely occupied watersheds and is a lifeline for millions of population. It has very important role in the health, spirituality and prosperity of the millions living on the Northern plains of India. The river is of utmost importance for drinking, irrigation, power generation and religious pilgrimages. It has been heavily polluted due to deforestation, sewage and industrial waste, excessive use of pesticides and fertilizers and disposal of dead bodies at Varanasi. Nitrogen (N) and phosphorus (P) being the critical nutrients in the surface waters, influence the energy and matter fluxes which are fundamental to all ecosystem processes which control biomass, distribution and abundance of the organisms[1]. In the aquatic ecosystem, the structure and dynamics of the phytoplankton are mainly controlled by the physico-chemical parameters[2]. Changes in physico-chemical parameters substantially affect the species that live in the ecosystems[3]. Natural variability and anthropogenically induced global climate changes are modifying aquatic and terrestrial environments, which in turn affect the sustainable and optimum utilization of resources for the ever-increasing population of human beings[4].

Climate change which acts as multiple stressors leads to synergistic or cumulative impacts on aquatic environments. Contaminant influxes to aquatic ecosystems will be enhanced by an increase in temperature and alterations in precipitation patternand this will make aquatic organisms more susceptible to the effects of pollutants. The effect on biotic components will be synergistic and cumulative. Overall, this will result in bio-magnification and higher contaminant loads in aquatic ecosystems. Physico-chemical parameters and sediment delivery can be altered by human interference and hence, shift the population and diversity of microbes and degradation of ecosystems[5]. The functioning of microbes is highly sensitive to variation in physico-chemical parameters of sediments which predicts ecological integrity of rivers and helps in

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understanding the impact of several stresses on biodiversity and functioning of aquatic (freshwater) ecosystems[6,7]. In aquatic ecosystems, increased influx of solar ultraviolet radiation (UVR) (100-400 nm) due to the enhanced anthropogenically released atmospheric pollutants results in degradation of the Earth's ozone layer and this has aroused serious concern regarding the effect of increased UVR on aquatic ecosystems. UV-B (280-315 nm) carries <1% of total solar energy and can penetrate to significant depths in oceans and lakes[8].

Changes in UV regimes due to varying stratospheric ozone would produce cumulative effects on function and structure of aquatic ecosystem[9]. Aquatic organisms in the upper photic zone are exposed to solar UVR which can affect cellular structures and biomolecules and may interfere with certain biochemical and physiological metabolism/ responses such as orientation, motility and may block enzymatic reactions[10]. UV-B radiation alters biomolecules and induces production of reactive oxygen species (ROS) in the cell[11,12]. However, environmental changes also modulate the effects of UVR. Aquatic organisms/microorganisms have developed various protective mechanisms/strategies for nullifying the harmful/deleterious effects of UVR. Apart from various effective repair mechanisms, phytoplankton use UV-screening/absorbing biomolecules such as scytonemin and mycosporine-like amino acids (MAAs) to overcome damage induced by UVR to photosynthetic apparatus, DNA and proteins. Higher MAAs concentration in the phytoplankton under the ozone 'hole' showed comparatively lesser photosynthetic quantum yield inhibition by UV-B from those present outside the areas which are ozone-depleted, showing their protective potentials[13,14]. Many species produce sun screening pigments/ compounds that filter out UV wavelengths and MAAs and scytonemin are important class of such compounds[15]. For making sound decisions regarding future of marine ecosystems, global C cycle and production systems, understanding and prediction of effects of global climate change on the phytoplankton which constitute the basis of most aquatic food chains, is extremely important[16].

This study aims to evaluate phytoplankton response to the cumulative effects of solar UVR and physico-chemical factors on the biosynthesis of MAAs and scytonemin and their characterization from phytoplankton inhabiting some of the polluted sites of the river Ganges at Varanasi. This work will help in better understanding of the role of various environmental factors including pollution on the diversity of phytoplankton and biosynthesis of photoprotective compounds in phytoplankton inhabiting polluted sites of aquatic ecosystems. This is possibly the first study of its kind in Varanasi which is an ancient city having religiously important holy river, the Ganges.

Materials and Methods

Description of sampling site

The sampling site encompasses urban areas of Varanasi, which is situated in the Eastern Gangetic plain of Northern part of India (82°15'E-84°30'E and 24°35'N-25°30'N) (Fig. 1A). Total five sites, namely Assi Ghat, Jain Ghat, Bhadaini Ghat, and Shiwala Ghat were selected for river quality monitoring (Fig. 1B). These sites are closely situated within a range of 500 m and have almost similar environmental conditions. These Ghats represent polluted sites as here domestic sewage is being discharged in the river. During summer day mean temperature varied between 26.6ºC to 44.2ºC and relative humidity between 27% and 83%. In Varanasi, prominent sources of pollution in the river Ganges are domestic sewage, industrial effluents, agricultural/urban runoff, dead bodies' cremation, land-use change and atmospheric deposition.

Measurements

Water samples were collected between May-June 2016 and these months represent the extreme summer season in India. Sampling was done from the rivers during the course of study. Planktonic net of 10 µm mesh size (Plankton Sampling Kit, Fieldmaster®, Wildco) was used for collecting samples of phytoplankton and samples were preserved in formalin (4%) for further analysis. Five replicates were collected in plastic bottles for physicochemical parameters analysis between 10.00 AM-12.00 noon from the depth ranging between 15-25 cm. In summer, the temperature ranged between 29-46ºC. Standard protocols[17] were followed for sampling and processing of samples from sampling sites to the laboratory. Samples were brought back to the laboratory within 7 h of the collection in cold packs for further analysis.

Chemistry of water

Samples were collected from different sites from below the surface (15-25 cm) in plastic containers (acid rinsed) for analyzing NO_3^-, NH_4^+, PO_4^{3-}, dissolved

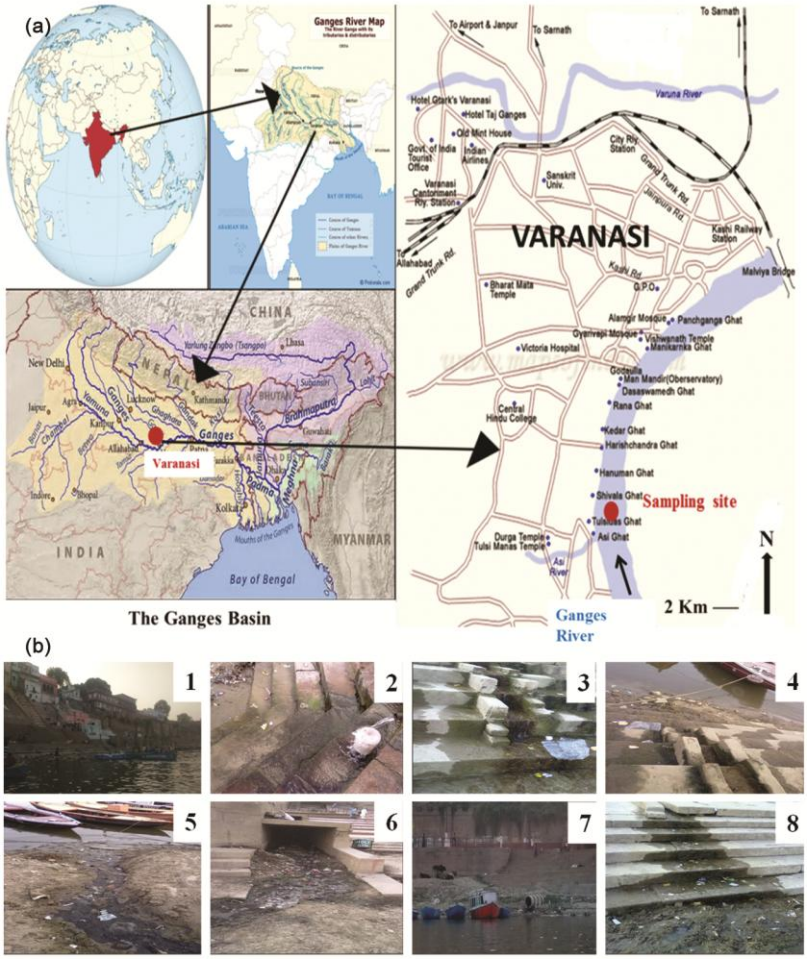

Fig. 1 — Selected study sites for Ganges river quality monitoring (A) Photographs of Ghats of Varanasi showing different polluted sites. Jain Ghat (1, 2) Bhadaini Ghat (3, 4) Shivala Ghat (5, 6, 7) and Assi Ghat (8).

organic carbon (DOC) and dissolved inorganic nitrogen (DIN) (NO_3^- + NH_4^+) using standard protocols[17]. Quantification of NO_3^--N was done using brucine sulphanilic acid method[18] and NH_4^+-N was determined by Nesseler's reagent[19]. Quantification of dissolved reactive phosphorus as orthophosphate was determined by ammonium molybdate method[20]. Estimation of DOC was done using $KMnO_4$ digestion method by mixing a known volume of filtered water with acidified N/80 $KMnO_4$ and samples were incubated at 37ºC[21]. After the collection of water samples, the temperature was measured with centigrade (0-110ºC) thermometer placed inside the water sampler. A portable pH meter was used for determining the pH of water samples and measurement was done immediately after the sampling for avoiding the chemical and biological reactions between the sample and atmosphere which could alter the pH[22]. Dissolved oxygen (DO) and biological oxygen demand (BOD) were determined by standard methods[17]. For analyzing DO, fixation of samples was done with the help of alkali-iodide-azide and $MnSO_4$ solution (2 mL each) and further analysis was donein the laboratory following Winkler's modified iodide-azide method. On bringing the samples (in iceboxes) to the laboratory, further analysis of chemical oxygen demand (by the dichromate reflux method using a Ferroin indicator), BOD (incubation at 25ºC for 5 days in the dark), acidity and alkalinity (by the potentiometric titration method with the help of a pH meter and diluted H_2SO_4 and NaOH) was done.

Biomass and composition of phytoplankton community

Measurement of phytoplankton biomass was done in terms of chlorophyll (Chl) *a* which comprises about 1.5% of dry organic matter of algae[17]. Extraction of Chl *a* was done in 90% acetone and quantification was done spectrophotometrically[19]. Identification of

phytoplankton was done morphologically through the stereo microscope (Labomed) and further examination of organisms was done using image binocular microscope. Photography of organisms was done using Dewinter-2011 scientific digital camera and Dewinter software was used for their analysis. Tentative identification of phytoplankton belonging to different classes was made with the help of standard reference books and published papers. Members of cyanophyceae were identified by taxonomic keys given by Desikachary[23] and Komárek *et al.*[30], bacillariophyceae were identified by Botes[26] and Sarode & Kamat[24]. The members belonging to chlorophyceae were identified by book written by Anand[25] Bellinger & Sigee[28], Munshi *et al.*[29] and Suthers & Rissik[30] whereas members of chrysophyceae were identified by Munshi *et al.*[29] and Suthers & Rissik[30].

Extraction of phycobiliproteins

Phycobiliproteins (PBPs) were extracted from phytoplankton following method of Sinha *et al*.[31]. Centrifugation was performed at 1500×g for 10 min at room temperature to harvest cells followed by washing them twice with 0.75 M potassium phosphate buffer (pH 7.0). Then the cells were resuspended in the minimum amount of the same buffer and 1 mM phenylmethanesulfonylfluoride (PMSF), 5% (w/v) sucrose and 10% (w/v) ethylenediaminetetraacetic acid (EDTA) were added. Disruption of cells was carried out with Mortar and Pestle and the resulting suspension was subjected to repeated cycles of freeze-thaw for extraction of PBPs. The debris was removed by centrifugation (15,000×g for 30 min) and the supernatant was subjected to spectrophotometric and fluorescence analysis.

Spectrophotometric and fluorescence analysis of phycobiliproteins

UV-Vis spectrophotometer (U-2910, 2 J1-0012, Hitachi, Tokyo, Japan; 1 cm of path length) was used for recording absorption values of PBPs. The data were analyzed by UV solution software provided by the manufacturer. Fluorescence spectrophotometer (G9800AA, Agilent Technologies, Cary Eclipse, USA) was utilized for recording fluorescence of PBPs subunits phycocyanin (PC) and phycoerythrin (PE) with excitation at 563 and 615 nm for PE and PC resulting in an emission at 575 and 642 nm, respectively.

Extraction and partial purification of UV screening compounds MAAs and scytonemin

Cells were harvested by centrifugation and 100% high performance liquid chromatography (HPLC)-grade methanol was used for extracting MAAs by incubating it overnight at 4°C. The aliquots were then centrifuged at 8000 g for 5 min. Supernatants, which were taken in fresh Eppendorf tubes were evaporated at 38°C by vacuum evaporator. Redissolved the residues in sterile double-distilled water (DDW) (600 µL) and added chloroform (100 µL) along with gentle vortexing. Centrifuged the mixture at 8000 g for 5 min and transferred the uppermost water phase (Photosynthetic pigment-free) carefully into fresh Eppendorf tubes and filtered the solution through sterilized syringe filters (0.2 µm pore-size) (Axiva Sichem Biotech., New Delhi)[32]. Extraction of scytonemin was done in methanol/ethyl acetate (1: 1, v/v) by incubating the samples overnight at 4°C followed by sonication (2011-Sonic, cycle 30%, Power 40%) for 4 min. Samples were centrifuged at 10000 g for 5 min and supernatants were evaporated at 38°C in a vacuum evaporator and redissolved in 1:1 (v/v) methanol:ethyl acetate (500 µL)[33]. Finally, the samples were filtered through sterilized syringe filters (0.2 µm pore-size) (Axiva Sichem Biotech., New Delhi) prior to the HPLC analysis.

These filtered samples were further subjected to HPLC analysis (Waters 2998, Photodiode Array, pump L-7100, USA). For HPLC, a reverse phase semi-preparative column was used which was equipped with a Licrospher RP 18 column and guard (5-µm packing; 250 mm×4 mm inside diameter).

HPLC and electrospray ionization-mass spectrometry (ESI-MS) analysis of UV protective compounds

Further analysis of partially purified UV-screening compounds (Scytonemin and MAAs) was done using the HPLC system. MAAs sample (50 µL) was injected into the column of the HPLC system by an autoinjector (Waters 717 plus). Acetic acid (HPLC grade) (0.02%) constituted the mobile phase which ran isocratically with a flow rate of 1.0 mL min^{-1}. The photodiode-array detection (PDA) scan wavelength ranged from 200-400 nm and detection wavelength for MAA was at 330 nm. HPLC Purified MAAs was subjected to ESI to produce protonated molecules. Amazon SL mass spectrometer (Bruker Daltonics Inc., Bremen, Germany) was used for recording mass spectra. Induction of $(M+H)^{1+}$ formation occurred at cone voltage of 30 V and had a mass range of 100-500 *m/z*. Based on *m/z* values and previously determined retention times, precursor ions were specifically selected for MS/MS analysis which had capillary voltage of 5,500 V and temperature of 300°C. Manual mode was selected for MS/MS and fragmentation of the precursor ion was carried out by

collision-induced dissociation utilizing He (collision gas) at 40 V. Isolation width of 2 u was selected for precursor ions and scans were accumulated with variable RF signal amplitudes[32]. Calibration of *m/z* scale of the mass spectrum was done through the external calibration standard electrospray 'tuning mix' from Agilent Technologies (Santa Rosa, USA). Analysis of data was done using the software DataAnalysis 4.0 (Bruker Daltonics Inc., Bremen, Germany).

For scytonemin, the flow rate of elution was maintained at a 1.5 mL min^{-1} using the mobile phase consisting of solvent A (ultra-purewater) and solvent B (acetonitrile–methanol-tetrahydrofuran, 75:15:10, v/v). Gradient elution programme of 30 min was set with a linear increase (0-15 min) from solvent A (15%) to solvent B (100%) and 15-30 min at 100% solvent B. In the HPLC column, samples (50 µL) were injected and the detection wavelength for scytonemin was at 380 nm with PDA scan wavelength ranging from 250-750 nm. Identification of scytonemin was done in the solvent through its characteristic absorption maxima and appropriate retention time[34,33].

Results

Physical and chemical parameters

Assessment of the physico-chemical parameters of the river system is crucial for studying the effect of certain parameters on the occurrence and distribution of different components of biodiversity in the river Ganges. As the selected sites were closely located and had almost similar environmental conditions no significant variation was observed in the different physical and chemical parameters of the water samples. The physico-chemical parameters of water have been given in Table 1. The temperature being the most crucial factor influences biological, chemical and physical characteristics of different water bodies including river systems. The present study showed that the mean temperature of the water was 32.5ºC during the summer season. A similar pattern was observed for the electric conductivity of samples. No significant difference was observed between the pH values of sampling sites and showed that the water samples were slightly alkaline ranging from 7.6-7.9 (7.78 ± 0.076). For the determination of the water quality of water bodies, the value of DO is remarkable. Organic decomposition and rates of respiration are high during summer and DO values remain low where the rate of photosynthesis is high. The mean value of the DO was found to be 3.07 ± 0.107 mg L^{-1}. During the periods of the present study, the average concentrations of NO_3^-, NH_4^+ and PO_4^{3-} were found to be 257.2±3.6 µg L^{-1}, 26.2±0.88 µg L^{-1} and 82.6±2.83 µg L^{-1}, respectively. These values were found to be high in May and June when river flow was found to be lowest. A significantly higher concentration of average BOD (7.50±0.312 mg L^{-1}) and COD (510.3±8.50 mg L^{-1}) were recorded as microbial and enzyme activities were markedly higher in summer. The mean value of DOC in the sample was found to be 7.7±0.2 mg L^{-1}. Alkalinity and acidity play an important role in the determination of water quality of water bodies. Alkalinity refers to the acid neutralizing ability of the water which gives primarily a function of bicarbonate, carbonate and hydroxide content and acidity of water refers to the quantitative abilityfor reaction with a strong base to a designated pH. A significantly higher mean alkalinity (406±8.50 $CaCO_3$ mg L^{-1}) and acidity (44.96±2.83 $CaCO_3$ mg L^{-1}) values were reported in the water sample.The electrical conductance tells about the total ions per cm and is a numerical expression of the capacity of the water sample for carrying an electric current. G values were significantly higher, 454.33±6.11 µmho cm^{-1}.

Biomass and composition of phytoplankton community

During the entire sampling period, the phytoplankton compositions were recorded in the river Ganges at Varanasi which was classified into four classes. These were Bacillariophyceae (20), Chlorophyceae (17), Cyanophyceae (11) and

Table 1 — Basic biological and physico-chemical parameters of the river Ganges, Varanasi, India

Parameters*	Value (mean ± SD)
Mean water temperature (°C)	32.46 ± 0.493
pH	7.78 ± 0.076
DO (mg L^{-1})	3.07 ± 0.107
BOD (mg L^{-1})	7.50 ± 0.312
COD (mg L^{-1})	510.3 ± 8.50
Nitrate (µg L^{-1})	257.2 ± 3.6
Phosphate (µg L^{-1})	82.6 ± 2.83
Alkalinity ($CaCO_3$ mg L^{-1})	406 ± 8.50
Acidity ($CaCO_3$ mg L^{-1})	44.96 ± 2.83
Ammonium (µg L^{-1})	26.2 ± 0.88
Electrical conductivity (µmho cm^{-1})	454.33 ± 6.11
DOC (mg L^{-1})	7.70 ± 0.20
TDS (mg L^{-1})	418 ± 11.59
Chl *a* (µg L^{-1})	35.7 ± 0.458
Maximum mean temperature (ºC)	39.4 ± 2.7443
Relative humidity (%)	52.4 ± 17.5373
UV-B (µWcm^{-2})	6.505 ± 1.2023
Ozone (DU)	276.4 ± 6.50

*Value is an average of different samples collected.

Chrysophyceae (1) cumulating into 49 taxa belonging to mainly 34 genera (Table 2).

The members belonging to Bacillariophyceae and Chlorophyceae were the two dominant classes, which comprised up to 75% of the total phytoplankton. The diatoms dominated the phytoplankton and that too essentially during the summer month only. Chlorophyceae constituted the second most dominant phytoplankton and cyanophyceae formed a sizeable portion of phytoplankton. About 20 different species of Bacillariophyceae were observed. The species which were found dominant were *Nitzschia* spp., *Navicula* spp., *Pinnularia* sp., *Achnanthes afflinis*, *Cocconeis* sp., *Fragilaria* spp., *Frustulia* sp., *Gomphonema* spp., *Cyclotella* spp., *Aulacoseira* sp. (syn. *Melosira* sp.), *Pleurosigma* sp., *Synedra* spp. and *Leptocylindrus danicus* (Fig. 2A).

Chlorophyceae was found to be the second most abundant group of phytoplankton during the course of study. Most diversity was found in Chlorophyceae which constituted 12 genera and 17 species and contributed about 21% of the total population of phytoplankton (Fig. 2B).

The most common members were *Chlamydomonas* sp., *Scenedesmus* spp., *Acutodesmus* spp., *Coelastrium indicum*, *Coenochloris* spp., *Pediastrum* spp., *Actinastrum* sp., *Chlorella vulgaris*, *Dictyosphaerium* sp. *Heynigia* sp., *Eudorina* sp. and *Crucigenia tetrapedia*. The Cyanophyceae contributed to about 22% to the total phytoplankton population in the river Ganges, Varanasi (Fig. 3A). A total of eleven species (*Chroococcus* sp., *Oscillatoria* sp., *Microcystis* sp., *Anabaena* sp., *Nostoc* sp., *Scytonema* sp., *Arthrospira* sp., *Phormidium* sp., *Planktolyngbya* sp., *Aphanocapsa* sp., *Merismopedia* sp.) were recorded. Chrysophyceae was represented by a single genus *Mallomonas* sp. and contributed to only 2% to the total phytoplankton population. Biomass of phytoplankton was measured in terms of Chl *a* which was found to be higher i.e., 35.7±0.458 µg L^{-1}.

Evaluation of phycobiliproteins

PBPs (PC and PE) were also investigated in phytoplankton samples. Absorption spectrum (Fig. 4A) of PBPs showed two prominent peaks at 562 and 615 nm. Fluorescence emission spectra of PC and PE also confirmed the peaks at 642 and 575 nm, respectively (Fig. 4B and Fig. 4C) in the phytoplankton samples.

Analysis and characterization of UV screening compounds

UV-absorbing/screening compounds/pigment MAAs and scytonemin were extracted, partially purified and characterized from phytoplankton. Figure 3B shows the absorption spectrum of methanolic extract of phytoplankton, in which prominent peaks of MAAs (334 nm), Chl *a* (430 nm and 664 nm), carotenoids (473 nm) and biliproteins (616 nm) were observed.

The presence of MAAs was confirmed by HPLC chromatogram of the aqueous solutions and their corresponding absorption maxima. Prominent peaks at 1.25, 2.15, 2.96 and 3.63 min were recorded in HPLC analysis of partially purified MAAs (Fig. 5A and Fig. 5B) with UV absorption maxima (UVλ_{max}) at 334, 332.2, 309.6 and 320 nm, which were identified as shinorine (λ_{max} = 334 nm; Fig. 6A), palythinol (λ_{max}

Table 2 — Major group of phytoplankton community belonging to different classes collected from the river Ganges at Varanasi.

Bacillariophyceae	Chlorophyceae	Cyanophyceae	Chrysophyceae
Nitzschia diversa	*Chlamydomonas* sp.	*Anabaena* sp.	*Mallomonas* sp.
Nitzschia denticula	*Scenedesmus incrassatulus*	*Nostoc* sp.	
Nitzschia holsatica	*Scenedesmus quadricanda*	*Scytonema* sp.	
Navicula confervacea	*Scenedesmus* sp.	*Arthrospira* sp.	
Navicula radiosa	*Acutodesmus* sp.	*Phormidium* sp.	
Pinnularia sp.	*Acutodesmus* sp.	*Planktolyngbya* sp.	
Cocconeis sp.	*Coelastrium indicum*	*Chroococcus* sp.	
Achnanthes afflinis	*Coenochloris* sp.	*Oscillatoria* sp.	
Fragilaria pinnata	*Coenochloris fottii*	*Microcystis* sp.	
Fragilaria arcus	*Pediastrum simplex*	*Aphanocapsa* sp.	
Frustulia rhomboides	*Pediastrum duplex*	*Merismopedia* sp.	
Gomphonema geminatum	*Actinastrum* sp.		
Gomphonema longiceps	*Chlorella vulgaris*		
Cyclotella meneghiniana	*Dictyosphaerium* sp. *Heynigia* sp.		
Cyclotella kuetzingiana	*Eudorina* sp.		
Aulacoseira sp. (syn. *Melosira* sp.)	*Crucigenia tetrapedia*		
Pleurosigma sp.			
Synedra ulna			
Synedra sp.			
Leptocylindrus danicus			

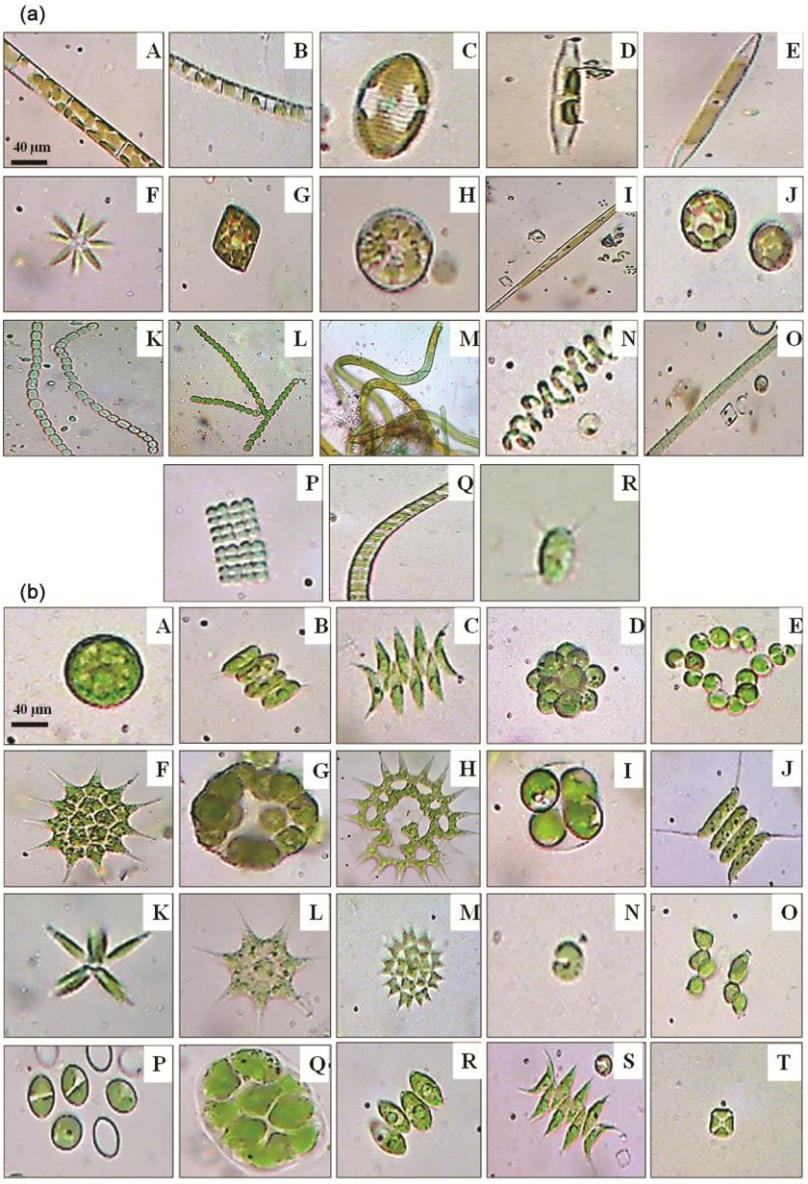

Fig. 2(A) — Microphotographs of phytoplankton belonging to Bacillariophyceae (A-J) (A) *Leptocylindrus danicus* (B) *Aulacoseira* sp. (C) *Navicula* sp. (D) *Nitzschia* sp. (E) *Fragilaria* sp. (F) *Nitzschia holsatica* (G) *Cyclotella* sp. (H) *Cyclotella meneghiniana* (I) *Pleurosigma* sp. (J) *Cyclotella* sp.; Cyanophyceae (K-Q) (K) *Anabaena* sp. (L) *Nostoc* sp. (M) *Scytonema* sp. (N) *Arthrospira* sp. (O) *Phormidium* sp. (P) *Merismopedia* sp. (Q) *Planktolyngbya* sp.; Chrysophyceae (R) *Mallomonas* sp. (B) — Microphotographs of phytoplankton belonging to Chlorophyceae (A-T) (A) *Chlamydomonas* sp. (B) *Scenedesmus incrassatulus* (C) *Acutodesmus* sp. (D) *Coelastrium indicum* (E) *Coenochloris* sp. (F) *Pediastrum* sp. (G) *Coenochloris* sp. (H) *Pediastrum simplex* (I) *Coenochloris fottii* (J) *Scenedesmus quadricanda* (K) *Actinastrum* sp. (L) *Pediastrum* sp. (M) *Pediastrum duplex* (N) *Chlorella vulgaris* (O) *Dictyosphaerium* sp. (P) *Heynigia* sp. (Q) *Eudorina* sp. (R) *Scenedesmus* sp. (S) *Acutodesmus* sp. (T) *Crucigeniate trapedia.*

=332.2 nm; Fig. 6B), mycosporine-glycine ((λ_{max} = 309.6 nm; Fig. 6C) and palythine (λ_{max} = 320 nm; Fig. 6D), respectively.

We observed only major fractions of MAAs having UVλ_{max} at 334 nm and 332.2 nm and 309.6 nm which were collected for carrying out ESI-MS analysis which revealed a prominent ion peak of protonated molecule ($[M^+ H]^+$) *m/z* value of 333.3, 303.5 ig. 7A) and 246.4 (Fig. 7B) revealed the identity of shinorine, palythinol and mycosporine-glycine, respectively.

In contrast to MAAs, scytonemin was found in high concentration in the sampled phytoplankton. HPLC chromatograms of extracted scytonemin from phytoplankton showed a prominent peak at the retention time of 1.90 min (Fig. 8A) with an absorption maximum at 386 nm (Fig. 8B).

Discussion

The present study aims to assess phytoplankton distribution and analysis along withthe characterization of photoprotective biomolecules such as MAAs and scytonemin from selected sites of the river Ganges, India. Phytoplankton in their natural habitats such as in river systemare exposed to UVR coming in the solar radiation along with other abiotic stresses such as temperature, pH, NO_3^-, NH_4^+, BOD, COD, alkalinity, acidity, etc. which affect their community structure (density, diversity, and occurrence) and productivity[35]. Results from this study showed enhanced values of Physico-chemical parameters (temperature, NO_3^-, PO_4^{3-}, DOC, acidity, alkalinity, etc.) and the presence of photoprotective compounds MAAs and scytonemin from the samples.

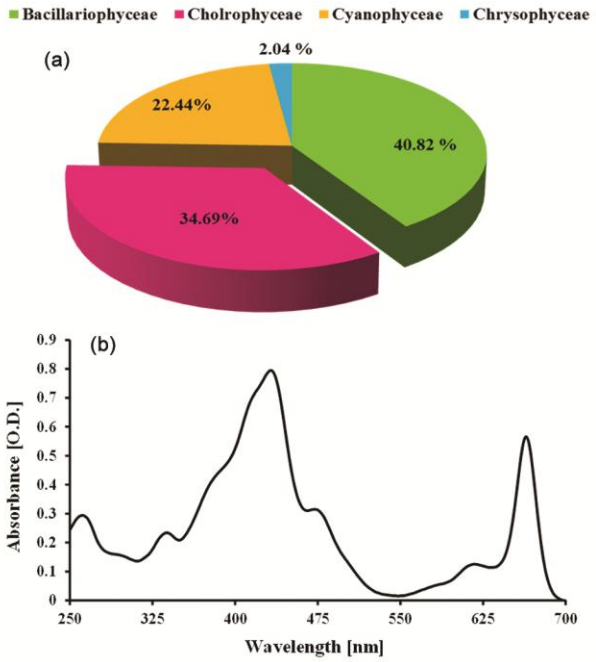

Fig. 3(A) — Diversity of phytoplankton belonging to different groups in the river Ganges at Varanasi (B) Absorption spectrum of phytoplankton (methanolic extract) exhibiting the peaks of mycosporine-like amino acid (334 nm), Chl *a* (430 nm and 664 nm), carotenoids (473 nm) and biliproteins (616 nm).

Another major factor of concern in any aquatic ecosystem is "pollution" in different forms. The holy river the Ganges is highly polluted in the plains and Varanasi being one of the most populated cities of eastern Uttar Pradesh is the major source of domestic sewage in the Ganges. As per reports of CPCB (2013)[36], approximately 46 MLD of untreated and 141 MLD of treated sewage are flushed into the river Ganges from Varanasi city. An increased concentration of sewage gets discharged in the river during May and June[37]. Hence, for our study, we have selected those Ghats where domestic sewage was being discharged in the river, which represent the nutrient-enriched condition as indicated by enhanced values of certain physico-chemical parameters.

Prediction of the distributional pattern of phytoplankton communities will be affected in the future due to multifaceted simultaneous alterations in the environmental conditions of their habitats, posing a bigchallenge to the scientific community and it is vital for prediction of function and services of such

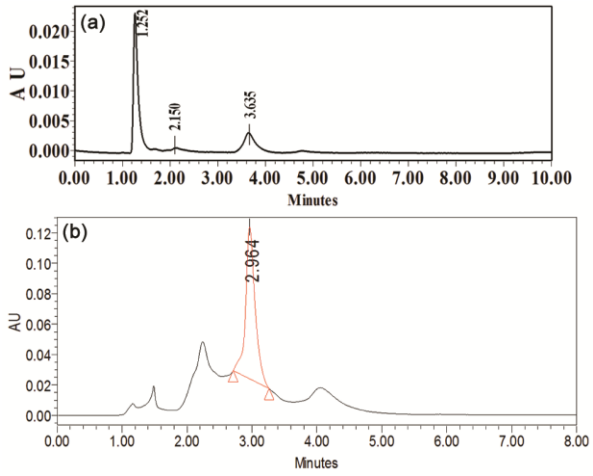

Fig. 5 — Partially purified MAAs from phytoplankton inhabiting the Ganges. HPLC chromatogram showing the typical peak of MAA at retention times of (A) 1.26 min (Shinorine), 2.12 min (Palythine) and 3.64 min (Palythinol) and (B) 2.96 min (Mycosporine-glycine).

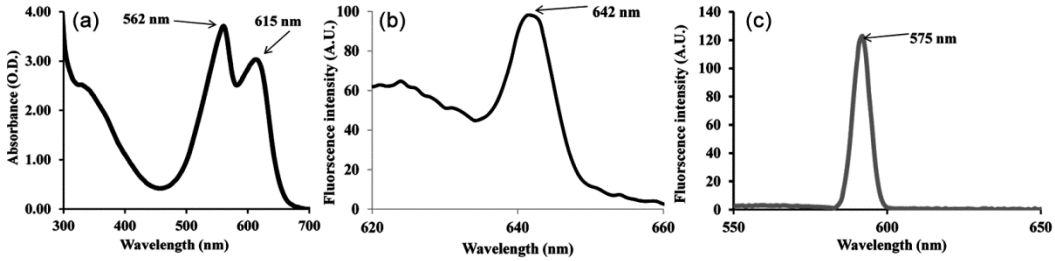

Fig. 4 — Absorption spectrum (A) and Emission spectra of PC (B) and PE (C) of PBPs respectively of phytoplankton collected from the river Ganges at Varanasi.

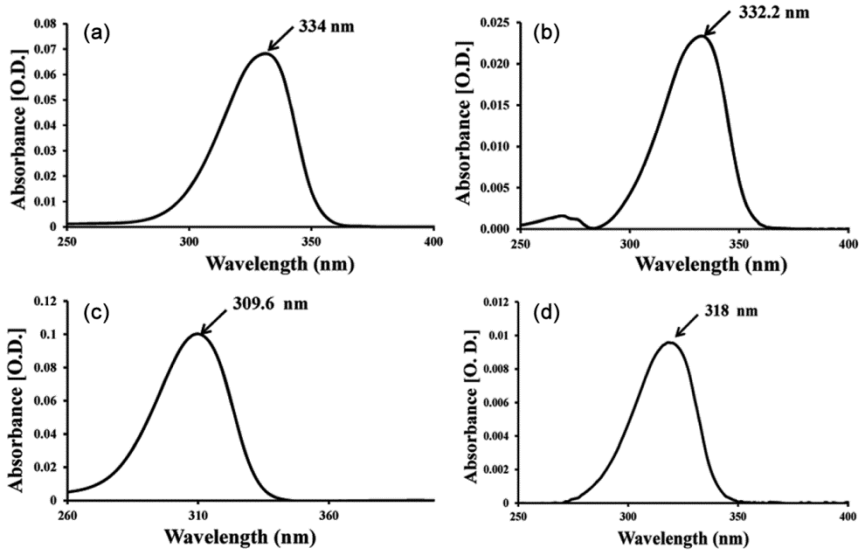

Fig. 6 — Absorption spectra of partially purified MAAs from phytoplankton separated by HPLC (A) Shinorine (B) Palythinol (C) Mycosporine-glycine and (D) Palythine.

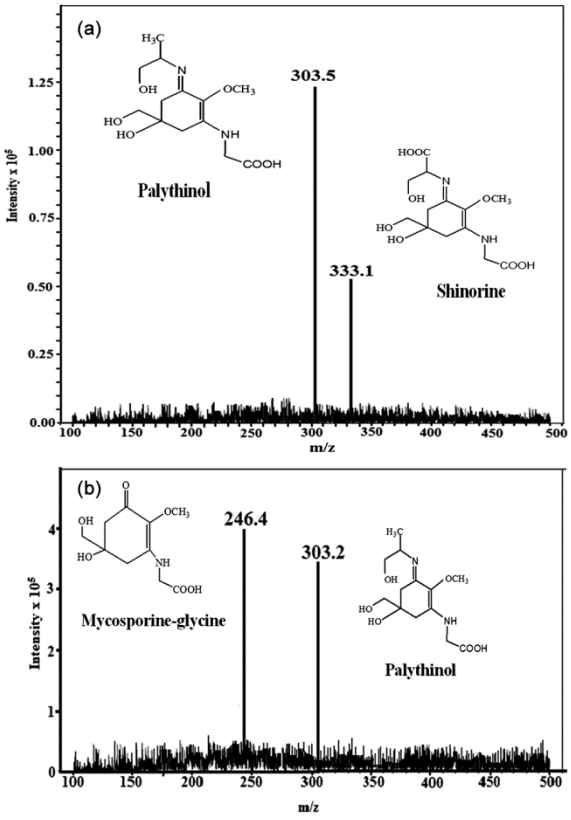

Fig. 7 — Electrospray ionization-mass spectra showing peaks with *m/z* values of 303.5 and 333.3 (A), 246.4 and 303.2 (B) of the HPLC purified MAAs confirming the purified MAAs as Palythinol, Shinorine and Mycosporine glycine.

ecosystems and their conservation[38,39]. Recently, for making advancements in this field several research directions and approaches have been suggested. These approaches would help in increasing the predictive power including the collection of experimental data of major functional traits on phytoplankton such as responses related to growth and production on nutrient enrichment, solar radiation including UVR, ocean acidification, warming and combination of different traits with distributional patterns along different environmental gradients[38]. The most revealing part of the bioindicator-based methods for identification of the ecological health of aquatic systems is the algal/cyanobacterial species richness, abundance, diversity,and biomass which we have tried to accessin the present study[40,41].

We found that in the selected sites of the river Gange's phytoplankton community were dominated by Bacillariophyceae followed by Chlorophyceae and Cyanophyceae which might be correlated to their ability to synthesize UV-absorbing compounds such as MAAs and scytonemin conferring them additional tolerance towards multiple stress factors present at their habitats.

The MAA profile of phytoplankton has been reported to contain MAAs i.e., shinorine, mycosporine-glycine, palythinol and palythine. HPLC-purified MAAs, when subjected to ESI-MS, revealed an ion peak of ($[M + H]^+$) at *m/z* 333.3, 303.5 and 246.4, which was in accordance with the previous results of ESI-MS analysis for MAAs in phytoplankton[42]. The compounds were identified as shinorine (λ_{max} = 334 nm; *m/z* 333.3), palythinol (λ_{max} = 332.2 nm; *m/z* 303.5), mycosporine-glycine ((λ_{max} = 309.6 nm; *m/z* 246.4) and palythine (λ_{max} = 320 nm), respectively.

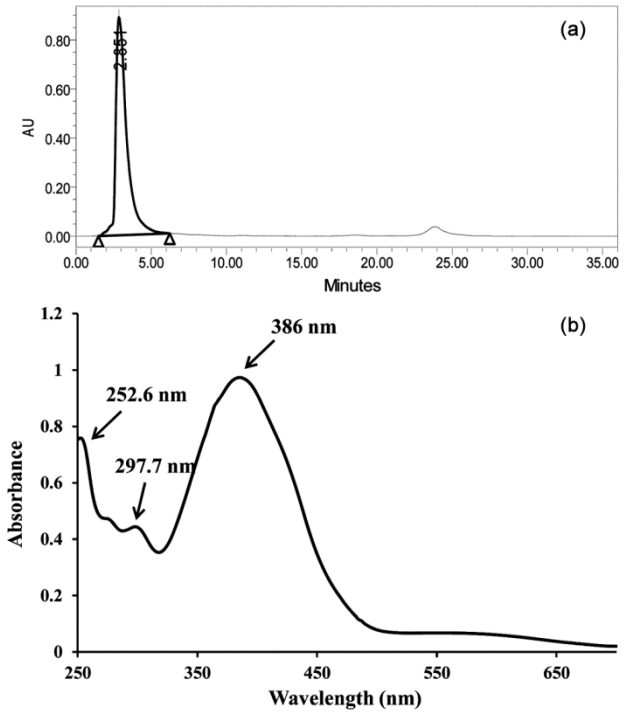

Fig. 8(A) — HPLC chromatogram of scytonemin, partially purified from phytoplankton inhabiting the river Ganges at Varanasi, showing the typical peak at retention time of 2.85 min (B) Absorption spectrum of purified scytonemin from phytoplankton showing maximum absorbance at 386 ± 2 nm.

Table 3 — MAAs identified in phytoplankton inhabiting the river Ganges.

MAA	Retention time (min)	λ_{max} (nm)	*m/z*
Shinorine	1.252	334	333.3
Mycosporine-glycine	2.964	309.6	246.4
Palythinol	3.635	332.2	303.5
Palythine	2.150	318	-

Palythine was not detected in ESI-MS analysis (Table 3). This is the first report for the occurrence of four MAAs i.e., shinorine, palythinol, mycosporine-glycine as well as palythine in the phytoplankton inhabiting the river Ganges, Varanasi. In contrast to MAAs, a significantly higher amount of scytonemin was observed in the studied phytoplankton. HPLC chromatograms of extracted scytonemin revealed the presence of a prominent peak at the retention time of 2.85 min having anabsorption maximum at 384 ± 2 nm, which was found to be consistent with the earlier findings[43,44].

Temperature is another crucial factor which affects the biological, physical and chemical characteristics of aquatic systems. Photosynthesis, enzymatic efficiency, and other metabolic activities are regulated by ambient temperatures[45]. Increasing temperature enhances metabolic activities/functions in phytoplankton and it was found that it may help in overcoming UVB-inflicted damages by accelerating the repair strategies/mechanisms in the cells[10]. It was found that the structure of the phytoplankton community changed, when there was an increase in temperature from 16.5°C to 22.5°C[46].

NH_4^+ and BOD are important indicators for inferring the magnitude and nature of wastewater/ domestic sewage released[47]. In the present study, values of NH_4^+ and BOD were found to be exceeded by 26.2 µg L^{-1} and 7.50 mg L^{-1} in the river. High values of NH_4^+, NO_3^- and BOD during the low flow of river suggests that during the dry season of May and June, water quality in the river Ganges is controlled by the anthropogenic factors and among various factors point sources such as sewage/domestic water release is the prominent one. The case of DOCs, however, could be opposite because during summer its concentration becomes low[48] and in this study, the concentration was 7.7±0.2 mg L^{-1}. DOCs play very important role in water bodies by absorbing incoming UV-B and UV-A radiation. In phytoplankton, nutrient uptake is affected by UVR and it was found that solar UV-B reduces biomass of phytoplankton in marine ecosystems[49]. The total Chl *a* content of phytoplankton is frequently employed for predicting biomass of phytoplankton[50]. This means the Chl *a* content could serve as a reliable indicator/parameter of phytoplankton biomass, hence, in the present study we have expressed biomass of phytoplankton in terms of Chl *a* content. The requirements of photosynthetically active radiation for photosynthesis also expose the phytoplankton to the detrimental solar UVR. Constant exposure to UVR has compelled the phytoplankton to evolve various defensive mechanisms for overcoming the UV-inflicted damage during their course of evolution. Common UV mitigating strategies found in phytoplankton inhabiting water bodies are crust formation, vertical migration and the production of UV-screening compounds such as MAAs and scytonemin[51].

The present study indicates that the studied phytoplankton may protect themselves from deleterious effects UVR along with other environmental stressors by synthesizing compounds such as MAAs and scytonemin. These compounds serve as antioxidants and preventdamage to cells from UV-induced formation of ROS[52,53]. Only a few studies were available on the phytoplankton abundance/ diversity in the river Ganges at Varanasi, especially

nearby the polluted sites. Anand and Chaudhary[54] studied phenological diversity of chlorophyceae from the river Ganges at Varanasi. Phytoplankton biomass in the changing state of N: P: Si stoichiometry in the river Ganges was studied by Pandey and Yadav[1]. None of the previous studies have focused on UV-protective compounds from phytoplankton in the river Ganges. In different species of phytoplankton, the mitigating strategies for protection can differ, hence, UVR can be considered as an important driv er for species competition in phytoplankton community[11]. Consequently, all these environmental and anthropogenic factors may interact antagonistically and synergistically for altering physiological responses as compared to that of an individual stressor. Our understanding of how different stressors such as UVR, temperature, pH, NO_3^-, NH_4^+ and PO_4^{3-}, BOD, COD, and DO interact under different environmental conditions or in different regions is still limited, hence, proper knowledge of the mechanisms of combined effects of UVR and other stressors on the structure and functions of phytoplankton communities of water bodies is required.

Conclusion

Climate change impacts related to alterations in UVR regimes and other environmental interactions resulting fromclimate change are projected to have significant synergistic and/or cumulative effects on the function and structure of freshwater ecosystems. Workers continue to find out the loop holes in utilizing specific biomonitors for predictingtrophic status and water quality of river ecosystems. Co-occurrence of multiple interrelated environmental factors, climatic differences, hydrology, variable human controls and connectivity with other domains are major challenges for bio-monitoring of surface water over large spatial scales as these factors affect the performance of specific bio-monitoring tools. This study is the first to report the MAAs and scytonemin profiles of phytoplankton inhabiting the river Ganges which is exposed to various environmental factors including domestic sewage discharge. HPLC-PDA and ESI-MS analysis revealed the occurrence of four different MAAs, shinorine, mycosporine-glycine, palythinol and palythine, as well as indolic-phenolic biomolecule scytonemin, the UV screening pigment located in the cyanobacterial sheath, in the phytoplankton. This paper summarizes the interactive effect of various environmental factors on the sensitivity of phytoplankton in terms of the phytoplankton community, biomassand biosynthesis of UV protective compounds MAAs and scytonemin and their characterization using various biochemical techniques. Synthesis and accumulation of UV screening compounds in phytoplankton may serve asa possible mechanism of protection from UV-induced damage along with different environmental stresses.

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Conflict of Interests

Authors declare no conflict of interest.

Author Contributions

HA performed the experiments, analyzed the results, and wrote the MS. JP and Rajneesh helped in performing the experiments, writing and critically reviewing the MS. DKS helped in the sampling. AP, VS, DK helped in performing the experiments and statistical analysis. RPS designed the experiments, helped in data analysis, provided laboratory facilities and reviewed the manuscript.

Authors declaration

We declare that all images are original and not published anywhere.

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