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In situ assessment of metal toxicity in riverine periphytic algae as a tool for biomonitoring of fluvial ecosystems

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Abstract

The combined effects of seasonality and in situ heavy metal (Cu, Zn, Pb) enrichment on periphyton in a large river was experimentally studied using metal-diffusing substrates. Highest release rate and intracellular accumulation of metal ions (Cu, Zn and Pb) was examined in rainy and summer seasons, respectively. Release rate and intracellular accumulation showed the following order: Zn>Cu>Pb in the three seasons. Periphytic algae growing on these substrates showed intracellular accumulation of test metals (Cu, Zn and Pb) which inhibited growth in a concentration dependent manner, as evidenced by reduced cell number, species richness and Shannon index of the community. Concentration dependent decrease in Chlorophyll *a* and rise in Caro./Chl *a* ratio was found under Cu and Zn stress. Periphytic community in summer and

winter was found similar than rainy season and was found be dominated by diatoms followed by green and cyanobacteria. In comparison to green algae and cyanobacteria, diatom species, showed relatively higher % relative abundance under heavy metal stress than their respective control. Increased lipid body production and cell wall deformities in diatoms were found under Cu and Zn stress but not under Pb stress, possibly because Pb diffusion from the substrates was low. After 28 days of diffusion, metal release rates were near the control levels and periphyton parameters had recovered, demonstrating rapid response to changing conditions by the periphyton. The rapid recovery of the periphyton (within 14 days) indicates that periphyton biomonitoring may be useful for not only monitoring of stable conditions but also for changing conditions, such as monitoring recovery. The present study shows the utility of metal diffusing substrate and periphytic diatom community as an effective tool for biomonitoring (including recovery) of heavy metal pollution in the fluvial ecosystem.

1. Introduction

Metal toxicity to periphyton has been often studied in polluted habitats, like acid mine drainage, metal smelter waste waters, and metal contaminated waterbodies (Morin et al., 2008a, b; Pandey et al., 2016). In many of these studies, carried out mostly on fluvial systems, periphyton of metal impacted stations have been compared with those from the reference (unpolluted) station to get an insight into how metal pollutants affect this particular community as also how the community recovers after a certain distance downstream. Artificial attachment sites have been often used so as to provide uniform substrates for overcoming variability in natural substrates found in a waterbody. Glass slides, with rough surface to encourage periphytic colonization, has been the common choice of periphyton ecologists (Weitzel, 1979). Periphytic species richness, diversity, similarity and even multivariate tools have often been related with metal concentration in water

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(Pandey et al., 2016, 2017). Nevertheless, such studies do not give us an idea about how periphyton of an unpolluted or pristine aquatic system would respond in case of metal enrichment. Another disadvantage is that one cannot be certain if the observed effect is due to metal or some other environmental perturbation. For instance, flow conditions in running water environments are highly variable and hence can have tremendous impact on algal periphyton (Ghosh and Gaur, 1994): if the control and the metal impacted sites do not have identical flow regimes, we cannot compare periphytic algae of two such sites to derive plausible conclusions with regard to heavy metal toxicity.

Efforts have been made to study metal toxicity to periphyton in laboratory streams or outdoor channels (Serra et al., 2009; Pandey et al., 2015). One of the major advantages of such an approach is ease in experimentation. Another advantage is that the researcher is sure about the observed effects being elicited due to metal because appropriate controls are always considered. However, environmental conditions in the laboratory or outdoor channels are vastly different from those occurring in natural bodies of water. This is one of the serious limitations of the artificial channels.

Now we are confronted with the question as to how can we study the effect of a toxicant or nutrient on periphyton in situ in a river or lake. Enriching the entire body of water with the test chemical is not an environment-friendly proposition as it may disturb the ecology of the system. Incidentally, this has been done to prove the critical role of phosphorus in triggering cyanobacterial blooms and eutrophication (Schindler, 1974). A novel way of in situ studying effect of nutrients (such as, nitrogen and phosphorus) on periphyton was developed in the form of nutrient diffusing porous clay substrates (Ghosh and Gaur, 1994; Scott et al., 2009). In this kind of set up, there is no need to add nutrients to waterbody; rather nutrients diffusing out of porous clay substrate can be directly taken up by periphyton growing onto them and this may facilitate studying the effect of in situ nutrient enrichment on periphyton.

Prompted by earlier studies on nutrient diffusing substrates, as mentioned above and the work of Arnegard et al. (1998), a novel metal diffusing clay substrate was prepared (Pandey et al., 2014; Pandey and Bergey, 2018) and used to in situ study the effect of zinc, copper and lead on periphyton community of a river. The study was carried out in summer, rainy and winter seasons. For this purpose, artificial substrates were filled with metal solutions to assess their effects on periphyton. The artificial substrate could substantially release metal ions from its porous surface for studying the effect of metal toxicity to periphytic community.

2. Materials and methods

Study area

The study was carried out in the river Ganga at Varanasi (25° 18´ N and and 83° 1´ E; 82 m above m.s.l.). The study area lies in the Indo-Gangetic plains and is characterized by tropical climate greatly influenced by monsoon. During the course of the study, the atmospheric temperature was very harsh during winter (November to February; 6-24 ^{0}C during the coldest month January) and summer (March to June; 29-45^{0}C during the hottest month May); rainy season (end of June to October) was hot (33–43^{0}C during July) and humid. The annual total rainfall of Varanasi is ~1100 mm. Several sites were surveyed to find out one which is relatively not much influenced by human activities and where artificial substrates can be placed without any incident of vandals disturbing them. Another criterion for the selection of site was approachability and ease in sampling. Keeping all these points in mind, a small stretch of the river was identified near the campus of the Banaras Hindu University, in Garhwa ghat (Ramna) area, which is very close to the new bridge spanning the river Ganga. The site selected for the

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study lies before the main city and hence it is relatively free of pollution. At the study area, the width and depth of the river vary seasonally, attaining their maxima during the rainy season. At the place where the substrates were kept, the river was about 2 m deep. The bottom of the river is sandy.

Nutrient diffusing clay substrates have been used to in situ assess nitrogen and phosphorus enrichment effect on periphyton of rivers and lakes (Ludwig et al., 2008, Scott et al., 2009). Inspired by these reports, a metal diffusing substrate (MDS) was developed to assess the effect of metal enrichment on river periphyton (Fig. 1). Each MDS was made by fixing a circular porous clay tile (fired in brick kiln; diameter 14 cm and thickness 4 mm) to the wide mouth (diameter 13.5 cm) of a plastic funnel (capacity 670 ml) using an epoxy resin (m-seal; Pidilite Industries, Daman, India). Solutions of copper ($CuCl_2.2H_2O$), zinc ($ZnCl_2.5H_2O$) and lead ($PbCl_2$) were prepared in Milli-Q water using their analytical grade salts (Rankem, India). These solutions had 1(low), 2.5 (medium) and 5 (high) g l^{-1} concentrations of each test metal. Metal solution was filled in MDS through the open end of the funnel, which was subsequently closed with a replaceable rubber cork. Metal ions diffused out from numerous tiny pores on the surface of clay tiles when placed in water. The pattern of release of the test metal ions from MDS was determined in triplicate by keeping them in the river. MDS, filled with the selected concentrations of the metal solutions, were placed in the river, as described below, for estimating the rate of release of metal ions. Every week, MDS were sampled and the concentration of metal ion remaining in the solution was measured using an atomic absorption spectrophotometer (PerkinElmer, AAnalyst 800). These data were used to calculate the rate of release of the test metal ions from MDS.

Two liters of river water sample were collected at weekly interval in plastic bottles. Collected water was carried to the laboratory within 30 minutes and refrigerated. Physicochemical parameters such as pH (Hanna pHep® tester), conductivity (Milwaukee stainless steel probe) and temperature were directly measured in the river. The flow rate was measured by measuring the time taken by a thermocol float to travel a specified distance. The methods used for various analyses are described below. Nitrate- and nitrite-nitrogen, total phosphorus and dissolved silica was estimated by the methods given in Wetzel and Likens (1979). 50 mL subsample from the collected water was centrifuged at 4500 rpm for estimating metal concentration by using ICP-optical emission spectrometry (ICP-OES, GemCone™ nebulizer, dual-view optical system, Perkin-Elmer, Optima 7300 V, USA).

The experiment was carried out during three seasons: summer (May-June, 2010), rainy (August-September 2010) and winter (November-December 2010). Three replicates were considered for each treatment and the control. MDS filled with metal solution of concentrations specified above were placed 15 m away from the bank of the river Ganga with their clay tile surfaces lying horizontally about 2 cm below the water surface (Fig. 1). MDS were mounted on bamboo frames that were fixed in the river with the help of bamboos buried vertically deep in the river sediment. The bamboo frames were placed parallel to the direction of river flow because it ensured similar flow conditions for all MDS.

Collection and analysis of river water

Collection and study of periphyton

Sampling of periphyton was done 7, 14, 21 and 28 days after MDS were placed in the river by scraping ~ 40 cm^2 area of the colonized porous tile (each time a fresh area). Periphyton samples were collected in glass centrifuge tubes with the help of a blade and stiff brush. Collected samples were taken to the laboratory in ice-packed boxes within 30 min. In the laboratory, these samples were divided into two parts (10 ml each). One part (10 ml) was further divided into two parts (5 ml each), of which one part were quickly subjected to 90% acetone treatment for pigment extraction while the remaining 5 ml was fixed with 4% formaldehyde for identification and enumeration. The second part of the periphytic sample (10 ml) was used for estimating the intracellular accumulation of the test metals.

Fresh periphytic samples were also microscopically examined for identification of algae and cyanobacteria often at 450x magnification; higher magnification was used as and when required. Diatom frustules were examined after cleaning. Permanent slides of diatom frustules were prepared as per Pandey et al (2018). The method involved treating periphytic algal samples with 90% acetone and leaving it for one day for removing cytoplasmic content. Subsequently, the samples were dried and treated with concentrated H_2SO_4 till white fumes stopped emanating from the samples. Thereafter, samples were treated with hydrogen peroxide for 30 min, and then washed thoroughly with deionized water. After drying, samples were mounted in Pleurax mounting medium (Refractive index 1.73) onto glass slides for microscopic examination.

In the periphyton, individual diatom cells were identified as per Algaebase, AIDI (Pandey et al., 2016) and ANSP (2012) algal image database. In addition, monographs on diatoms by Patrick and Reimer (1966, 1975) and a manual on stream periphyton by Biggs and Kilroy (2000) were also consulted. Coccoid green algae were identified from Phillipose (1959). Cyanobacteria were identified from the monograph of Desikachary (1959).

Algal cells were counted to estimate densities with a Spencer's haemocytometer at 450x magnification in 1 ml (n=3) of composite periphyton specimens. Cell density data was used for examining biovolume estimation of the periphytic diatom community as well as for calculating

Journal Pre-proof

% relative abundance of different algal classes and dominant algal (diatoms, green algae and cyanobacteria) taxa under control and heavy metal stress.

The amount of chlorophyll *a* and carotenoids in periphytic samples were determined by extracting these pigments in a 90% alkaline acetone solution, measuring spectroscopic light absorbance (Ultrospec 2100 Pro UV/Visible spectrophotometer, Amersham Biosciences, UK) at 665 and 430 nm wavelengths and converting these measurements into biomass using the trichromatic equations by Wetzel and Likens (1979). Chl *a* content of periphyton was used as an estimate of the total biomass of the community. The ratio of carotenoids and Chl *a* was determined, as this may indicate heavy metal stress.

Protocols given by Pandey et al. (2014) was followed for estimating the intracellular concentration of Cu, Zn and Pb. Periphytic algal samples were washed with 2mM EDTA solution for 10 min and digested with concentrated HNO_3, H_2O_2 (80 %) and deionized water in 1:1:3 ratios on a hot plate at 90 °C (Bates et al., 1982). The residue was dissolved in 2% (v/v) nitric acid and the final volume adjusted to 10 ml before measuring metal concentration with an atomic absorption spectrophotometer (Perkin-Elmer, AAnalyst 800).

The number and volume of lipid bodies (LBs) were determined in 50 cells of a taxon as per Pandey et al. (2015). Deformities (DFs) in diatom frustules were examined (in 500 frustules) in fresh as well as through their permanent slide at 1000 x magnification as per Falasco et al. (2009) and Pandey et al. (2014).

Statistical analysis

The Shannon index, species richness and Jaccard's index of periphytic diatom community was estimated using “PAST” software (Natural History Museum, University of Oslo) (Hammer et al.,

Journal Pre-proof

Journal Pre-proof

2001). Data were statistically analyzed by one-way analysis of variance (ANOVA) followed by Tukey's HSD test for comparing various means.

3. Results

Physico-chemical characteristics of river water

Table S1 shows some important physicochemical characteristics of river water during three time periods. The current velocity showed tremendous fluctuations; it was maximum during the rainy season, but summer and winter season showed relatively lower current velocities. The rainy season not only had the highest flow rate, it also showed much greater fluctuations in current velocity. Insofar as the pH of the water is concerned, it was slightly above 7 in a majority of cases; no clear cut seasonal pattern was discernible. The conductivity was maximum during the summer season in comparison to the other two times of sampling. Nitrate-nitrogen occurred at higher concentration during summer in comparison to the other two seasons of sampling. Total phosporus concentration also varied seasonally. It was the maximum during the rainy season followed in decreasing order by winter and summer. Dissolved silica content was around 10 mg l^{-1} in most of the cases, but was maximum during the rainy season. The table makes it amply clear that low concentrations of heavy metals did not vary much in the river water.

Metal release rate from MDS and intracellular metal content

The rate of release of metal ions from MDS was studied in relation to time, and the data are presented in Figs. 2, S1 and S2. The rate of release of copper ions was a function of its concentration in MDS (Fig 2). Greater the concentration of metal ion inside MDS, greater was the rate of release of the test metal ion from MDS. Another important pattern has been the effect of time on the release of metal ions. The release of copper occurred very rapidly during the first week and then it gradually declined with the passage of time and considerably lower rates were seen in the third and the fourth week of sampling (Fig. 2). The initial curve was steeper at the highest considered concentration of copper in MDS. Another important pattern evident in the experiment has been marked influence of season on the rate of release of copper ions. It was the maximum during the rainy season followed in decreasing order by summer and winter seasons. The rate of release of zinc could be found in Figure S1. the general pattern of the rate of release was similar to that of copper. It was maximum during rainy season and there was a clear-cut effect of the time duration on the rate of release. The release rate of lead was low relative to the release rates for copper and zinc (Fig. S2); however, the general pattern showed the effect of concentration and time.

Intracellular accumulation of the test metals by the algal periphyton was also studied and the data could be found in Figs. 1, S1 and S2. Intracellular concentration of copper increased concomitantly with increase in concentration of copper in MDS (Fig. 1). It was maximum at the highest tested concentration of copper and the lowest at the lowest tested concentration in MDS. In almost all the cases, the intracellular level of copper attained maxima in the first week, and thereafter it declined at all the tested concentrations of copper in MDS. Insofar as the effect of season is concerned, maximum intracellular copper accumulation occurred in the summer season and the minimum during the rainy and winter seasons. The intracellular accumulation of zinc also followed a pattern (Fig. S1) which broadly matched with that obtained for copper. It deserves mention that zinc was intracellularly accumulated much more in comparison to copper and lead. There was clear-cut effect of metal concentration in MDS and time. The periphytic community accumulated lower intracellular concentrations of lead than concentrations of copper

Journal Pre-proof

Journal Pre-proof

and zinc and this lower level of lead is consistent with the lower release rate of lead from the MDS (compare Fig. S2 with Figs. 1 and S1).

Periphytic community structure

Periphytic growths could be seen on clay substrates placed in the river (Fig. 3). Colonization of algae onto clay tiles started with the appearance of yellowish-cottony patches intermixed with a few green patches which persisted for a time period of two weeks. The general pattern of colonization remained similar in the control and metal-filled MDS. Periphytic biomass on clay tiles at any point of time was a function of immigration, growth and loss due to emigration or other factors. The periphytic colonization on the clay tiles was found quickest in the summer season followed by winter and was the slowest during the rainy season. After two weeks of colonization, green patches became more marked overshadowing the yellowish cottony patches in the third week. In the fourth week all the substrates were covered with periphytic growths of various colours ranging from yellow to green, blue green and brown.

After substrates were placed in the river, periphytic colonization was initiated by a mixture of adnate diatoms and small coccoid green algae. Colonization in the control (without metal) was visible to the naked eye after two days while MDS filled with metal solution, depending on the concentration of metal, took a slightly longer time to attain the same stage. The initiation of algal colonization was well marked by interspersed yellowish-green circular patches of algae on the clay surface of MDS. Besides diatoms, filamentous green algae were also visible with stalked and non-stalked diatoms colonizing MDS surface. Unicellular coccoidal green algae were also seen in the periphytic algal community along with a few cyanobacterial cells. Periphytic communities of summer, winter and rainy seasons were compared using Jaccard's similarity coefficient, and cluster dendrograms were prepared (Fig. S3). The figure makes it clear that the community of the rainy season was vastly different from that of the summer and winter seasons. Summer and winter communities showed great resemblance with each other.

Fig. 4 depicts the cell density and total biovolume of the periphytic community during the three seasons. Initially, during the first week of colonization, the periphytic community showed a relatively smaller number of cells, which later on followed an increasing trend in the subsequent weeks attaining the maximum value in the fourth week of the experiment. Accrual of algal periphyton was the lowest during the rainy season and the maximum during the summer in the case of the control substrates. The cell density was the highest in the case of the control substrates but declined in the case of periphyton developing on the metal-filled MDS. Whereas copper and zinc caused substantial toxic effects, lead was the least toxic. There was a distinct concentration-dependent effect of the test metals on the cell density as also the total biovolume of the periphytic community. Maximum inhibition of the cell density and total biovolume occurred at the highest tested concentration of the metal, and the minimum at the lowest tested concentration. It is interesting to note that the effect of metal concentration was particularly marked in the first two week of colonization. However, it became less prominent during the latter part of the experiment. In almost all the cases, especially from the third week onwards, the cell number and biovolume of the periphyton of the control and various metal treatments do not appear to be much different from each other. This was not the case with lead, as the communities developing on lead diffusing substrates resembled with the control from the second week onwards. Fig. 4 also reveals that the inhibitory effect of the test metals on the two parameters as lesser during the rainy season in comparison to summer and winter seasons.

The periphytic assemblage developing on the control and metal-diffusing substrates comprised individuals belonging to three major groups: Bacillariophyta (diatoms), Cyanophyta (which are now preferred to be called as Cyanobacteria) and Chlorophyta (green algae) (Table S2). Fig. 5 shows relative abundance of these groups in the assemblage after 7 and 28 days of metal exposure, as also in the control assemblage for the same time period. The members of Bacillariophyta dominated the periphytic assemblage in the control as well as various treatments. Cyanobacteria became particularly abundant during the summer season. Green algae and cyanobacteria became less abundant during the rainy season; on the other hand, diatoms were most abundant (relative abundance > 80%) during this time period. Cyanobacteria responded in a definite manner to metal enrichment. The relative abundance of cyanobacteria declined in metal-filled MDS. The declination was concentration-dependent; it was greater at the highest tested concentration of various test metals. It was particularly marked in the case of the one week old community. Although the decline in cyanobacterial relative abundance by metal treatment was not marked after 28 day exposure to the test metals, the relative contribution of cyanobacteria was the greatest in the last week (28 days) of the experimental period in the case of the control also. In a majority of cases, in the control and various treatments, the relative abundance of diatoms slightly decreased during the later part of the experiment. During the initial stage of the experiment (7 days), the relative proportion of green algae was slightly increased on MDS releasing metal ions. However, it became almost similar to that of the control during the later part of the experiment.

Species richness and Shannon diversity of the control and metal-exposed periphytic community could be found in Fig. 6. The data have been presented for two time periods, i.e., 7th and 28th day, for the three seasons because presenting all the data was considered cumbersome and not necessary. Species richness was maximum in the case of the community growing on the control substrates. The control substrates showed variations in species richness in the three

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seasons. It was the minimum during the rainy season and the maximum in the summer. Species richness of the community declined with increase in the level of metal enrichment in each of the season. This particular effect was evident in the first week. In the case of the 28-day old periphytic community developing on metal diffusing substrates, the effect of concentration was not marked as the communities developing at various concentrations of a test metal and the control had almost similar species richness. The declination of species richness was substantial in the case of copper and zinc, but very mild in the case of lead exposed communities.

Species diversity also showed a pattern which showed decline, in comparison to the control, dependent on the concentration of metal being released. Like species richness, Shannon diversity also declined during the rainy season. The concentration-dependent decline in species diversity was not marked in 28-day old periphytic community. Hence, the general pattern for species diversity and species richness closely match each other.

Figs. S4 to S6 show relative abundance of common taxa in the 14-day old community encountered on the control and various metal treatments during the three seasons. Fig. S4 shows that the relative abundance of *Achnanthes exigua* was slightly enhanced by metal enrichment; higher the concentration of metal, greater was the increase in relative proportion of this particular species. The response was not metal specific; it was rather concentration dependent. *Achnanthidium minutissimum* relative abundance increased following increase in metal concentration. *Cymbella cymbriformis* showed mixed response. Its relative abundance initially increased following low copper and zinc enrichment; however, decrease could be observed subsequently. Lead could not elicit this kind of effect. The relative abundance of *Fragilaria capucina* also increased with increase in the level of the test metals released by the substrates. Lead was the least toxic to this particular diatom species.

Journal Pre-proof

Gomphonema parvulum was another diatom whose relative abundance increased following metal treatment (except Pb exposure) (Fig. S5). In the case of copper and zinc releasing substrates, the increase was evident only at the lowest tested concentration of these test metals. Its relative abundance was very low during the rainy season and moreover metal enrichment further reduced it. *Navicula recens* showed great sensitivity to the test metals. Its relative contribution declined with increase in concentration of metal being released by the substrates. *Nitzschia linearis* was another diatom that showed tolerance to the test metals as its relative abundance increased with increase in the amount of metal ions released by MDS. *Pinnularia conica* completely disappeared from the community during the rainy season, although it was present at other times. This diatom also showed some tolerance to copper and zinc as evident by enhancement of its relative abundance. However, lead did not exert significant on its relative abundance.

Ulnaria ulna, another common large sized diatom species, showed a pattern (Fig. S6) broadly resembling that of other metal sensitive species. Its relative abundance consistently and gradually declined with increase in concentration of metal in the medium. *Chlorella vulgaris* responded positively, although slightly to metal enrichment as was evident by increase in its relative abundance especially during winter and summer months. The relative abundance of this alga during the rainy season did not show any specific pattern; it remained almost similar but considerably lower than at other time periods. The relative abundance of *Scenedesmus quadricauda* fluctuated slightly, but it increased in several instances of metal treatment. *Chroococcus limneticus* was slightly enhanced by low concentrations of metal ions; but higher concentrations of the test metals were inhibitory. Its relative abundance was greatly decreased in all the cases, including control, during the rainy season.

Journal Pre-proof

Pigments and oil globules

The amount of chlorophyll *a* and the ratio of carotenoids to chlorophyll *a* of the periphytic community are presented in Table 1. The table makes it amply clear that metal stress (except Pb exposure) reduced chlorophyll *a* content of the periphytic community. The reduction was strictly concentration dependent. Zinc and Cu caused marked inhibition of chl *a* content of the community; however, Pb did not affect chlorophyll content of the community. Insofar as the ratio of carotenoids to chlorophyll *a* is concerned, both Cu and Zn brought about its enhancement which was dependent on the concentration of metal being released by the substrate. The greater the concentration of metal, the higher was the ratio of chlorophyll *a*: carotenoids. Lead did not bring about appreciable change in the ratio of these two pigments.

In diatom species, oil globules (also referred to as lipid droplets) were observed (Fig. 7); they were fewer in the case of the control. However, their number and size (% biovolume relative to that of the entire cell) substantially increased under Cu stress (Fig. 7; Table S3). This phenomenon was regularly observed in various diatom species under Cu and Zn stress during the three seasons; however, a regular and consistent trend in relation to the concentration of metal in MDS was observed only in eight of them, namely, *Achnanthes exigua*, *Navicula gregaria*, *Navicula recens*, *Nitzschia amphibia*, *Nitzschia palea*, *Fragilaria capucina*, *Pinnularia conica* and *Ulnaria ulna* (Table S3). In many of the species listed in the table, the number of oil globules did not change considerably. However, tremendous fluctuations in biovolume of oil globules occurred under metal stress. It is particularly interesting to note that the relative contribution of oil globules to total cellular biovolume was as much as 70% in some cases of metal treatment of *Achnanthes exigua*. At the other extreme is *Ulnaria ulna*; the control cells did not show any oil globule, but metal treatment triggered the formation of small oil globules.

Nitzschia linearis also did not show any oil globule in the control; however, they did appear under copper stress. Lead was not efficient in inducing the formation of oil globules.

Deformities

Deformities in diatoms frustules were examined in the control and metal treatments in the three seasons (Table 2). In the three seasons, in comparison to the control, higher % deformed frustules were examined under Cu, Zn and Pb treatments, which was also found to be concentration dependent . However, under Cu and Zn treatments % deformities were more prevalent than under Pb treatments.

4. Discussion

Prompted by earlier studies on nutrient diffusing substrates (Fairchild and Lowe, 1984; Pringle and Bowers, 1984) and the preliminary work of Arnegard et al. (1998), a unique metal diffusing porous clay substrate was developed which was cheap and ensured in situ study of the effect of metal enrichment on periphyton (Pandey and Bergey, 2018). The substrate could very well release metal ions throughout the period of the experiment; the rate of release was very high in the beginning owing to large difference in concentration of metal inside MDS and water which facilitated rapid diffusion of metal ions from the clay surface. But the diffusion of metal ions from the porous clay surface considerably slowed down with the passage of time. This seems to have primarily happened due to lowering of concentration of metal inside, although clogging of some of the pores on the clay surface cannot be ruled out. A similar pattern of release of nutrient ions has been earlier observed by researchers who deployed chemical diffusing substrates in natural waters to study the effects of nitrogen and phosphorus enrichment on periphytic algal communities (Scott et al., 2009). Of the three metals selected for the study, Cu and Zn could easily diffuse out of the substrate, whereas Pb showed an extremely low rate of diffusion. This

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variability may be related with greater effective radius of divalent ionic form of Pb (119 pm) than that of Cu (73 pm) or Zn (74 pm). The rate of release of Cu, Zn and Pb ions from MDS increased with their concentration in the MDS. The rate of release was the greatest during the rainy season coinciding with the maximum current velocity during this period. Apparently, high current velocity quickly swept away metal ions which were released by the substrate so as to facilitate the release more of them from the surface during the rainy season.

Periphytic algae were able to colonize the substrate following a pattern which broadly resembled with that obtained by other researchers (Steinman and McIntire, 1990; Pandey et al., 2014). Metal diffusing substrates showed a similar pattern of colonization to control substrates; however, the pace of colonization was slower on metal exposed substrates. The test metals released by the substrates were taken up and intracellularly accumulated by periphytic organisms. The rate of release of metal ions was maximum in the first week and therefore greatest intracellular accumulation was registered during the first week. The release of metal ions slowed down in subsequent weeks and this led to reduced transport of metal ions into the cell. Concurrently, multiplication of periphytic cells also diluted the intracellular metal content with the passage of time. The present observations showing intracellular accumulation of high concentrations of the test metals by the periphytic community are in consonance with several earlier researchers. For instance, Duong et al. (2008) and Morin et al. (2008a) noted accumulation of high concentrations of cadmium by periphytic biofilm growing in metal enriched environment. Similarly, Pandey et al. (2014) reported high intracellular accumulation of heavy metals in the periphytic diatom community colonised on metal diffusing substrates deployed in the river Ganges. Pandey and Bergey (2016) also found high build up of heavy metals (Cu and Zn) in the periphytic diatom communities collected from metalliferous sites of Rajasthan, India.

It has been pointed out by several researchers that metal content of biofilm can serve as a reliable parameter for biomonitoring of metal concentration in water. (Newman et al., 1985; Morin et al., 2008a). In most of the previous studies, metal content in water has been related with total metal accumulated by periphytic biomass. This approach may not be very appealing because in metal toxicity studies it is the intracellular metal which is responsible for toxic effects. Behra et al. (2002) related metal content in water with intracellular metal content in periphytic biomass. The present data are also based on intracellular metal content. In the present study, the intracellular metal content, although increased initially in the first week, declined in the later part of the experiment. This relates very well to concomitant decrease in the release of metal ion by MDS. Arini et al. (2012a) similarly noted decline in metal content of metal-loaded periphytic biofilm that were transferred from metal contaminated to the reference site. In a mesocosm study also Morin et al. (2008b) could establish correlation between cadmium accumulation and dissolved cadmium concentration.

The present study showed deleterious effects of the test metals on periphytic community. The cell density and biovolume of the community followed a decreasing trend with increase in concentration of metal being released by the clay substrates. Even chlorophyll *a* content per unit area showed a metal concentration-dependent decreasing trend. The present observations are in agreement with previous studies on metal toxicity to algal communities. For instance, Nirmala Kumari et al. (1991) found algal abundance to be inversely related with concentration of metal ions in a river system. Furthermore, Hill et al. (2000) noted decrease in concentration of chlorophyll *a* following increase in metal level in a river impacted by mining operations. Zn and

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Cd elicited more deleterious impact on chlorophyll a content of younger film than the older one (Ivorra et al., 2000). Many other workers have also reported reduced periphytic biomass at elevated concentrations of metals in stream mesocosms (Sigmon et al., 1977; Hedtke, 1984). Conversely, de la Peña and Barreiro (2009) found pollutant load to be weakly related with chlorophyll, and not at all related with the biomass. Metal pollution could not significantly alter total standing crop of the phytoplankton community of a river (Montiero et al., 1995). Whitton and Kelly (1995) and Stevenson and Pan (1999) considered biomass to be an undependable criterion for the assessment of water quality. Notwithstanding various arguments made by previous researchers, as mentioned above, the biomass parameters clearly reflected metal concentration in the present study. However, some variation of results may be due to the development of a periphyton community that is tolerant to the local metals concentration (i.e., due to a change in species composition).

In the case of the periphytic assemblage developing on the control substrates, cyanobacteria were the most abundant during summer, which is characterized by high temperature and light intensity. This observation agrees well with several previous reports of high temperatures and optimal growth of cyanobacteria (Robarts and Zohary, 1987; Paerl, 1988). In fact, cyanobacterial blooms have frequently been encountered in summer from many parts of the world (Paerl, 1988). In the present study, the organisms belonging to cyanobacteria showed greater sensitivity to the test metals compared to organisms belonging to other groups. On the other hand, green algae slightly increased in relative abundance following metal enrichment. The apparent increase in green algae may have been an artifact of using relative abundance because of reductions in other groups and less impact on green algae (but possibly no actual increase). Diatoms as a group showed intermediate response. Hence the present observations agree well with Singh and Rai (1990) who noted the following order of Zn sensitivity to phytoplankton: Cyanophyta > Chlorophyta > Bacillariophyta. Takamura et al. (1989) also observed greater sensitivity of cyanobacteria to Cu, Cd and Zn, whereas green algae were relatively tolerant to the test metals. Nirmala Kumari et al. (1991) in a study of phytoplankton of metal contaminated river reported the following order of metal tolerance in algal groups: Chlorophyceae > Bacillariophyceae > Cyanophyceae > Euglenophyceae. Whitton (1970) and Foster (1982) also observed green algae, especially the members of Ulotrichales, remarkably tolerant to heavy metals. On the contrary, Corcoll et al. (2012) found cyanobacteria to be tolerant to zinc in a microcosm experiment, and Ivorra et al. (2000) and Kumar et al. (2012) also noted great tolerance of cyanobacterial mats to metal pollution. Genter et al. (1987) noticed greater tolerance to Zn in green algae than in diatoms and shifts in algal community from diatoms to filamentous green algae and then to unicellular green algae with elevation of concentration of zinc.

Development of tolerant communities may also explain some of the differences in results among studies. Major changes to community were reductions in species richness and diversity (Shannon index). The effect showed concentration dependence. This obviously happened due to disappearance or reduction in relative abundance of metal sensitive species from the community. Niyogi et al. (2002) also reported a negative relationship between diatom diversity and stress imposed by acid mine drainage. de la Peña and Barreiro (2009) studied the impact of abandoned mine drainage on water quality and periphyton of nearby streams. Severely polluted sites had diatom assemblages with lowered richness. Morin et al. (2008a, b) were able to relate Shannon diversity index with the extent of pollution. Arini et al. (2012a, b) observed reduction of species diversity of diatoms. On the contrary, Hirst et al. (2002) studied variations in metal concentration and diatoms in 51 streams located in metal-mining areas of Wales and Cornwall, U.K. but could

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not observe significant relationship between species diversity, species richness and evenness with metal concentration in streams. Similarly, de la Peña and Barreiro (2009) observed higher species diversity at moderately polluted site in comparison to the reference site. However, a majority of earlier workers have reported decrease in number of species and reduced diversity of metal exposed algal communities (Sharipova et al., 2007; Pandey et al., 2014, 2015; Pandey and Bergey, 2016).

As already pointed out above, metal enrichment had tremendous impact on the components of periphytic community; some species showed tolerance whereas others were sensitive to the stress. *Achnanthidium minutissimum*, one of the abundant species in the community, showed remarkable tolerance to the test metals. Its relative abundance increased with increasing release of the test metals from the substrate. *A. minutissimum* is one of the few species that colonizes a bare surface and is considered to be a good indicator of habitats disturbed by physical extremes or by pollutants (Rott, 1991). A majority of earlier studies show relative abundance of this species at elevated concentrations of metals in water (Cattaneo et al., 2004; Luís et al., 2011; Arini et al., 2012b; Cantonati et al., 2014). Yoshiaki et al. (2004) showed increased relative abundance of *A. minutissimum* with rising concentrations of copper, lead and zinc, and concluded that it was tolerant to metals due perhaps to its smaller size. However, Luís et al (2009) considered it to be neutrophilous and avoided high metal concentrations. On the other hand, a few researchers believe it to be sensitive to heavy metals (Sabater, 2000; Blanck et al., 2003). *A. minutissimum* can tolerate large variations in pH (Verb and Vis, 2000; Luis et al., 2011) and grows well at high current velocities (Duncan and Blinn, 1989). Interestingly, Sgro et al. (2007) regarded this diatom as an indicator of clean water. It has been pointed out that *A. minutissimum* and other small diatoms, firmly attached to the substrate, are often entrapped in organic matrix which may impede the passage of ions, including metal ions, into the frustule thereby protecting them from metal toxicity (Burkholder et al., 1990). In the same context, it needs to be mentioned that *A. minutissimum* is a species complex, which showed morphological and ecological variations (Potapova and Hamilton, 2007), as a result of which differences in metal tolerance might also occur. *Achnanathes exigua* was another diatom which was found tolerant to the test metals. Ruggiu et al. (1998) studied diatom remains in cores taken out from sediments of a subarctic Italian lake and found *Achnanthes* spp. to be tolerant to metal pollution. Hill et al. (2000) also observed the dominance of *Achnanthes* at a river site most impacted by mining operations. *Cymbella cymbriformis* showed tolerance to low and medium tested concentrations of the test metals. Roch et al. (1985) found it to be common in a lake impacted by mining activities.

Fragilaria capucina was another diatom slightly tolerant to the test metals. Pandey et al. (2015 and 2016) also reported dominance of *F. capucina* under Zn exposure (field and laboratory conditions) but not under Cu stress. Conversely, Bere and Tundisi (2012) reported *F. capucina* to be sensitive to metal pollutants in a laboratory mesocosm. *Gomphonema parvulum* also showed tolerance to the test metals. The ability of *G. parvulum* to tolerate metal enrichment agrees well with several previous researchers. In a laboratory grown phytoplankton community, *Gomphonema parvulum* has been found to be zinc tolerant (Loez et al. 1995). Metal pollution caused a shift in dominance of the phytoplankton community of a river in favour of *G. parvulum* and some other taxa (Montiero et al., 1995). Duong et al. (2008) also found *Gomphonema parvulum* to be cadmium tolerant. In fact, it was one of the dominant organisms in outflow from a mining tailing dam (Sabater, 2000). On the contrary, Bere and Tundisi (2012) recently reported it to be metal sensitive. *Pinnularia conica* also showed metal tolerance in the present study. Gold

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et al. (2002) observed *Pinnularia* sp. at sites containing high concentrations of cadmium and zinc; several other researchers have found *Pinnularia* spp. to be tolerant to heavy metals (Hirst et al., 2002; Pandey et al., 2014; Pandey et al., 2016). Absence during the rainy season was another noteworthy feature of *P. conica* observed in the present study. Since rainy season had high current velocity, this particular species may have poor colonization success in high flow. *Ulnaria ulna* showed marked sensitivity to the test metals. Another notable feature of this taxon observed during the present study was its sensitivity to high current velocity; it failed to establish during the rainy season.

Among green algae, *Chlorella vulgaris* and *Scenedesms* sp. showed tolerance to high concentrations of test metals. Metal tolerance of these two taxa has been reported earlier by several researchers. For instance, in a laboratory grown phytoplankton community, Loez et al. (1995) found *C. vulgaris* to be zinc tolerant. Stokes et al. (1973) also found *Chlorella* growing in a lake contaminated by smelter wastes to be metal tolerant. In a study of phytoplankton of river Moosi, Nirmala Kumari et al. (1991) also reported *C. vulgaris* as a metal tolerant species. The other green alga, *Scenedesmus quadricauda* has also been found to be copper tolerant growing well in Portugal reservoirs receiving copper-enriched mine effluent (Oliveira, 1985). Pandey et al. (2015, 2016) also found *Scenedesmus* sp. from chronically polluted water to be metal tolerant.

Diatoms accumulate a lot of lipids in their cell, and a large proportion of cellular volume may be occupied by them in the form of lipid droplets, also referred to as oil globules (Pandey et al., 2017). Keeping this in mind, the lipid droplets were enumerated and their volume determined in the present study. The test metals induced the formation of lipid droplets in diatom cells; higher the concentration of metal greater was the number and volume of lipid droplets. The present observations are in agreement with several other researchers who noted greater lipid

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accumulation in diatoms and algae under metal (Pandey et al., 2015; Pandey and Bergey, 2016; Gautam et al., 2017; Pandey et al., 2017) and diverse environmental stresses (Sharma et al., 2012). The lipid droplets had the largest volume at the highest tested concentration of the test metals thereby suggesting greater lipid accumulation by them. Since the change in number and volume of lipid droplet showed distinct relationship with metal concentration, these two may also be tested more vigorously for their application as a criterion in metal biomonitoring.

In the present study, morphological deformities in periphytic diatoms were more prevalent under metal stress than the control, which was found to be in agreement with available literature. For example, Pandey et al. (2014) reported significantly higher % deformed frustules in the diatom community under Cu, Zn and Pb stress in the River Ganges and also found positive and statistically significant relationship between % deformed frustules and different metals (Cu, Zn and Pb) exposure. Cantonati et al. (2014) also reported deformities in *Achnanthidium minutissimum* in the samples collected from different parts of Europe, Russia and America impacted with heavy metals and considered deformities in this diatom species as a biomonitoring tool for heavy metal contamination.

Published reports also showed that the different diatom metrics examined were act together to counter metal stress in periphytic diatoms. For example, Pandey et al. (2016) examined LBs and DFs together in diatom frustules (*Fragilaria capucina*, *Nitzschia linearis* and *N. amphibia*) from metalliferous (Cu and Zn) sites of India. Gautam et al. (2017) reported LBs and DFs together in the cells of *Gomphonema pseudoaugur* from metal (Pb and Se) contaminated waterbodies of India. Under laboratory conditions, Pandey et al. (2015) reported induction of DFs and LBs in the diatom cells (*Navicula gregaria*, *Nitzschia linearis* and *N. amphibia*) under Cu and Zn stress.

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This study also demonstrates the in situ effects of metal toxicity followed by recovery of periphyton (using traditional algal parameters and non-taxonomical parameters) in the time period of 28 days. Pandey and Bergey (2018) also examined more or less similar results of periphyton recovery under similar sets of experiment conditions. Under stress, recovery response of periphyton depends upon various factors such as, seasons, nutrients availability, production of extra-polymeric substances (EPS) and emigration and immigration rates of algal cells (Stevenson and Peterson, 1991; Pandey and Bergey, 2018). Short-life span, flexible life-history and good dispersal abilities of periphytic algae also plays significant role in understanding recovery response of periphyton (Steinman and McIntire, 1990). Thus, recovery response of periphyton indicates not only monitoring of stable conditions but also for changing conditions, such as monitoring recovery of aquatic ecosystems.

Finally, as shown by our study as well as those of other researches, we conclude that changes in diatom assemblages, both taxonomic and morphological features, will be an excellent and specific indicators of metal contamination. This study may also helpful in developing proper restoration practices by using periphyton that helps in protecting the integrity of pristine fluvial ecosystems.

5. Conclusions

The metal diffusing substrate designed in the present study is cheap, environment-friendly and reliable, and could be used for in situ study of effects of metals and all other toxic chemicals on periphytic community. However, the chemical, i.e., ion or molecule, whose effect one intends to assess on periphyton should be small enough to diffuse out of the clay surface. In the present study, the periphytic diatom community responded in a definite manner to metal stress, which leads to taxonomical (cell density, biovolume, chl a, caro./chl *a* ratio, species

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composition, and biodiversity indices), morphological (deformities) and physiological (lipid bodies) alterations and metal diffusing substrates should be possible to employ it for biomonitoring of waterbodies impacted by heavy metals.

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Table 1. Concentration of chlorophyll *a* and the ratio of carotenoids to chlorophyll *a* in periphytic community exposed to the test metals.

Treatment	7 days		28 days	
	Chl *a* (µg cm^{-2})	**Carot./Chl *a***	**Chl *a* (µg cm^{-2})**	**Carot./Chl *a***
Control	0.95±0.12	0.86±0.12	4.88±0.21	0.98±0.15
Cu^L	0.85±0.21	1.00±0.14	4.81±0.22	0.99±0.24
Cu^M	0.55±0.08	1.57±0.21	4.87±0.21	1.16±0.21
Cu^H	0.15±0.02	1.86±0.25	4.85±0.25	1.21±0.22
Zn^L	0.92±0.10	0.93±0.14	4.85±0.18	1.21±0.24
Zn^M	0.67±0.08	1.02±0.08	4.84±0.15	1.11±0.25
Zn^H	0.40±0.05	1.42±0.09	4.87±0.27	1.05±0.21
Pb^L	0.95±0.13	0.93±0.11	4.85±0.16	1.00±0.18
Pb^M	0.95±0.19	1.02±0.10	4.88±0.17	1.05±0.16
Pb^H	0.95±0.20	0.95±0.13	4.90±0.16	1.04±0.14

Table 2. Seasonal response pattern of attached algal community under in situ heavy metal enrichment expressed in terms of % deformity. Values given in the table represent the % deformities values on 7 and 28 days. Superscripts L, M and H denotes Low (1 g l^{-1}), Medium (2.5 g l^{-1}) and High (5 g l^{-1}) concentrations of Cu, Zn and Pb filled inside metal diffusing subtrates. For more detail see figures 1,S1 and S2.

Treatments	% Deformity		
	Winter	**Summer**	**Rainy**
Control	0.02-0.07	0.02-0.09	0.05-0.11
Cu^L	0.65-0.03	1.0-0.11	0.72-0.04

Cu^{M}	1.68-0.21	2.1-.25	1.55-0.22
Cu^{H}	2.45-0.32	3.0-0.22	2.72-0.15
Zn^{L}	0.57-0.12	0.8-0.12	0.52-0.05
Zn^{M}	1.83-0.30	2.0-0.14	1.92-0.22
Zn^{H}	2.29-0.22	2.50-0.18	2.52-0.29
Pb^{L}	0.18-0.04	0.21-0.05	0.15-0.03
Pb^{M}	0.27-0.025	0.25-0.07	0.29-0.02
Pb^{H}	0.42-0.05	0.35-0.09	0.49-0.07

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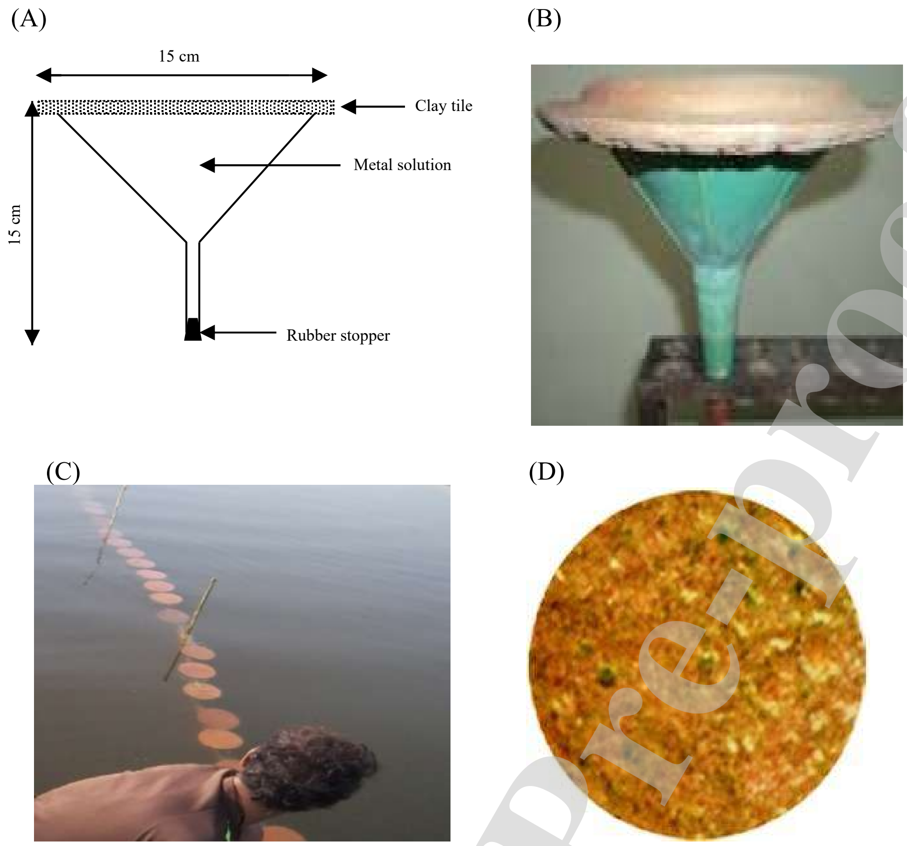

Fig. 1. (A) Diagrammatic representation of the design of metal diffusing substrate (MDS); (B) Photograph of an MDS; (C) Deployment of MDS in the river; (D) Periphytic colonization on the porous clay surface of MDS.

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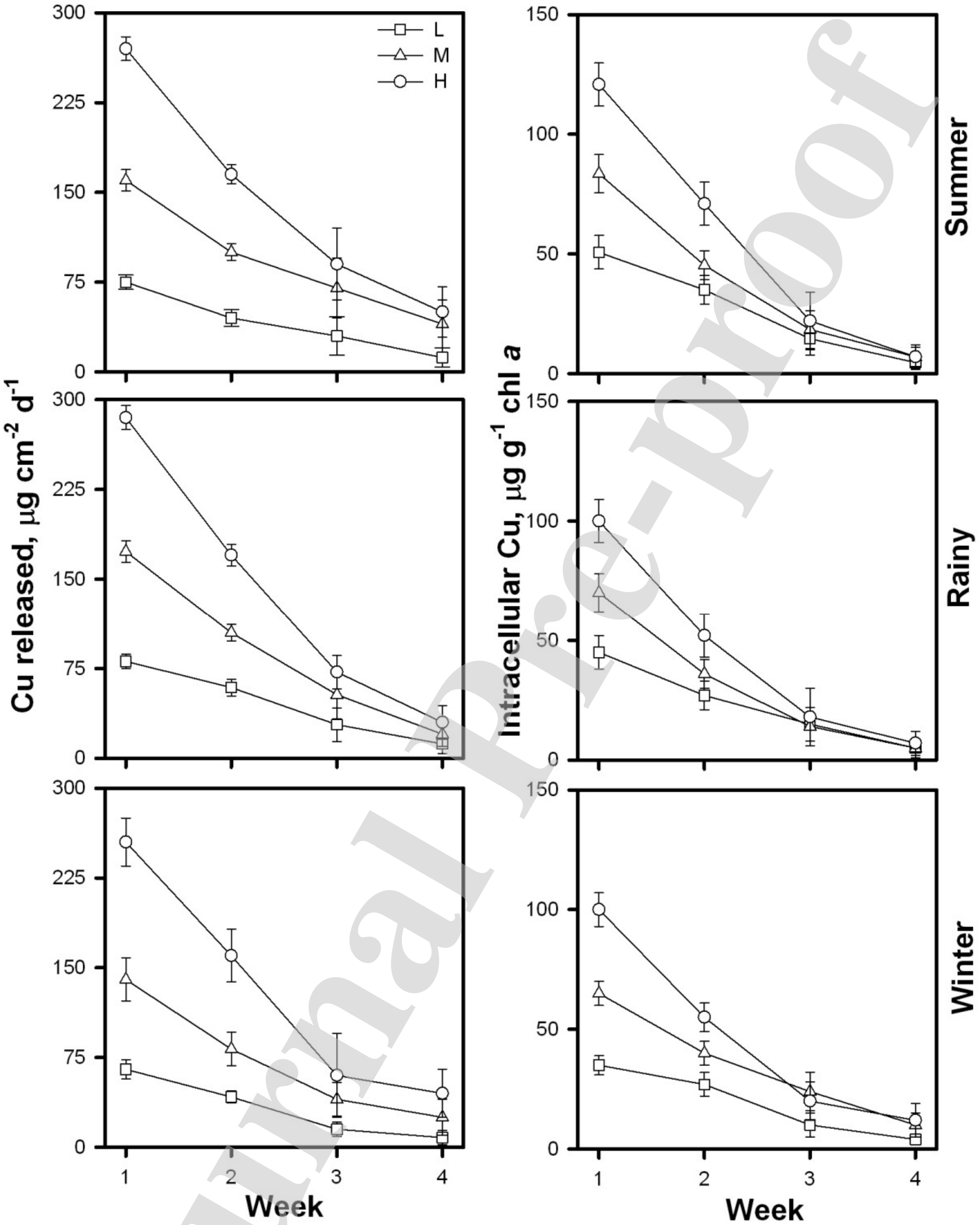

Fig. 2. Seasonal variations in the rate of release of Cu from MDS filled with low (L; 1 g l^{-1}), medium (M; 2.5 g l^{-1}) and high (5 g l^{-1}) concentrations of Cu, and intracellular Cu content in the periphyton. Vertical bars show standard error of means.

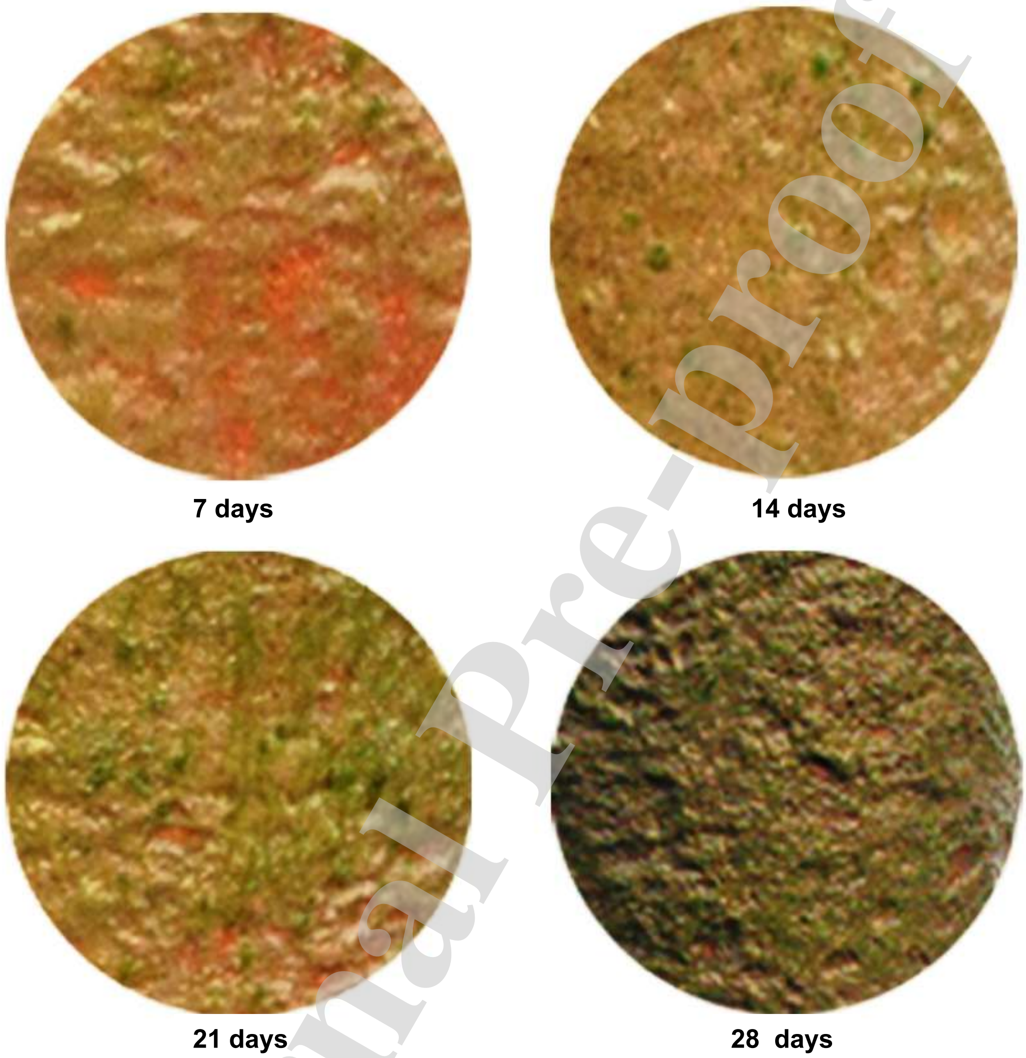

Fig. 3. The pattern of periphytic colonization onto clay surface of MDS. The period of the experiment was May to June, 2010.

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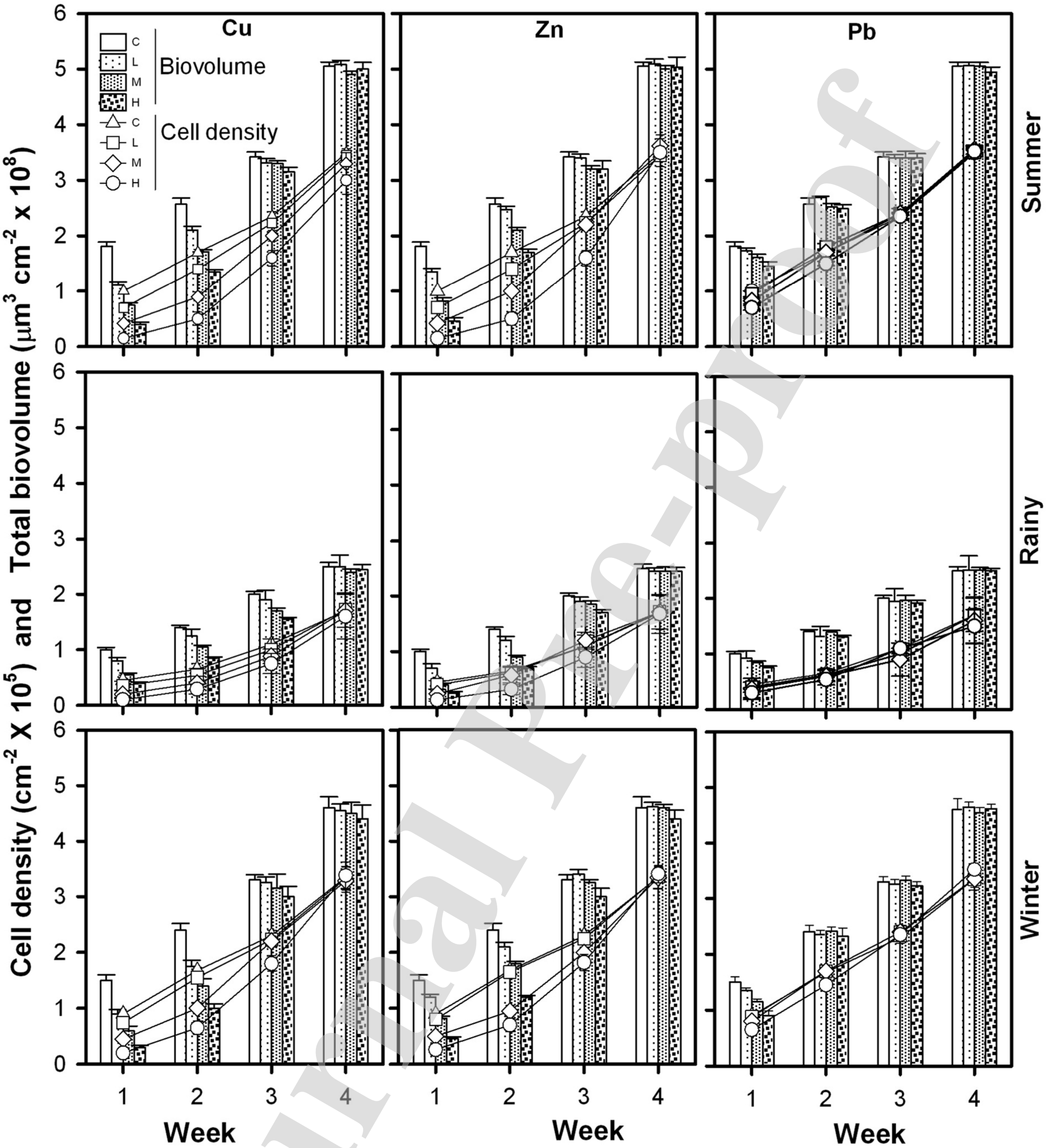

Fig. 4. Cell density and total biovolume of periphytic algal assemblages in the three seasons at low, medium and high concentrations (see Figs. 1, S1 and S2 for details) of the test metals. In the figure bars represent total biovolume while line plot represent cell density.

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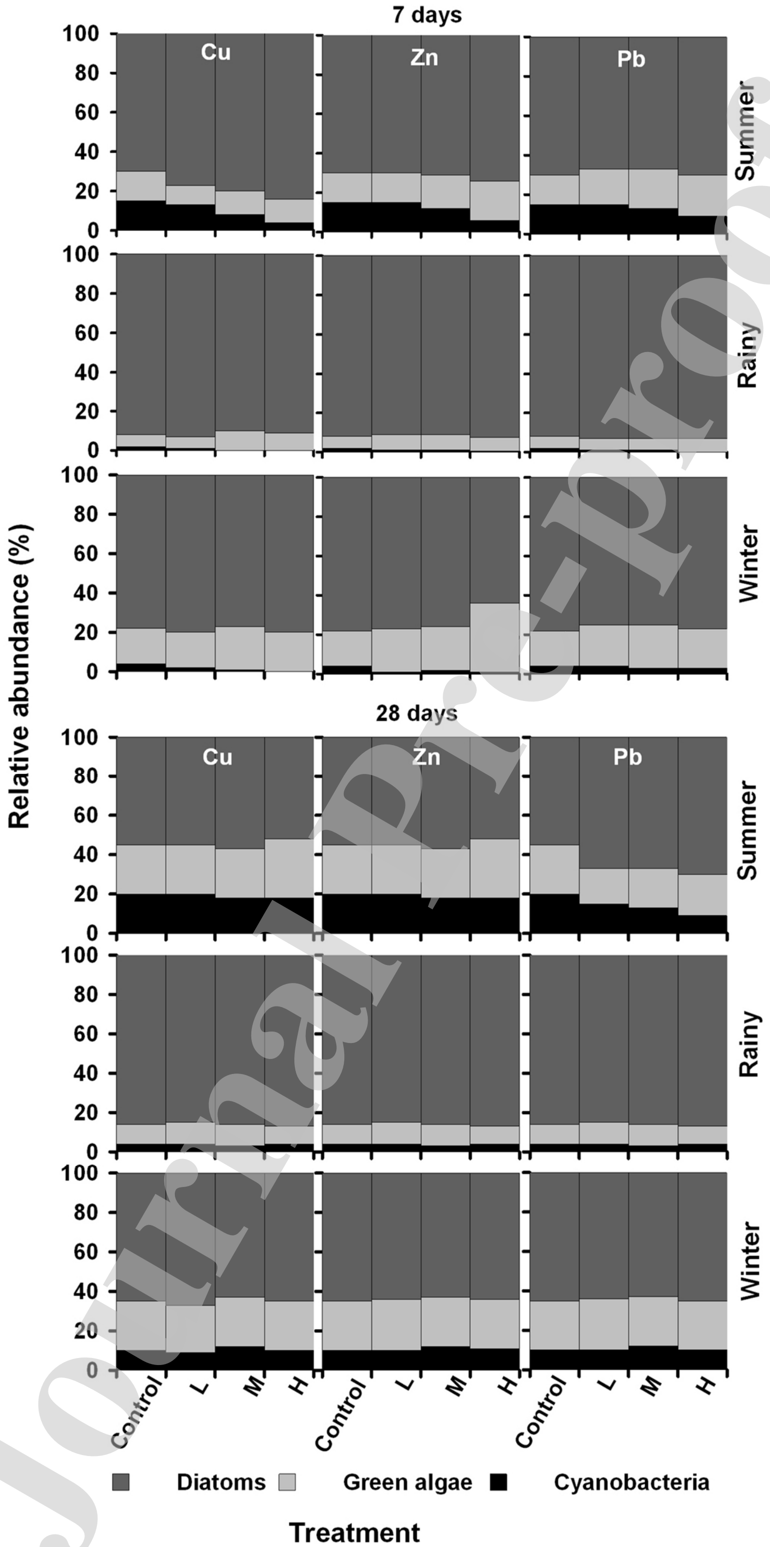

Fig. 5. Seasonal variations in the relative abundance of various groups in the periphyton at various concentrations of the test metals in MDS.

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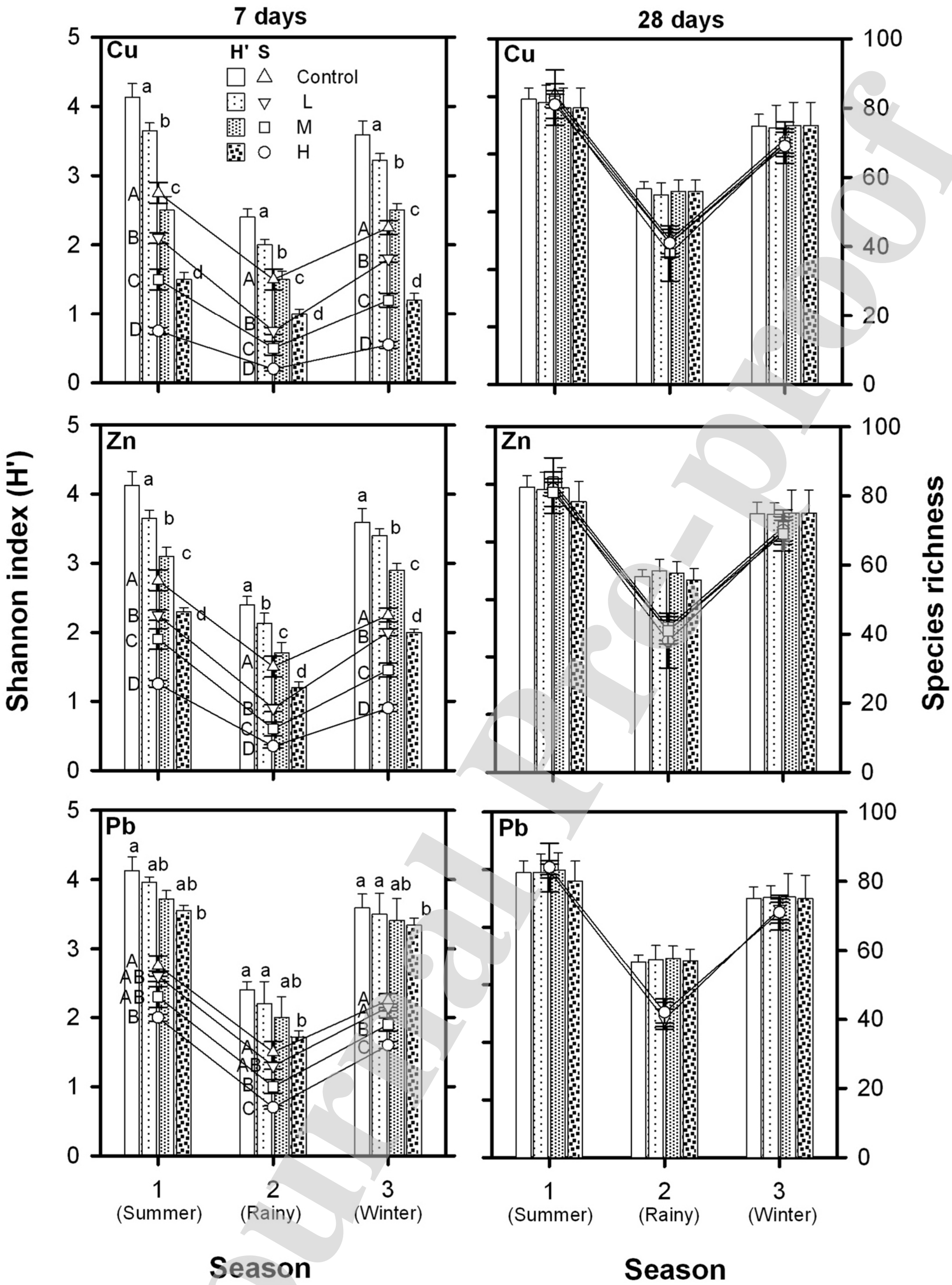

Fig. 6. Species richness and Shannon index (H') (species diversity) of the periphytic algal assemblage exposed to low, medium and high concentrations (see Figs. 1, S1 and S2 for details) of test metals for 7 and 28 days. Data bearing different letters for different seasons are significantly different (p< 0.05; Tukey's HSD test) from each other.

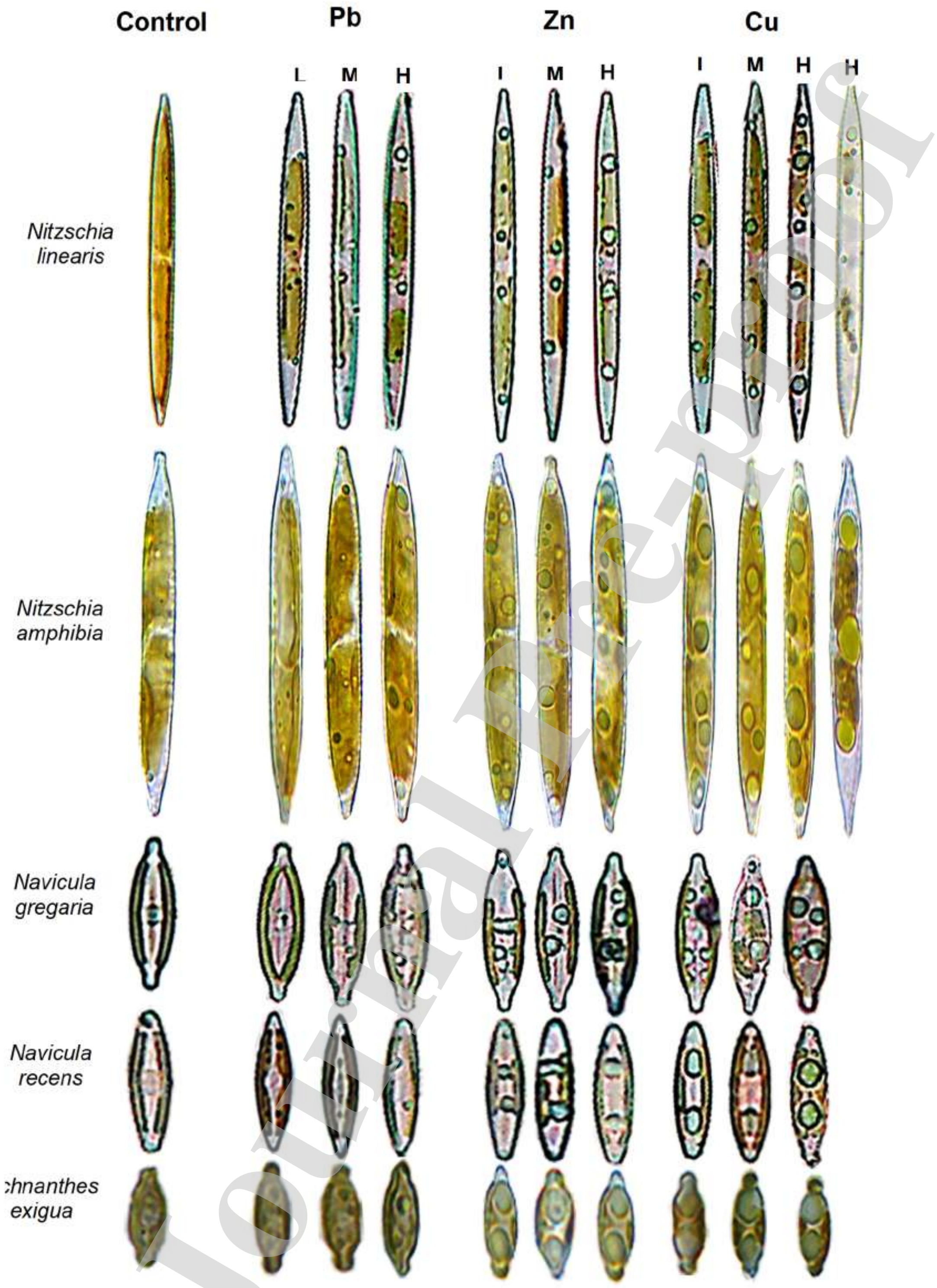

Fig. 7. Lipid droplets in live diatoms of the periphytic algal community in the control and metal treatments (L, M and H respectively represent low, medium and high concentrations of test metals; see Figs. 1, S1 and S2 for details).

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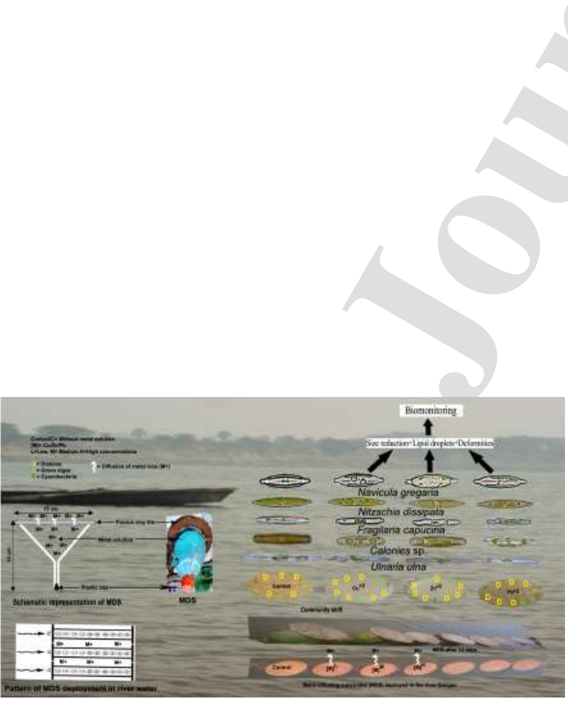
Biomonitoring
Size reduction+Lipid droplets+Deformities
Navicula gregaria
Nitzschia dissipata
Fragilaria capucina
Calonies sp.
Ulnaria ulna
Schematic representation of MDS
MDS
Community shift
Pattern of MDS deployment in river water

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Highlights

- Metal diffusing substrates tested Cu, Zn & Pb toxicity to riverine periphytic algae.
- Taxonomic algal and diatom parameters were impacted by metal stress.
- Metals induce lipid bodies and morphological abnormalities in periphytic diatoms.

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Author contributions

Lalit Kumar Pandey: Conceptualization, Investigation, Methodology, Formal analysis, Writing - original draft, Writing - review & editing and Funding acquisition.

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Declaration of interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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