De: Carmen Espinosa
A: Lorenzo Proia
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Title: Effects of the interaction between nutrient concentration and DIN:SRP ratio on geosmin production by freshwater biofilms
Journal: Science of the Total Environment

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Science of the Total Environment

Effects of the interaction between nutrient concentration and DIN:SRP ratio on geosmin production by freshwater biofilms

--Manuscript Draft--

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Abstract:	The global increase of cyanobacterial blooms occurrence has been associated with the presence of compounds that generate earthy and musty odour in freshwater systems, among which geosmin stands out. The lack of information on the factors associated to geosmin production by benthic organisms has driven the development of this study, whose main goal is to determine the effects of nutrient concentration and DIN:SRP ratio on geosmin formation and release. The experiment was performed in 18 microcosms under controlled conditions for 21 days, using a natural biofilm suspension from Ter river (NE, Spain) to promote biofilm settlement. Six treatments were set crossing three DIN:SRP ratios (A = 4:1, B = 16:1 and C = 64:1) with two nutrient concentrations (Low and High). After 7 days of experiment, geosmin was detected in biofilm, being higher under high nutrient concentration and low DIN:SRP ratio conditions. In this treatment, geosmin in biofilm reached its maximum concentration at day 16 (3.8 ± 0.9 ng/mg), decreasing at the end of the experiment (21d) due to cyanobacteria detachment and geosmin release into the water (136 ± 6 ng/L). Overall, this experimental study showed that high nutrient concentration and low DIN:SRP ratio favoured the Oscillatoria genus development within biofilm communities, generating the optimal conditions for geosmin production. The interaction between these two factors was demonstrated to be a potential driver of benthic geosmin production and release, and should be monitored and controlled in rivers exploited for drinking water purposes.
Response to Reviewers:	Reviewer #1: I note and welcome the changes you have made in response to the comments from myself and the other reviewer. The figures are greatly improved from the original submission. Much clearer and more easily interpretable. However, I note there remain a number of English and grammar errors. It appears that there has been an oversight in proof reading since the majority of the revisions have been made and I suggest that the whole article is reviewed thoroughly for such errors. Whilst I acknowledge that a major revision has already been made to this manuscript, I consider that further minor corrections are still necessary prior to the acceptance of this ac Thank you very much for the positive feedback. We have proceeded to correct the

Effects of the interaction between nutrient concentration and DIN:SRP ratio on geosmin production by freshwater biofilms

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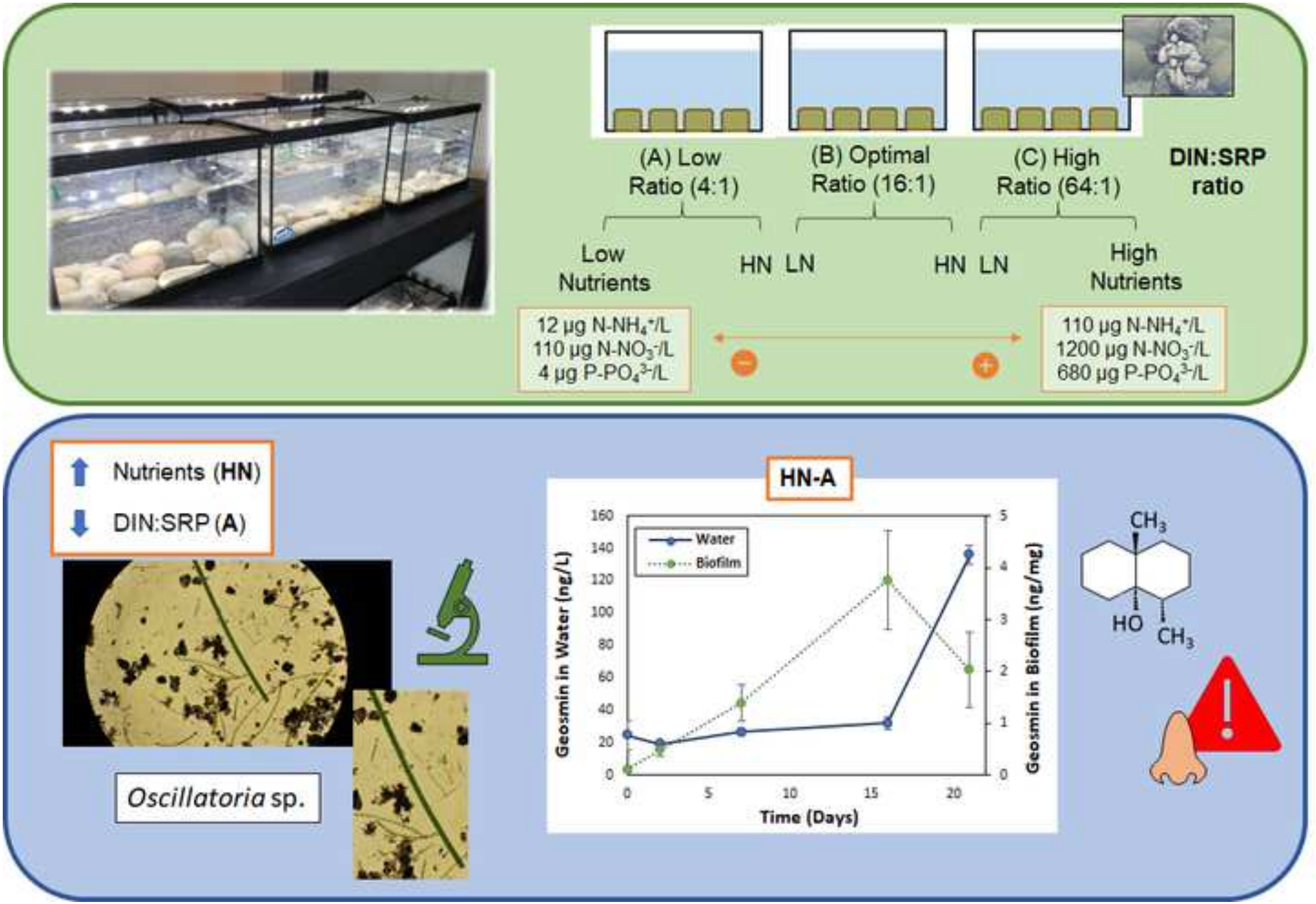
Graphical Abstract



Highlights (for review : 3 to 5 bullet points (maximum 85 characters including spaces per bullet point)

Highlights

- Geosmin episodes are associated with benthic and planktonic cyanobacterial blooms.
- Low DIN:SRP ratio and high nutrient concentration favor *Oscillatoria* sp development.
- *Oscillatoria* sp produces geosmin under low DIN:SRP ratio and high nutrient conditions.
- Phosphorus concentration is a key factor in geosmin production by benthic cyanobacteria.
- Changes in DIN:SRP due to climate change and human pressures favor geosmin production potential

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Keywords: Geosmin; *Oscillatoria* sp.; DIN:SRP ratio; nutrient concentration; biofilm; microcosms.

1. Introduction

In recent decades, anthropogenic activities have severely degraded streams and rivers worldwide. Excessive nutrient load is considered one of the major threats leading to substantial alterations in the overall functioning and structure of the ecosystem (Doods and Smith, 2016). These effects are of greater importance in rivers affected by situations of water scarcity (i.e. Mediterranean streams), where water flow can be extremely reduced, thus generating less dilution capacity and a greater increase in nutrient concentration (Karaouzas et al., 2018). The variations in nutrient export induced by anthropogenic activities can deeply modify the ratios between dissolved inorganic nitrogen (DIN) and soluble reactive phosphorus (SRP) (von Schiller et al., 2008), with the consequent exposure of freshwater microbial communities to significant fluctuations of both nutrients' concentration and balance over time (Artigas et al., 2015). These modifications of nutrients availability and balance can provoke a

marked increase of algal communities' growth in aquatic environments, leading to cyanobacterial blooms typical of eutrophicated ecosystems.

Abundant growth of benthic and planktonic cyanobacteria in freshwaters exploited for drinking water purposes has been correlated with the appearance of secondary metabolites, such as geosmin and 2 – methylisoborneol (MIB) (Espinosa et al., 2020; Lee et al., 2017), affecting the organoleptic characteristics of water and influencing both odor and taste, with a consequent negative impact in the perception of the population about the tap water quality (Ding et al., 2014). Although no health risk has been linked to these Taste and Odor compounds (T&Os), they are perceived by humans at very low levels of concentration (Smith et al., 2009), resulting in consumer complaints and representing a challenge for drinking water companies. Among the existing T&Os, geosmin has been identified as the main metabolite leading to bad taste and odor in drinking waters (Watson et al., 2016). This metabolite is a volatile bicyclic terpenoid, produced by certain cyanobacterial cells during the exponential phase of growth and released into the water as a consequence of cell death and/or biomass decomposition (Kim et al., 2018; Lee et al., 2017). Although some heterotrophic bacteria have been identified as potential geosmin producers (such as the actinomycete bacteria *Streptomyces* sp. and the fungi *Penicillium* sp.), they usually have a terrestrial origin or are present in sediments and foodstuffs. And while they can also be related to some geosmin episodes in aquatic systems, cyanobacteria are still considered the main geosmin producers in freshwater ecosystems (Lukassen et al., 2019; Olsen et al., 2016). The majority of these geosmin-producing cyanobacteria are benthic or epiphytic (70%), while the rest are planktonic (Jüttner and Watson, 2007). In the group of geosmin-producing cyanobacteria, *Oscillatoria* sp., *Lynghya* sp., *Symploca* sp. and *Dolichospermum* sp. have been identified as the most common genera in freshwater ecosystems (Smith et al., 2009). These genera have also been described as MIB producers, although only a few species have the ability to produce both geosmin and MIB (Jüttner and Watson, 2007). Even if numerous episodes have been reported all over the

73 world, the main triggers driving the production of these compounds by cyanobacteria in
74 freshwaters are not deeply understood yet (Tung et al., 2008).

75 Fluvial biofilms are complex microbial communities formed by different groups of organisms,
76 which together with extracellular enzymes and detritus are enclosed within a polymeric matrix
77 (Romaní, 2010). The effects of nutrient concentration on the biofilm community of running
78 waters have been widely documented describing that, depending on the nutrient
79 concentrations, the stoichiometry of the benthic biofilm can differ, conjointly with the nutrient
80 uptake rates (consequence of changes in nutrient demand) modifying the microbial
81 interactions between the autotrophic and heterotrophic biofilm compartments (Price and
82 Carrick, 2016; Bechtold et al., 2012). Furthermore, deviation from Redfield ratio (106C:16N:1P)
83 has been used as an indication of nutrient limitation for algal growth (Redfield et al., 1963).
84 Specifically, the N:P ratio of 16:1 is used as a reference to differentiate P-limitation (N:P>16)
85 from N-limitation (N:P<16) although this value may differ between algal and cyanobacterial
86 groups (Geider and La Roche, 2002; Sabater et al., 2016). Therefore, water N:P ratios may
87 control population dynamics and the coexistence of species in river biofilms.

88 Previous studies indicated that environmental factors such as temperature, light and nutrient
89 availability influence geosmin production (Alghanmi et al., 2018; Lee et al., 2017). However,
90 most of these were carried out on single species cultures under highly controlled conditions
91 (Parinet et al., 2010; Li et al., 2012; Suurnäkki et al., 2015). On the other hands, some field
92 studies have suggested that high nutrient concentrations, low nitrogen to phosphorus ratio
93 (N:P) and relatively low NO_3:NH_3 ratio can be important factors potentially promoting algal
94 growth, and favoring the cyanobacterial production of secondary metabolites such as geosmin
95 and MIB (Olsen et al., 2016; Harris et al., 2016). Most of these studies have focused on
96 phytoplanktonic organisms, whereas very few have deal with the identification of geosmin
97 production drivers in natural river benthic communities. A field study performed in the

Llobregat river (NE Spain) pointed out that the nutrient imbalance (TN:TP = 10) could have favored geosmin production by biofilms (Vilalta et al., 2003). However, field studies alone are not enough to establish causal relationships between environmental factors and biological responses and need to be confirmed under controlled conditions. Therefore, there is still a lack of information about the independent and combined effects of the availability and imbalance of nutrients on triggering geosmin production by benthic microorganisms growing within biofilm communities.

Nowadays, the water quality of more and more rivers is deteriorating due to the increase in the nutrients' concentration derived from anthropogenic activities. As has been described in reservoirs, this nutrient increase can lead to the uncontrolled growth of cyanobacteria, which in shallow rivers develop mainly as benthic. These rivers can be a source of water for drinking water treatment companies, so the drivers behind cyanobacteria growth, such as nutrient concentrations and DIN:SRP ratio, need to be evaluated in order to generate useful information to understand and predict the occurrence of geosmin episodes in these freshwater ecosystems.

This study was designed to explore the independent and combined effects of the DIN:SRP ratio and nutrient concentrations on the biofilm structure-function, and its relationship with the geosmin production and release. To this end, an experiment was carried out in controlled laboratory microcosms exposing natural biofilm communities to three different DIN:SRP ratios - low (4:1), optimal (Redfield, 16:1) and high (64:1) - under two nutrient concentration levels (low and high) in water. Although some studies have been carried out evaluating geosmin production by different cyanobacteria strains in laboratory cultures, to the best of our knowledge this is the first study evaluating the independent and combined effect of the DIN:SRP ratio and nutrient concentration on geosmin production by a natural benthic biofilm. The main outcomes of this study could be very useful for catchment managers and water

utilities, helping them to understand under which nutrient conditions they should expect geosmin in water used for drinking purposes, improving their response time in geosmin treatment and reducing consumer discomfort and complaints.

2. Materials and Methods

2.1. Experimental design and sampling

Eighteen microcosms consisting of 6L glass aquariums (length x width x height 26 x 15 x 17 cm) were used to evaluate the biofilm functional and structural response under different nutrient concentrations and DIN:SRP ratios in water. Each microcosm, containing 16 scraped and autoclaved stream cobbles, was filled with 3L of artificial water. Artificial water was prepared to simulate a pristine stream as described in Ylla et al. (2009) and was obtained by dissolving pure salts in distilled water creating a chemical composition of 14.96 mg/L Na^{+}, 10.81 mg/L Ca^{2+}, 0.52 mg/L K^{+}, 0.40 mg/L Mg^{2+}, 9.71 mg/L SO_4^{2-}, 12.45 mg/L SiO_3^{2-}, 19.67 mg/L Cl^{2-} and 14.52 mg/L HCO_3^{-}. To maintain these conditions, the water of the microcosms was renewed every two to three days. Each aquarium had a submersible pump (EDEN 105, Eden Water Paradise, Italy) to promote oxygenation and water circulation. The photoperiod was set at 12h light: 12h dark using LEDs (LENB 135-lm, LENB/14.97/11.98), and the room temperature was set at 16°C.

The experiment was carried out during a geosmin episode that occurred in March 2019 in the Ter river (NE Spain), coinciding with the presence of benthic *Oscillatoria* mats (authors, personal observation). From this river, a natural biofilm suspension was obtained by scrapping several cobbles randomly selected along a 50m river stretch located at the water collection point of the water drinking company Aigües de Vic (X440478; Y4648779 UTM31N), and 15 mL of this suspension was inoculated in each microcosm to promote biofilm colonization. During the colonization period (three weeks), a new inoculum was added after each water renewal to favor biofilm settlement in the microcosms (Vendrell-Puigmitja et al., 2020).

After the colonization period, the exposure period started. Six treatments (n = 3) were set by crossing three DIN:SRP ratios (Low = 4:1 (A), Optimal = 16:1 (B) and High = 64:1 (C)) with two nutrient concentrations (Low and High) (**Table 1**). Specifically, these treatments were low nutrient concentration and low DIN:SRP ratio (LN-A), low nutrient concentration and optimal DIN:SRP ratio (LN-B), low nutrient concentration and high DIN:SRP ratio (LN-C), high nutrient concentration and low DIN:SRP ratio (HN-A), high nutrient concentration and optimal DIN:SRP ratio (HN-B) and high nutrient concentration and high DIN:SRP ratio (HN-C). DIN:SRP ratios were determined as DIN (Dissolved Inorganic Nitrogen) divided by Soluble Reactive Phosphorus (SRP) in molar quantities. DIN concentration was determined as the sum of ammonium ($N\text{-}NH_4^+$) and nitrate ($N\text{-}NO_3^-$) concentration. Nutrient concentration and DIN:SRP ratios were chosen to cover a range representative of Ter river basin variation, whose nutrient concentration ranges between 12 – 300 µg $N\text{-}NH_4^+$/L, 100 – 2000 µg $N\text{-}NO_3^-$/L, and 10 – 950 µg $P\text{-}PO_4^{3-}$/L (authors, field study; Water Catalan Agency, 2020). During the exposure period, which lasted for 21 days, nutrient concentration in each aquarium was maintained at each water renewal, which were done every two to three days. Nitrogen concentration was adjusted using nitrate and ammonium standard solutions (1 g/L PanReac-AppliChem), and the phosphorus concentration was adjusted using a phosphate concentrated stock solution (10 mM $P\text{-}PO_4^{3-}$) obtained by dissolving pure salts (KH_2PO_4, PanReac-AppliChem) into deionized water.

Before and after each water renewal during both the colonization and exposure periods, physico-chemical parameters were measured with portable probes: temperature, dissolved oxygen concentration and saturation (YSI professional plus, YSI Incorporated, USA), pH (XS pH7+ DHS) and electrical conductivity (XS COND 7+). From each microcosm, water samples were taken and filtered through 0.2 µm nylon membrane filters (Merck Millipore) before the analysis of SRP and DIN forms (ammonium and nitrate). All samples were stored at -20°C until analysis.

Biofilms were sampled just before the beginning of the exposure period (t0) and after 2, 7, 16 and 21 days. Three cobbles were randomly sampled from each microcosm at each sampling day. The photosynthetic efficiency and the phototrophic community composition were measured directly with an amplitude modulated fluorometer (Mini-PAM fluorometer, Walz, Effeltrich, Germany) and a BenthoTorch portable fluorometer probe (bbe Moldaenke, Schwentinenta, DK) respectively. After that, each cobble was scrapped in 45 mL of water from the same microcosm to obtain a biofilm suspension. Aliquots of this suspension were used to analyze geosmin concentration in biofilm, Chlorophyll *a* (Chl *a*), ash free dry mass (AFDM), algal taxonomic composition and extracellular enzymatic activities (AEE). AEE samples were analyzed the same day and the rest were stored frozen (-20°C) until analyses, except aliquots for geosmin concentration, which were stored at -80°C and taxonomic samples, which were fixed with formalin (2%) and stored at 4°C. The area of the scraped cobbles was obtained by drawing the surface on aluminum foil and recalculating depending on the weight (Graham et al., 1988).

Water samples were also taken at each sampling time to analyze nutrients and geosmin concentrations in water. Additionally, on the last day of the experiment, a nutrient uptake capacity experiment was performed with the colonized cobbles present in the microcosms. Samples for geosmin quantification in water were stored at 4°C in dark conditions until the analysis, which was performed within 48 hours after collection to avoid degradation. Water samples for nutrient analysis were filtered through 0.2 µm nylon membrane filters (Merck Millipore) and frozen at -20°C until analysis.

2.2. Biofilm samples

2.2.1. Geosmin

The concentration of geosmin in biofilm was quantified following the protocol described by Espinosa et al. (2020). Briefly, a headspace solid phase micro-extraction was performed. To

obtain 50 mL of total volume, 5 mL of the biofilm sample were decanted in a 100 mL opaque reaction vial containing (i) 45 mL of saline solution (10 g of NaCl in 45 mL of sterile dH_2O) and (ii) a magnetic stir bar. The samples were frozen (-80°C) and thawed five times to facilitate cell breakage and geosmin release in the aquatic phase. A 65 µm PDMS/DVB fiber was injected into the headspace of the sample vial, and the vial was placed on a magnetic stirrer inside an oven at 60°C for 25 min to extract geosmin from the samples. Geosmin characterization was performed using a GC/MS instrument (ISQ – TRACE GC ULTRA). Geosmin was separated using a capillary column (Sigma Aldrich SPB®-5 Capillary GC Column) measuring 30 m x 0.25 mm with a film thickness of 0.25 µm. Carrier gas was helium at a flow rate of 1 mL/min. Geosmin was desorbed by exposing the fiber in the GC injection port for 6 min at 250°C. A 0.75 mm internal diameter glass liner was used, and the injection port was in splitless mode. The temperature program was isothermal for 3 min at 70°C, raised to 200°C at a rate of 10°C/min and, finally, to 280°C at a rate of 30°C/min, with a hold time of 4 min. The transfer line to the mass spectrometer and the ion source was 250 and 200°C respectively, the scan was in SIM mode, with the scanned fragments being 111, 112, 125, 164 and 182 m/z. The analytical detection limit was 2.5 ng/L, and the precision of the method was evaluated with the relative standard deviation (RSD ≤20%). Geosmin concentration in biofilm was expressed as ng geosmin/mg AFDM.

2.2.2. Chlorophyll *a*

Algal biomass in biofilms was estimated from the analysis of Chlorophyll *a (*Chl *a*) concentration. Chlorophyll *a* was extracted from the sample with 90% acetone over a period of 12 h in the dark at 4°C. Acetone extracts were filtered through 0.7 µm glass fiber filters (GF/F filters, Whatman International) and Chlorophyll *a* concentration was determined spectrophotometrically measuring the absorbance at 430, 665 and 750nm (NanoPhotometer™

P-360, IMPLEN) following the method described by Jeffrey & Humphrey (1975). Chlorophyll *a* concentration was expressed as µg/cm^2.

2.2.3. AFDM

Total biofilm biomass was measured as ash free dry mass (AFDM). The aliquots of biofilm suspension were filtered through pre-combusted (4 h at 500°C, Carbolite muffle ELF 11/14B) and pre-weighed filters (47-mm GF/F Whatman glass-fiber filters, 0.7 µm pore size), then dried for 72 h at 60°C (Forced air oven, MEMMERT IFE500) in order to calculate dry mass (DM). Afterwards, samples were combusted at 500°C (Carbolite muffle ELF 11/14B) for 4 h, and then weighed again. The differences in filter mass before and after drying were calculated for DM (72 h at 60°C) and after combustion (4 h at 500°C) subtracted from DM to obtain AFDM. Results were standardized by the scratched biofilm surface of the cobbles and expressed as g/m^2.

2.2.4. Algal taxonomic composition in biofilm

Algal identification was done following Lange Bertalot (2001), counting a minimum of 400 cells and measuring at least 6 vision fields per sample. Cell biovolume was obtained following the procedure described in Hillebrand et al. (1999). To calculate the biovolume, the length and width of a minimum of ten individuals, randomly selected, were measured for each genus. The optical microscope (Nikon Eclipse 600W) using phase-contrast and Nomarski differential interference contrast optics at a magnification of 400 increments was used, counting and measuring the cells of at least 6 fields of vision per sample. The values were transformed as described in Ricart et al. (2009), using the surface of the sample, the dilutions needed, and the number of cells counted, and were expressed as µm^3/cm^2.

2.2.5. Extracellular Enzymatic Activities

Leucine-aminopeptidase (EC 3.4.11.1) and phosphatase (EC 3.1.3.1–2) activity was measured by using fluorescent-linked substrates (L-leucine-7-amino-4-methylcoumarin hydrochloride [AMC] used for peptidase and 4-methylumbellyferyl-phosphate [MUF] for phosphatase) following the protocol described in Romaní & Marxsen (2002). The fluorescence was measured at 380/460 nm excitation/emission for MUF and at 380/460 nm excitation/emission for AMC using the Spark® multimode microplate reader (TECAN). Values are expressed as µmol MUF/cm^2·h and µmol AMC/cm^2·h.

2.3. Water samples

2.3.1. Geosmin

To analyze geosmin concentration in water samples, 50 ml of each sample and 10 g of NaCl were added to a 100 mL opaque reaction vial. To extract the geosmin in water a 65 µm PDMS/DVB fiber was used. Separation and analysis of the extracted volatile compound was performed in a GC/MS instrument (ISQ – TRACE GC ULTRA), as described in section 2.2.1. Geosmin concentration in water was expressed as ng/L.

2.3.2. Nutrients

SRP concentration in water samples was measured using the protocol described by Murphy & Riley (1962). DIN concentration was determined as the sum of ammonium ($N\text{-}NH_4^+$) and nitrate ($N\text{-}NO_3^-$) concentration. $N\text{-}NH_4^+$ was analyzed following the protocol described in Reardon et al. (1966), and $N\text{-}NO_3^-$ was determined following the method described in Rand et al. (1976). For all these colorimetric determinations, the absorbance was measured with the spectrophotometer NanoPhotometer™ P-360, INPLEM.

2.3.3. Nutrient Uptake

The nutrient uptake capacity of biofilms was calculated by measuring ammonium, nitrate and phosphate temporal decay after a controlled spike in microcosms. The artificial water of each microcosm was renewed to ensure that the starting conditions of the aquariums were optimal. Each microcosm was then spiked with an appropriate volume of a phosphate concentrated stock solution (10 mM $P\text{-}PO_4^{3-}$) obtained by dissolving pure salts (KH_2PO_4, PanReac-AppliChem) into deionized water, and ammonium (NH_4^+, 1 g/L standard solution, PanReac-AppliChem) stock solution in order to quadruple the basal concentration of nutrients (Proia et al., 2017). The biofilms were then incubated for 4 hours under controlled temperature and light conditions. Aliquots of water were taken at 1, 5, 30, 60, 120, 180 and 240 minutes after spiking, immediately filtered through 0.2 µm nylon membrane filters (Merk Millipore) and stored at -20°C until analysis. The uptake capacity (U, $1/m^2 \cdot min$) was calculated as (**Eq. 1**):

$$U = \frac{\frac{\left[(C_i \cdot V_i) - (C_f \cdot V_f)\right]}{C_i \cdot Vi}}{A \cdot T_f} \qquad (Eq.\ 1)$$

where C_i = initial nutrient concentration; V_i = initial volume; C_f = final nutrient concentration; V_f = final volume; A = biofilm's area and T_f = residence time.

2.4. Data treatment

Before the statistical analysis, the Kolmogorov – Smirnov test was performed to verify that the variables fulfilled the conditions of normal distribution; and if they did not, they were logarithmically transformed. Physicochemical and biological data were evaluated using the two-way repeated measures analysis of variance (ANOVA) in SPSS Statistics version 21, with nutrient concentration and DIN:SRP ratio as factors and sampling date (time) as repeated measure. In addition, physicochemical and biological data was evaluated on each sampling date by a two-way ANOVA, with the DIN:SRP ratio and nutrient concentration as independent variables, and geosmin in water (ng/L), geosmin in biofilm (ng/mg), cyanobacteria and diatoms

biomass (µg/cm^2), Chlorophyll *a* (µg/cm^2), photosynthetic efficiency (Yeff) and PHO (µmol MUF/cm^2·h) as dependent variables. The post-hoc test performed was the Bonferroni test. Pearson correlation coefficient tests were carried out to explore the relationship between the variables. Statistical significance was set at $p < 0.05$ for all tests performed. The distribution of the variables according to the treatments was evaluated using a principal component analysis (PCA) performed with RStudio software (version 3.6.0).

3. Results

3.1. Physicochemical parameters

The physicochemical conditions in the microcosms remained stable throughout the experiment, with water temperature varying insignificantly around 16°C, slight alkaline pH and low electrical conductivity (**Table 1**), and without significant differences among them despite the treatments (repeated measures ANOVA).

3.2. Geosmin concentration

3.2.1. Geosmin in biofilm

Geosmin concentration in biofilm (**Figure 1. A., Table 2**) varied significantly over time among microcosms, with the HN-A treatment being the one which significantly differed from the others (**Table 3**).From day 7 to the end of the experiment, geosmin concentration in biofilm was significantly the highest in the HN-A treatment (**Table 2**). Under these conditions, geosmin concentration increased gradually, reaching its maximum (3.8 ± 0.9 ng/mg) at t=16d and decreasing thereafter to 2.0 ± 0.7 ng/mg (t=21d, **Figure 1.A.**). Under HN-B conditions, the biofilm did not produce geosmin during the whole experiment.

3.2.1. Geosmin in water

At the beginning of the exposure period (t=0d), geosmin was detected in all treatments at low concentrations (27.4 ± 6.7 ng/L), without any significant difference among microcosms (**Figure 1.B.**, **Table 2**, **Table 3**). After 7 days of treatment, geosmin concentration in water was significantly higher in those treatments with a lower DIN:SRP ratio (Bonferroni test: $p < 0.005$) independently of the nutrient concentration. However, from then on, the interaction between the two factors had a significant effect on geosmin concentration. At the end of the experiment (day 21), both nutrient concentration and the DIN:SRP ratio, as well as their interaction showed a significant effect on geosmin concentration in water. Specifically, the microcosms developed under the lowest DIN:SRP conditions showed higher geosmin concentration in water compared to the other ratios (Bonferroni test: $p < 0.001$), and the highest geosmin concentration (136 ± 6 ng/L) was found under HN conditions (**Figure 1.B.**). The only treatment where geosmin was not detected at the end of the experiment was HN-B.

3.3. Biofilm attributes

3.3.1. Structural parameters

3.3.1.1. Community composition

During the experiment, the phototrophic community composition of the biofilm (cyanobacteria, green algae and diatoms) varied over time (**Table 2**). Specifically, the DIN:SRP ratio significantly influenced the cyanobacteria biomass and the relative diatoms and cyanobacteria abundances over time, while the diatoms biomass was affected by the nutrient concentration and its interaction with the DIN:SRP ratio (**Table 3**).

One week after the beginning of the exposure period (t=7d), the cyanobacteria biomass was statistically different between treatments depending on the DIN:SRP ratio, being higher under low DIN:SRP treatments (Bonferroni test: $p < 0.01$). At day 16, nutrient concentration and its interaction with the DIN:SRP ratio also had a significant effect on cyanobacteria biomass, being

337 significantly higher under low DIN:SRP treatments (Bonferroni test: $p < 0.01$) and resulting the
338 highest in HN-A treatment (1.63 ± 0.36 µg chl*a*/cm^2). A similar trend was observed for the
339 cyanobacteria relative abundance. The highest value of abundance was found in the HN-A
340 treatment at t=16d (48 ± 2%), although at the end of the experiment, this value decreased to
341 31 ± 7% (**Figure 2**). The opposite pattern was observed for the diatom's relative abundance.

342 At the end of the experiment (t=21d) the algal taxonomic community in the biofilm was
343 evaluated (**Figure 3.**). The relative abundance of *Oscillatoria* sp. was higher under low DIN:SRP
344 ratio conditions compared with the other ratios (Bonferroni test: $p = 0.001$), reaching the
345 highest value (45 ± 10%) under the HN-A treatment. The cyanobacterium *Oscillatoria* sp. and
346 the diatom *Melosira* sp. biovolume varied among microcosms depending on the DIN:SRP ratio
347 ($F = 18.9$; $p < 0.001$ for *Oscillatoria* sp., $F = 17.5$; $p < 0.001$ for *Melosira* sp.) and its interaction
348 with nutrient concentration ($F = 5.0$; $p < 0.05$ for *Oscillatoria* sp., $F = 5.0$; $p < 0.05$ for *Melosira*
349 sp.). In addition, a strong negative correlation was found between *Oscillatoria* sp. and *Melosira*
350 sp. biovolume (Pearson's correlation: $r = -0.914$; $p < 0.001$). The *Oscillatoria* sp. biovolume was
351 also positively correlated with geosmin concentration in water and biofilm (Pearson's
352 correlation: $r = 0.886$; $p < 0.001$ and $r = 0.888$; $p < 0.001$, respectively).

353 #### 3.3.1.2. Chlorophyll *a*

354 Chlorophyll *a* concentration varied significantly throughout the experiment depending on the
355 treatment (**Table 2**, **Table 3**). At the end of the experiment (t=21d), both factors and their
356 interaction showed a significant effect on the biofilm Chlorophyll *a* content (*Supplementary*
357 *Figure*), with higher concentration under the optimal DIN:SRP ratio conditions compared with
358 high DIN:SRP ratio treatment (Bonferroni test: $p < 0.005$). It was also observed that under HN
359 conditions the biofilm had a higher production of Chlorophyll *a*, with the HN-B treatment being
360 the one showing the highest concentration (18.3 ± 0.9 µg/cm^2) (**Table 2**).

361 ### 3.3.2. Functional parameters

3.3.2.1 Photosynthetic capacity

The photosynthetic capacity of the biofilm (expressed as Yeff) (**Table 2**) was affected by the DIN:SRP ratio, showing at t=16d significant lower values under low DIN:SRP treatments (0.563 ± 0.025) compared to the optimal DIN:SRP ratio (0.646 ± 0.016) (Bonferroni test: $p < 0.05$) (**Table 3**). Five days later, nutrient concentration also affected the Yeff values, with the lowest being found for the biofilms developed under high nutrient concentrations and low DIN:SRP ratio conditions (0.529 ± 0.046).

3.3.2.2. Nutrient uptake

Phosphate uptake (or release) capacity was significantly affected by nutrient concentration ($F = 5.4$, $p < 0.05$) and its interaction with DIN:SRP ratio ($F = 25.4$, $p < 0.001$). In general, the uptake capacity was higher in LN conditions (0.20 ± 0.06 $mg/m^2 \cdot min$) than under HN conditions (0.09 ± 0.04 $m^{-2} \cdot min^{-1}$), except for the high DIN:SRP ratio that showed the opposite trend. The ammonium uptake capacity was significantly affected by nutrient concentration ($F = 25.2$, $p < 0.001$), with higher uptake capacities under LN conditions (0.37 ± 0.07 $m^{-2} \cdot min^{-1}$) compared with HN (0.13 ± 0.004 $m^{-2} \cdot min^{-1}$). Nitrate uptake capacity did not differ among microcosms.

3.3.2.3. Extracellular Enzymatic Activities

The phosphatase activity (PHO) of the biofilms varied throughout the experiment (**Table 3**). Specifically, at t=16d, the biofilms under high DIN:SRP ratio conditions showed higher PHO than those of the optimal DIN:SRP ratio treatment (Bonferroni test: $p < 0.05$) (**Table 2**). At the end of the experiment, the PHO values were different depending on the nutrient concentration and DIN:SRP ratio, with the high DIN:SRP ratio treatment being significantly different from the others (Bonferroni test: $p < 0.05$), and higher under HN conditions (72.0 ± 18.4 µmol $MUF/cm^2 \cdot h$). For the leucine-aminopeptidase activity, no significant differences were observed among microcosms over time.

Both nutrients uptake/release rates and enzymatic activities showed differences between treatments depending on the nutrient concentration under which the biofilm developed, without any relationship with the production and release of geosmin.

3.3.3. Overall relationships between biofilm responses and geosmin dynamics

The Principal Component Analysis (**Figure 4**) shows how the parameters evaluated are distributed according to the different treatments. It emphasizes that the X axis (explaining the 49.8% of variance) separates the HN-A treatment from the rest, being the one with the highest concentration of geosmin in water and in biofilm, greater cyanobacteria biomass, and higher presence of *Oscillatoria* sp. The Y axis (17.8% of variance) separates the optimal (B) and high (C) DIN:SRP ratio treatments according to the concentration of nutrients (LN or HN), since these conditions affected the enzymatic activities and the nutrients uptake/release capacities of the biofilms.

4. Discussion

This study demonstrates that, both nutrient concentration and DIN:SRP ratio are important drivers of geosmin occurrence in freshwaters, since they favor the growth of geosmin-producing organisms in biofilm communities and promoting intracellular geosmin formation and its subsequent release to the water column.

The biofilm community structure began to change 7 days after the nutrient manipulation started, mainly because of the different DIN:SRP ratios. In particular, cyanobacteria increased its relative abundance, becoming predominant in the biofilms exposed to low DIN:SRP ratio conditions, especially under high nutrient concentration (HN-A) (**Figure 2**). These results agree with recent studies describing how the phosphorus availability excess may stimulate the magnitude of cyanobacteria blooms (Jankowiak et al., 2019). Moreover, it has been described that certain cyanobacteria can store essential metabolic nutrients in the cytoplasm, especially

410 under eutrophic conditions (Felisberto et al., 2011). For example, phosphorus can be
411 accumulated in intracellular polyphosphate granules, whereas under nitrogen-limited
412 conditions some cyanobacteria have also shown a high capacity to fix it (Felisberto et al.,
413 2011). Different studies, performed mainly in lakes, have pointed out some nutrient thresholds
414 to control the growth of cyanobacteria, such as the TP value, which had to be between 20 –
415 100 µg TP/L (Li et al., 2018; Sharma et al., 2011). Stroom and Kardinaal (2016) reported that
416 below 0.03 mg TP/L, the risk of cyanobacteria dominance is 10%, increasing to 40% at 0.07 mg
417 TP/L, whereas another study indicated that N levels can be also important, 0.8 mg TN/L being
418 the threshold to limit the growth rate of *Microcystis* dominated blooms (Xu et al., 2014). The
419 results obtained in this study point out that similar values can be fixed as nutrient thresholds
420 for cyanobacteria growth in rivers, together with the N:P ratio, since the N:P ratio value should
421 be maintained at a level of at least 10, but preferably above 50 – 64, thereby reducing the
422 likelihood for N-limiting situation which may favor cyanobacteria dominating blooms (Li et al.,
423 2018). It should be noted that in our study we have evaluated the N:P ratio as DIN:SRP, while
424 in the studies of lakes and reservoirs the ratio is TN:TP.

425 At the end of the experiment (t=21d), cyanobacteria abundance in the HN-A treatment
426 decreased, arguably because of cell degradation. It has been demonstrated that the growth
427 phase of different cyanobacterial strains could last from 8 to 24 days before entering the
428 stationary or degradation phase (Kruskopf & Du Plessis, 2006; Jindal et al., 2011).
429 Nevertheless, the conditions were still optimal for the cyanobacteria community, which were
430 present in this treatment, with *Oscillatoria* sp. accounting for almost 45% of the total biofilm
431 biovolume, a rather high value compared to its presence in other treatments (**Figure 3**). The
432 growth phase of the Oscillatoria genus varies depending on the species. Kruskopf & Du Plessis
433 (2006) observed that *Oscillatoria simplicissima* reached the fast growth phase after 8 days,
434 whereas *Oscillatoria formosa* could grow exponentially for 24 days and before starting the
435 stationary phase (Jindal et al., 2011).

436 Changes at the structural level were reflected in functional parameters with photosynthetic
437 efficiency (Yeff) being significantly lower under low DIN:SRP ratio. This could be explained by
438 the higher presence of cyanobacteria in these treatments, since some studies have found that
439 cyanobacteria usually show low photosynthetic efficiency values compared with the rest of the
440 algal community (Espinosa et al., 2020; Luimstra et al., 2018).

441 In this study, the highest geosmin concentration in both biofilm and water was registered
442 under low DIN:SRP ratio combined with high nutrient concentration (HN-A), thus confirming
443 the important role of these variables as major drivers of the biofilm geosmin production and
444 release. Moreover, our results corroborate that cyanobacteria, and more specifically
445 *Oscillatoria* sp., are responsible for geosmin production in freshwater biofilm communities. In
446 fact, the linear regression analyses performed on cyanobacteria biomass (µg chl*a*/cm^2) and
447 geosmin occurrence in biofilms and water show a significantly high relationship (**Figure 5**). In
448 our study, geosmin concentration pattern differed between the two compartments evaluated
449 (water and biofilm) over time (**Figure 1**). In HN-A treatment, geosmin in biofilm increased
450 earlier than in water (**Figure 1.A.**), and reached its maximum at t = 16d resulting in 99.1 ± 0.6%
451 of the total geosmin detected (both water and geosmin). This was the maximum geosmin
452 concentration observed in biofilm and coincided with the greater cyanobacteria abundance in
453 the biofilm (**Figure 2**). In the same treatment, the geosmin concentration in biofilm decreased
454 substantially at the end of the experiment, accounting for 86.6 ± 5.1% of total geosmin
455 detected. This was reflected in a marked increase of dissolved geosmin concentration in water
456 (**Figure 1.B.**). The decoupling observed between the presence of geosmin in biofilm and its
457 release to the water could explain the lower R^2 value obtained in the linear regression analysis
458 between cyanobacteria biomass and geosmin in water. This pattern could be related with the
459 cyanobacteria life cycle, and more specifically to their optimal geosmin production (associated
460 with the growth phase) and release time (linked to biomass decomposition and/or cell lysis),
461 since it has been described that many cyanobacteria species can produce geosmin, although

462 they may not actively release it until cell lysis occurs (Kim et al., 2018; Sabater et al., 2014).
463 Furthermore, the relative amounts of intra and extracellular portions of geosmin may also vary
464 considerably with cell age, environmental conditions and among different species (Alghanmi et
465 al., 2018). The results obtained in our study agreed with other studies carried out with
466 cyanobacterial cultures, which indicated that the highest production of geosmin occurred in
467 the late exponential growth phase, with release starting during the stationary phase and full
468 release occurring with cell's death and lysis (Saaudon et al., 2001). Another study performed
469 with *Anabaena* sp. cultures found that about 85 – 95% of the total geosmin was concentrated
470 in the cell rather than released in the cultivation medium (Alghanmi et al. 2018). A similar
471 trend was observed by Ho et al. (2012), who found that >98% of the total geosmin was
472 intracellular in untreated *Anabaena circinalis* rich waters. Nevertheless, all these studies were
473 carried out with monospecific cyanobacterial cultures, whereas our work is the first one
474 demonstrating the role of nutrient concentration and DIN:SRP ratio as the main drivers of the
475 geosmin intracellular production and release to the water from producing organisms that are
476 part of a complex benthic microbial community.

477 At the structural level, Chlorophyll *a* concentration was affected by both factors and its
478 interaction, with higher Chlorophyll *a* concentration under HN-B conditions. There was a
479 negative correlation between geosmin and Chlorophyll *a* concentration in the biofilm
480 (Pearson's correlation: $r = -0.535$; $p = 0.049$). This trend could be observed at the end of the
481 experiment (t=21d), when the highest concentration of Chlorophyll *a* (0.38 ± 0.01 ng/mg) was
482 measured in the treatment in which there was no geosmin production (HN-B) (**Figure 6**).
483 Geosmin and Chlorophyll *a* have the same metabolic pathway and it has been described that
484 when cyanobacteria start synthetizing geosmin, the production of Chlorophyll *a* decreases or
485 even stops (Cai et al., 2017). Other studies have shown that under elevated nitrate
486 concentrations, a greater amount of Chlorophyll *a* is synthesized, with a corresponding
487 decrease of geosmin synthesis (Saadoun et al., 2001). This partially agrees with our results,

since under elevated nitrogen conditions, we found the highest Chlorophyll *a* concentration. However, when phosphorus concentration also increased (lower DIN:SRP ratio), Chlorophyll *a* concentration was lower and geosmin levels increased (**Figure 6**). This evidence would confirm the fundamental role of phosphorus concentration in the geosmin production process.

The results of this study indicate that the interaction between the DIN:SRP ratio and nutrient concentration in water has a key role in the geosmin production and its release by cyanobacteria in the biofilm. Therefore, freshwater systems affected by high nutrient concentration and with an imbalance of phosphorus over nitrogen are of special concern because they are susceptible to experience geosmin episodes. These episodes could lead to bad odor and taste situations on surface waters, potentially affecting the customers trust and being a huge problem for water utilities in those rivers exploited for drinking purposes.

5. Conclusions and perspectives

Overall, this experimental study shows that both nutrient concentration and DIN:SRP ratio has a clear effect on the biofilm community structure and function, and consequently on the geosmin formation in the biofilm and its subsequent release into water. Low DIN:SRP ratio and high nutrient concentration favored the appearance of the *Oscillatoria* genus, generating the optimal conditions for geosmin production. However, it should be noted that the conclusions of this study are limited to the nutrient levels evaluated, without knowing whether the increase in cyanobacterial biomass as a function of the DIN:SRP ratio and the nutrients concentration is linear, exponential or follows some other pattern. Therefore, further investigations on this topic should evaluate wider variation of these factors to establish the trend of these behaviors with greater precision.

Our results could help drinking water companies in the forecasting and management of geosmin episodes in rivers and shallow reservoirs, where the main producers are benthic cyanobacteria, simply by carrying out the DIN:SRP ratio calculation. Furthermore, monitoring

513 the geosmin concentration in biofilm can lead to a notable increase in the ability to advance to
514 an episode of geosmin in water, since this study has shown that after a few days of detecting
515 high concentrations of geosmin in biofilm, it is released into the water. However, today
516 implementing this analysis in the laboratories of drinking water treatment companies could be
517 expensive and time-consuming. Nevertheless, given the remarkable relationship established
518 between geosmin in biofilm and cyanobacteria, by including the evaluation of biofilm
519 community's development in the regular monitoring, drinking water companies could detect
520 the increase of cyanobacteria abundances which, in a situation of higher nutrient availability
521 and low DIN:SRP ratio, could trigger the appearance of geosmin in water later on.

522 The need to predict and manage these geosmin episodes is especially important in areas with
523 Mediterranean climatic conditions under the current global change context, which is expected
524 to drastically reduce river flows as a consequence of more severe droughts. In this context, our
525 results strongly suggest a potential increase of geosmin episodes, which will be favored by the
526 increase of nutrient concentration, consequence of the decreased dilution capacity during
527 droughts.

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685

Table 1. Mean value and standard deviation (n=30) for the ammonium ($N\text{-}NH_4^+$ µg/L), nitrate ($N\text{-}NO_3^-$ µg/L), SRP ($P\text{-}PO_4^{3-}$ µg/L), the DIN:SRP ratio, pH, water temperature (°C), electrical conductivity (EC) (µS/cm), dissolved oxygen (DO) (mg/L), and oxygen saturation (%) for the treatments: low nutrient (LN) and high nutrient (HN); low ratio (A = 4:1), optimal ratio (B = 16:1) and high ratio (C = 64:1).

	Treatment					
	LN			**HN**		
	A	**B**	**C**	**A**	**B**	**C**
$N\text{-}NH_4^+$ (µg/L)	12.7 ± 2.7	12.6 ± 0.6	11.7 ± 1.7	110 ± 7	110 ± 6	106 ± 5
$N\text{-}NO_3^-$ (µg/L)	107 ± 15	108 ± 18	112 ± 5	1175 ± 112	1174 ± 90	1142 ± 24
$P\text{-}PO_4^{3-}$ (µg/L)	61.0 ± 4.8	18.2 ± 3.8	4.0 ± 0.6	682 ± 49	164 ± 9	42.0 ± 6.6
DIN:SRP	4.2 ± 0.2	14.9 ± 1.4	69.8 ± 7.2	4.4 ± 0.5	16.5 ± 1.1	64.8 ± 5.2
pH	8.1 ± 0.2	8.1 ± 0.2	8.1 ± 0.2	8.0 ± 0.3	8.0 ± 0.2	8.1 ± 0.2
Temperature (ºC)	16.8 ± 0.4	16.9 ± 0.3	16.8 ± 0.3	16.7 ± 0.3	16.8 ± 0.3	16.9 ± 0.3
EC (µS/cm)	164 ± 17	163 ± 14	158 ± 33	166 ± 13	164 ± 14	170 ± 12
DO (mg/L)	8.3 ± 0.5	8.0 ± 0.6	8.2 ± 0.5	8.2 ± 0.5	8.4 ± 0.5	8.0 ± 0.4
Saturation (%)	85 ± 5	84 ± 7	85 ± 5	84 ± 5	86 ± 5	83 ± 4

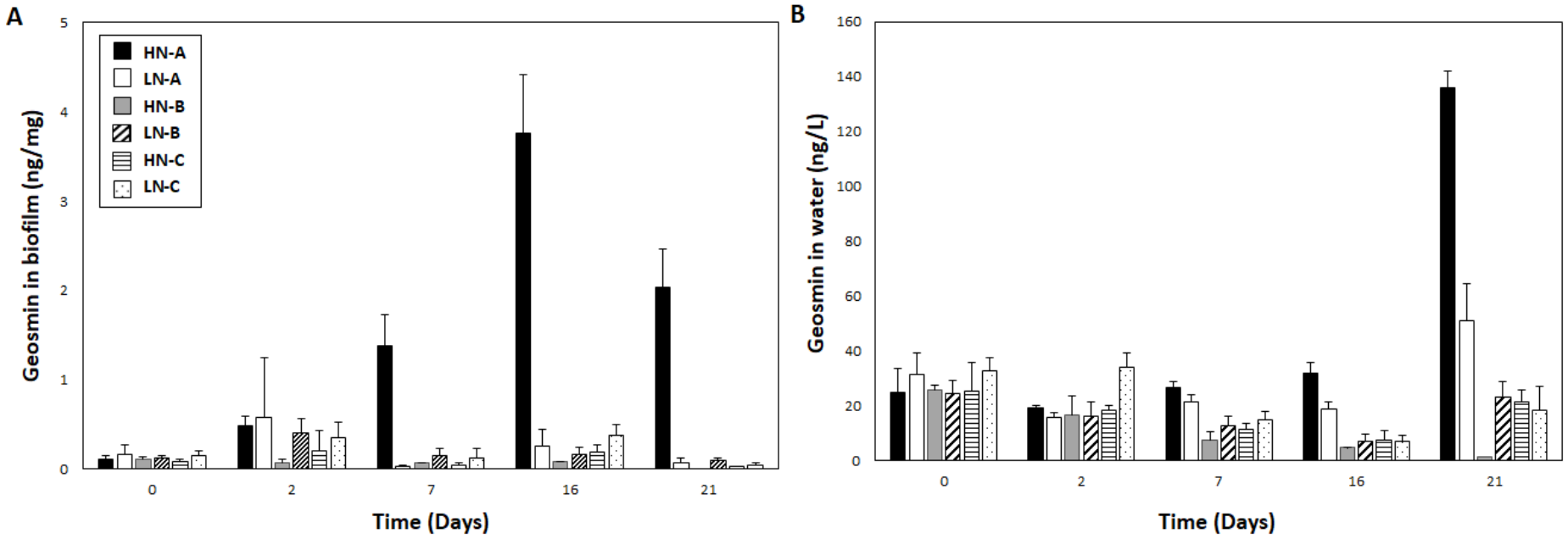
Table 2. Mean value and standard deviation for geosmin in water (ng/L), geosmin in biofilm (ng/mg), cyanobacteria and diatoms biomass (µg/cm^2), Chlorophyll *a* (µg/cm^2), photosynthetic efficiency (Yeff) and PHO (µmol MUF/cm^2·h) at the beginning of the experiment (not significant differences between treatments), and for the treatments: low nutrient (LN) and high nutrient (HN); low ratio (A = 4:1), optimal ratio (B = 16:1) and high ratio (C = 64:1), at times 7, 16 and 21 days.

	Treatment																		
	LN										**HN**								
			A			**B**			**C**			**A**			**B**			**C**	
	0d	**7d**	**16d**	**21d**	**7d**	**16d**	**21d**	**7d**	**16d**	**21d**	**7d**	**16d**	**21d**	**7d**	**16d**	**21d**	**7d**	**16d**	**21d**
Geosmin in water (ng/L)	27.4 ± 6.7	21.2 ± 2.9	18.7 ± 2.8	20.9 ± 13.6	12.8 ± 3.4	7.0 ± 2.8	23.3 ± 5.4	14.8 ± 2.9	7.2 ± 2.1	18.4 ± 8.7	26.6 ± 2.2	32.0 ± 3.9	135.8 ± 6.1	7.5 ± 3.1	4.8 ± 0.0	< 2.5	11.6 ± 1.9	7.5 ± 3.5	21.5 ± 4.2
Geosmin in biofilm (ng/mg)	0.10 ± 0.05	0.03 ± 0.01	0.26 ± 0.19	0.08 ± 0.04	0.16 ± 0.08	0.17 ± 0.07	0.10 ± 0.03	0.13 ± 0.1	0.38 ± 0.12	0.04 ± 0.03	1.39 ± 0.35	3.76 ± 0.96	2.03 ± 0.74	0.08 ± 0.00	0.09 ± 0.00	0.00 ± 0.00	0.04 ± 0.03	0.20 ± 0.07	0.03 ± 0.00
Cyanobacteria (µg chl*a*/cm^2)	0.43 ± 0.06	1.23 ± 0.71	0.61 ± 0.27	0.55 ± 0.06	0.28 ± 0.22	0.38 ± 0.21	0.48 ± 0.20	0.52 ± 0.30	0.52 ± 0.10	0.39 ± 0.20	1.95 ± 0.36	1.63 ± 0.36	0.87 ± 0.38	0.28 ± 0.16	0.57 ± 0.15	0.46 ± 0.05	0,66 ± 0.23	0.34 ± 0.31	0.59 ± 0.14
Diatoms (µg chl*a*/cm^2)	2.10 ± 0.60	2.68 ± 0.42	1.55 ± 0.82	1.61 ± 0.45	2.37 ± 0.99	1.62 ± 0.63	1.63 ± 0.35	3.00 ± 2.31	2.66 ± 1.29	1.61 ± 0.51	3.49 ± 0.40	2.14 ± 1.12	2.33 ± 1.90	1.65 ± 1.02	4.35 ± 1.05	2.99 ± 2.52	2.00 ± 0.51	1.66 ± 1.69	3.38 ± 0.75
Chlorophyll *a* (µg/cm^2)	3.90 ± 0.64	3.50 ± 1.31	3.10 ± 1.89	6.51 ± 0.51	2.63 ± 0.48	3.82 ± 1.19	4.20 ± 0.92	7.87 ± 0.88	4.14 ± 1.38	2.80 ± 0.70	2.87 ± 0.81	3.68 ± 0.32	8.68 ± 1.73	5.12 ± 2.45	7.99 ± 1.32	18.32 ± 0.97	5.10 ± 0.28	11.72 ± 2.31	10.00 ± 3.37
Yeff	0.589 ± 0.029	0.646 ± 0.064	0.581 ± 0.083	0.640 ± 0.053	0.614 ± 0.036	0.635 ± 0.068	0.680 ± 0.040	0.639 ± 0.047	0.555 ± 0.056	0.645 ± 0.033	0.630 ± 0.084	0.545 ± 0.042	0.529 ± 0.046	0.607 ± 0.083	0.657 ± 0.025	0.672 ± 0.031	0.633 ± 0.031	0.615 ± 0.036	0.631 ± 0.068
PHO (MUF/ cm^2·h)	19.7 ± 1.9	16.8 ± 7.7	16.5 ± 9.4	24.2 ± 4.2	13.0 ± 1.6	23.6 ± 12.9	29.3 ± 11.6	33.2 ± 8.5	42.0 ± 20.1	31.6 ± 11.7	17.4 ± 9.6	25.6 ± 9.2	35.9 ± 11.7	24.2 ± 8.3	17.5 ± 7.9	36.1 ± 6.5	26.9 ± 14.2	54.4 ± 28.8	72.0 ± 18.4

699 **Table 3**. Statistical F and p value for the two-way repeated measures ANOVA and the two-way
700 ANOVA on physicochemical and biological data. Sampling time t=0d and t=2d have not been
701 included since there were not statistical differences for any of the variables evaluated. The
702 factors evaluated were nutrient concentration (N), nutrient ratio (R) and their interaction
703 (NxR) as independent variables, and geosmin in water (ng/L), geosmin in biofilm (ng/mg),
704 cyanobacteria and diatoms biomass (µg chl*a*/cm^2), Chlorophyll *a* (µg/cm^2), photosynthetic
705 efficiency (Yeff) and PHO (µmol MUF/cm^2·h) as dependent variables.

		Repeated measures ANOVA		**ANOVA t = 7d**		**t = 16d**		**t = 21d**	
		F	p	F	p	F	p	F	p
Geosmin in Water (ng/L)	**N**	9.22	<0.001	0.34	n.s.	4.22	n.s.	21.65	0.001
	R	37.03	<0.001	22.54	<0.001	50.42	<0.001	126.28	<0.001
	NxR	15.30	<0.001	3.44	n.s.	7.47	0.01	50.05	<0.001
Geosmin in Biofilm (ng/mg)	**N**	26.34	<0.001	22.40	0.001	30.63	0.001	6.90	<0.05
	R	22.00;	<0.001	25.14	<0.001	39.07	<0.001	9.64	0.01
	NxR	24.89	<0.001	35.33	<0.001	39.95	<0.001	9.67	0.01
Cyanobacteria (µg chl*a*/cm^2)	**N**	2.80	<0.05	0.81	n.s.	5.08	<0.05	0.12	n.s.
	R	6.45	<0.001	5.28	<0.05	33.67	<0.001	3.44	n.s.
	NxR	1.53	n.s.	0.09	n.s.	7.75	<0.01	0.55	n.s.
Diatoms (µg chl*a*/cm^2)	**N**	0.98	n.s.	0.93	n.s.	1.75	n.s.	0.73	n.s.
	R	3.16	<0.001	4.39	<0.05	28.45	<0.001	3.44	n.s.
	NxR	1.76	n.s.	0.13	n.s.	12.00	<0.01	1.18	n.s.
Chlorophyll *a* (µg/cm^2)	**N**	41.04	<0.001	0.28	n.s.	14.82	<0.01	110.16	<0.001
	R	13.74	<0.001	12.08	<0.01	6.34	<0.05	14.53	0.001
	NxR	13.74	<0.001	6.57	<0.05	3.74	n.s.	22.19	<0.001
Yeff	**N**	3.72	0.01	0.78	n.s.	4.59	n.s.	2.06	n.s.
	R	4.86	<0.001	0.30	n.s.	6.00	<0.05	5.24	<0.05
	NxR	2.23	<0.05	0.02	n.s.	0.99	n.s.	3.16	n.s.
PHO (µmol MUF/ cm^2·h)	**N**	1.82	n.s.	0.17	n.s.	0.38	n.s.	11.17	<0.01
	R	0.73	n.s.	3.48	n.s.	4.96	<0.05	5.77	<0.05
	NxR	1.14	n.s.	1.22	n.s.	0.43	n.s.	3.29	n.s.

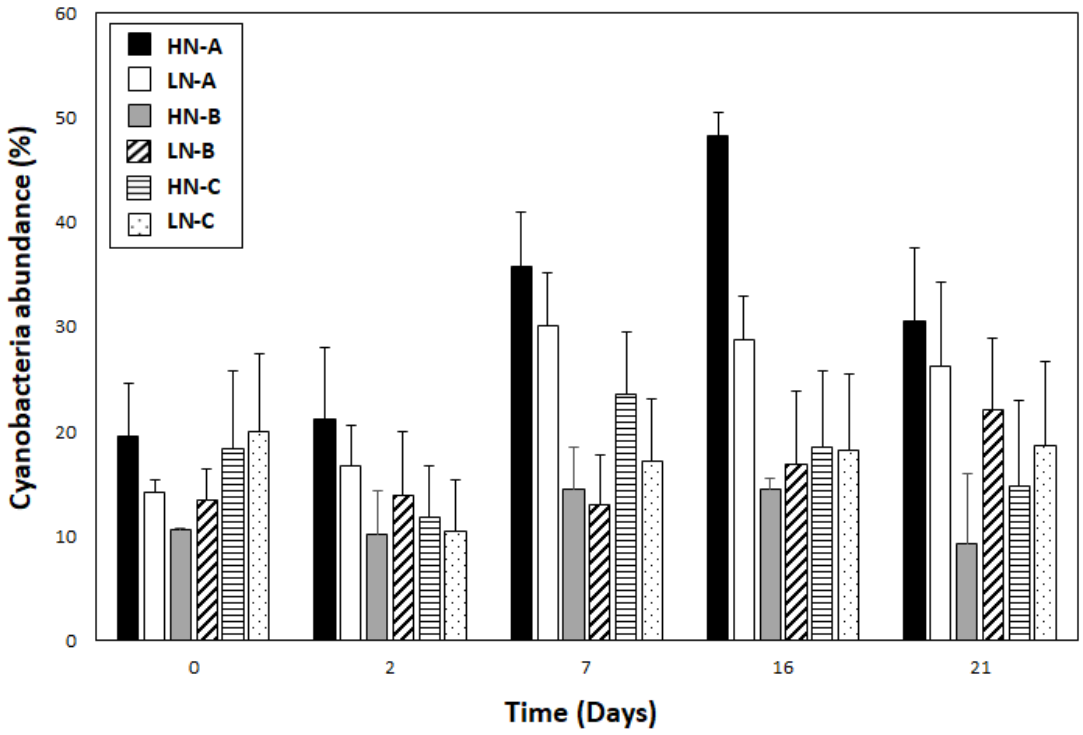
706



707

708 **Figure 1.** Mean values and standard deviation on sampling days t =0, 2, 7 16 and 21 days, for each treatment (HN-A, LN-A, HN-B, LN-B, HN-C and LN-C) for **A**.

709 Geosmin concentration in biofilm (ng/mg) and **B**. Geosmin concentration in water (ng/L).



710

711 **Figure 2.** Mean values and standard deviation of the cyanobacteria relative abundance (in %)

712 given by the BenthoTorch for the sampling days (t = 0, 2, 7 16 and 21 days) for each treatment:

713 HN-A, LN-A, HN-B, LN-B, HN-C and LN-C.

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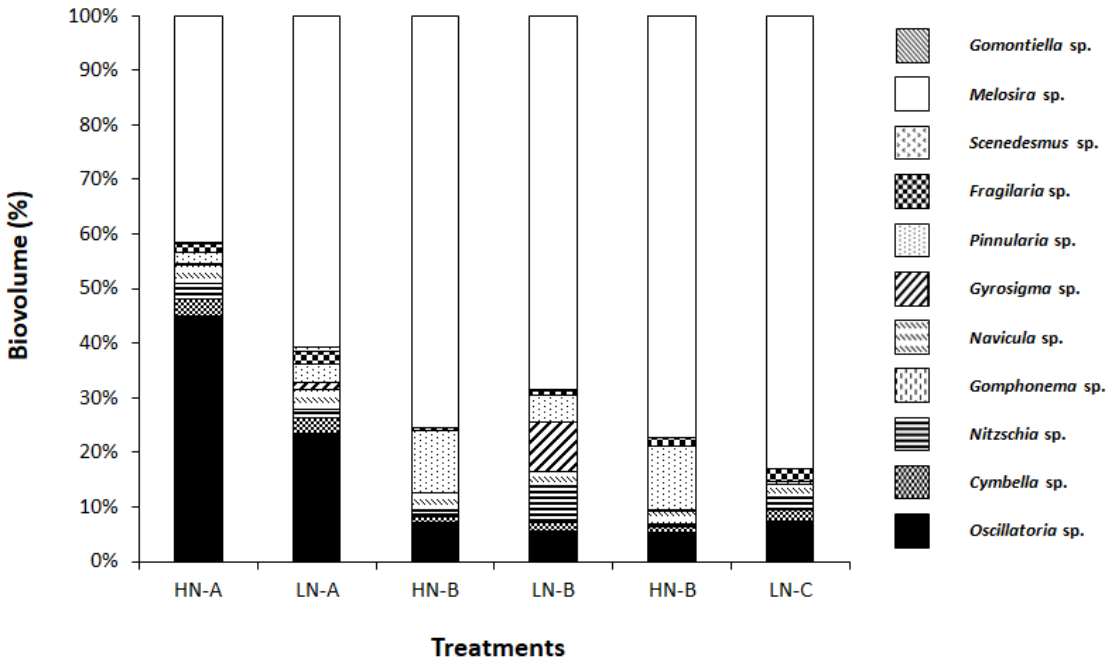

716

717 **Figure 3.** Relative taxa expressed as biovolume (in %) present in the biofilm of each treatment:

718 HN-A, LN-A, HN-B, LN-B, HN-C and LN-C.

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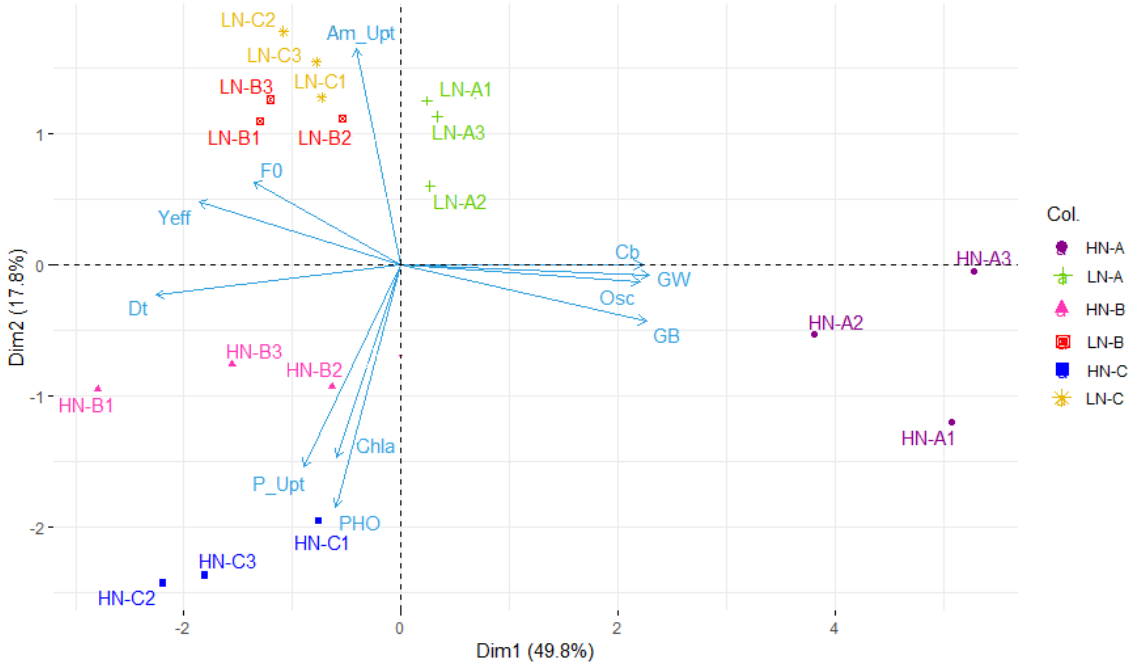
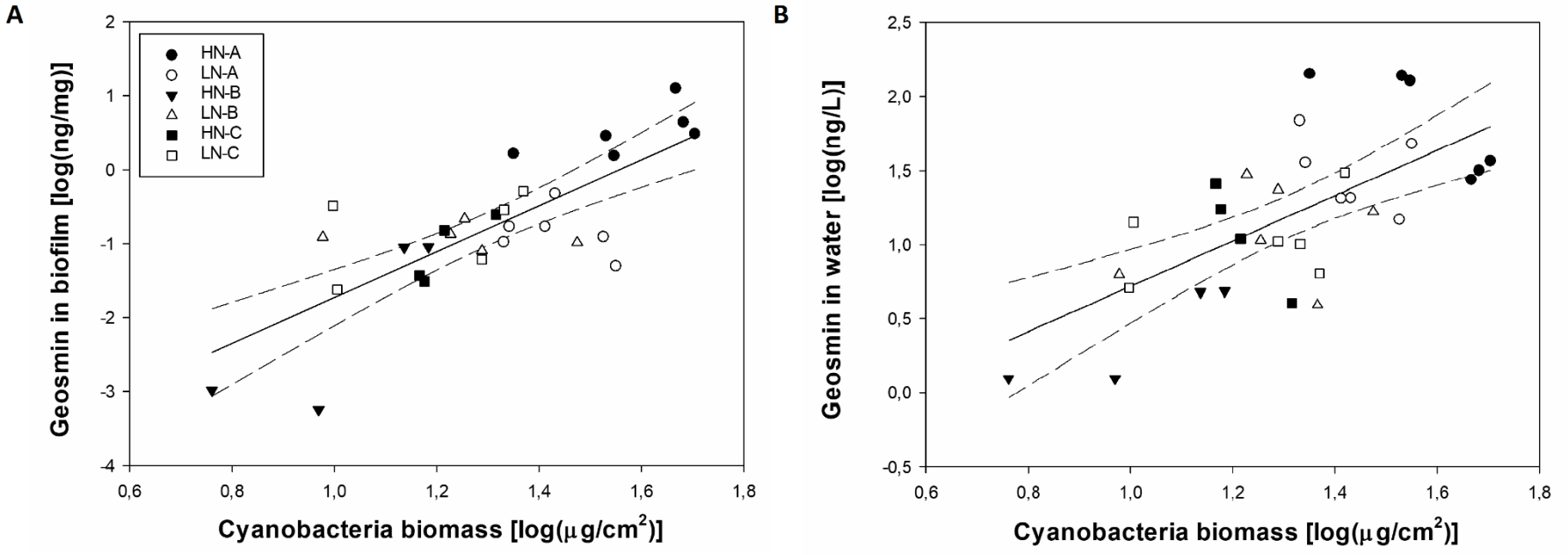


723

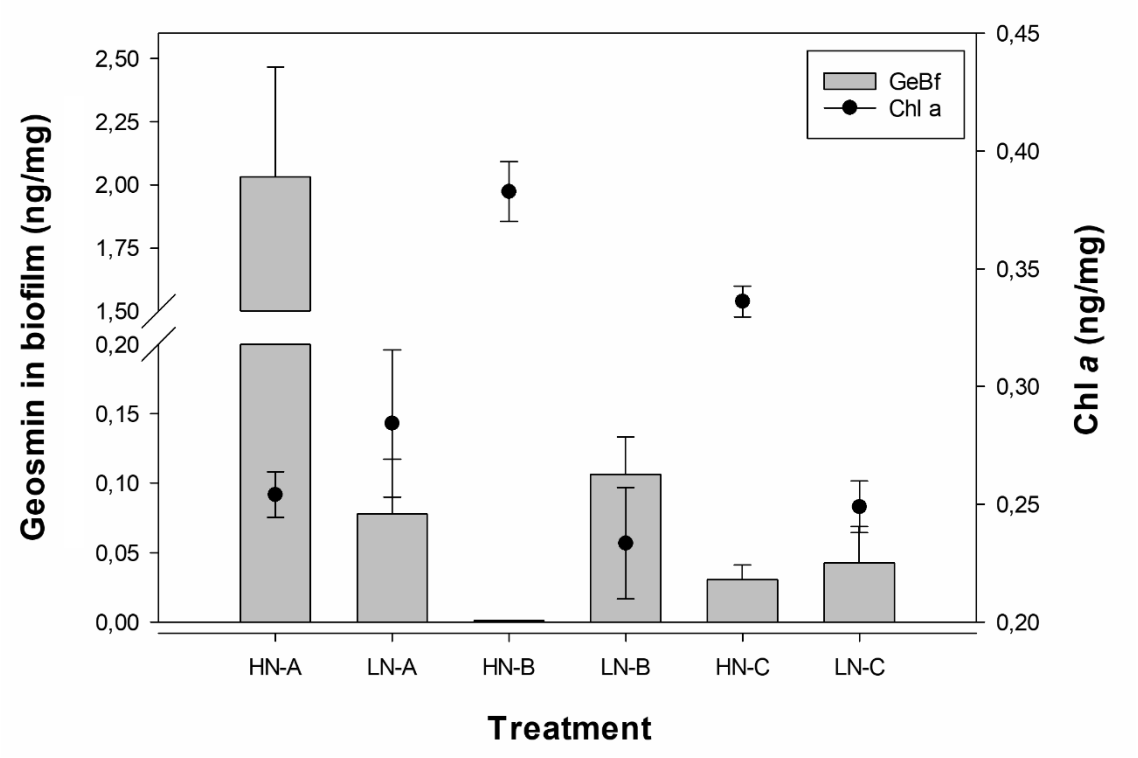
724 **Figure 4**. Principal Component Analysis showing treatments distribution based on the variables
725 evaluated. Axes 1 and 2 combined explain 67.6% of the variance. Treatments: HN-A, LN-A, HN-
726 B, LN-B, HN-C and LN-C. Variables: geosmin in biofilm (GB), geosmin in water (GW),
727 cyanobacteria biomass (Cb), diatoms biomass (Dt), *Oscillatoria* sp. (Osc), photosynthetic
728 efficiency (Yeff), Chlorophyll a (Chla), SRP uptake (P-Upt), ammonium uptake (Am_Upt) and
729 phosphatase activity (PHO).



730

731 **Figure 5.** Relationship between **(A)** log geosmin in biofilm (ng/mg) and log cyanobacteria biomass (µg/cm^2) (R^2 = 0.59, F = 40.35, p < 0.0001) and **(B)** log

732 geosmin in water (ng/L) and log cyanobacteria biomass (µg/cm^2) (R^2 = 0.43, F = 22.76, p < 0.0001), at t =16d and t =21d.



733

734 **Figure 6.** Mean values and standard deviation at t=21d for each treatment (HN-A, LN-A, HN-B,

735 LN-B, HN-C and LN-C) of the geosmin concentration in biofilm (ng/mg) vs. Chlorophyll *a*

736 concentration in biofilm (ng/mg).

Table

	Treatment					
	LN			HN		
	A	B	C	A	B	C
$N\text{-}NH_4^+$ (µg/L)	12.7 ± 2.7	12.6 ± 0.6	11.7 ± 1.7	110 ± 7	110 ± 6	106 ± 5
$N\text{-}NO_3^-$ (µg/L)	107 ± 15	108 ± 18	112 ± 5	1175 ± 112	1174 ± 90	1142 ± 24
$P\text{-}PO_4^{3-}$ (µg/L)	61.0 ± 4.8	18.2 ± 3.8	4.0 ± 0.6	682 ± 49	164 ± 9	42.0 ± 6.6
N:P	4.2 ± 0.2	14.9 ± 1.4	69.8 ± 7.2	4.4 ± 0.5	16.5 ± 1.1	64.8 ± 5.2
pH	8.1 ± 0.2	8.1 ± 0.2	8.1 ± 0.2	8.0 ± 0.3	8.0 ± 0.2	8.1 ± 0.2
Temperature (ºC)	16.8 ± 0.4	16.9 ± 0.3	16.8 ± 0.3	16.7 ± 0.3	16.8 ± 0.3	16.9 ± 0.3
EC (µS/cm)	164 ± 17	163 ± 14	158 ± 33	166 ± 13	164 ± 14	170 ± 12
OD (mg/L)	8.3 ± 0.5	8.0 ± 0.6	8.2 ± 0.5	8.2 ± 0.5	8.4 ± 0.5	8.0 ± 0.4
Saturation (%)	85 ± 5	84 ± 7	85 ± 5	84 ± 5	86 ± 5	83 ± 4

Table

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	Treatment																		
	LN										HN								
	A				B			C			A			B			C		
	0d	7d	16d	21d	7d	16d	21d	7d	16d	21d	7d	16d	21d	7d	16d	21d	7d	16d	21d
Geosmin in water (ng/L)	27.4 ± 6.7	21.2 ± 2.9	18.7 ± 2.8	20.9 ± 13.6	12.8 ± 3.4	7.0 ± 2.8	23.3 ± 5.4	14.8 ± 2.9	7.2 ± 2.1	18.4 ± 8.7	26.6 ± 2.2	32.0 ± 3.9	135.8 ± 6.1	7.5 ± 3.1	4.8 ± 0.0	< 2.5	11.6 ± 1.9	7.5 ± 3.5	21.5 ± 4.2
Geosmin in biofilm (ng/mg)	0.10 ± 0.05	0.03 ± 0.01	0.26 ± 0.19	0.08 ± 0.04	0.16 ± 0.08	0.17 ± 0.07	0.10 ± 0.03	0.13 ± 0.1	0.38 ± 0.12	0.04 ± 0.03	1.39 ± 0.35	3.76 ± 0.96	2.03 ± 0.74	0.08 ± 0.00	0.09 ± 0.00	0.00 ± 0.00	0.04 ± 0.03	0.20 ± 0.07	0.03 ± 0.00
Cyanobacteria (µg chl*a*/cm²)	0.43 ± 0.06	1.23 ± 0.71	0.61 ± 0.27	0.55 ± 0.06	0.28 ± 0.22	0.38 ± 0.21	0.48 ± 0.20	0.52 ± 0.30	0.52 ± 0.10	0.39 ± 0.20	1.95 ± 0.36	1.63 ± 0.36	0.87 ± 0.38	0.28 ± 0.16	0.57 ± 0.15	0.46 ± 0.05	0,66 ± 0.23	0.34 ± 0.31	0.59 ± 0.14
Diatoms (µg chl*a*/cm²)	2.10 ± 0.60	2.68 ± 0.42	1.55 ± 0.82	1.61 ± 0.45	2.37 ± 0.99	1.62 ± 0.63	1.63 ± 0.35	3.00 ± 2.31	2.66 ± 1.29	1.61 ± 0.51	3.49 ± 0.40	2.14 ± 1.12	2.33 ± 1.90	1.65 ± 1.02	4.35 ± 1.05	2.99 ± 2.52	2.00 ± 0.51	1.66 ± 1.69	3.38 ± 0.75
Chlorophyll *a* (µg/cm²)	3.90 ± 0.64	3.50 ± 1.31	3.10 ± 1.89	6.51 ± 0.51	2.63 ± 0.48	3.82 ± 1.19	4.20 ± 0.92	7.87 ± 0.88	4.14 ± 1.38	2.80 ± 0.70	2.87 ± 0.81	3.68 ± 0.32	8.68 ± 1.73	5.12 ± 2.45	7.99 ± 1.32	18.32 ± 0.97	5.10 ± 0.28	11.72 ± 2.31	10.00 ± 3.37
Yeff	0.589 ± 0.029	0.646 ± 0.064	0.581 ± 0.083	0.640 ± 0.053	0.614 ± 0.036	0.635 ± 0.068	0.680 ± 0.040	0.639 ± 0.047	0.555 ± 0.056	0.645 ± 0.033	0.630 ± 0.084	0.545 ± 0.042	0.529 ± 0.046	0.607 ± 0.083	0.657 ± 0.025	0.672 ± 0.031	0.633 ± 0.031	0.615 ± 0.036	0.631 ± 0.068
PHO (MUF/cm²·h)	19.7 ± 1.9	16.8 ± 7.7	16.5 ± 9.4	24.2 ± 4.2	13.0 ± 1.6	23.6 ± 12.9	29.3 ± 11.6	33.2 ± 8.5	42.0 ± 20.1	31.6 ± 11.7	17.4 ± 9.6	25.6 ± 9.2	35.9 ± 11.7	24.2 ± 8.3	17.5 ± 7.9	36.1 ± 6.5	26.9 ± 14.2	54.4 ± 28.8	72.0 ± 18.4

Table

		Repeated measures ANOVA		ANOVA t = 7d		t = 16d		t = 21d	
		F	p	F	p	F	p	F	p
Geosmin in Water (ng/L)	N	9.22	<0.001	0.34	n.s.	4.22	n.s.	21.65	0.001
	R	37.03	<0.001	22.54	<0.001	50.42	<0.001	126.28	<0.001
	NxR	15.30	<0.001	3.44	n.s.	7.47	0.01	50.05	<0.001
Geosmin in Biofilm (ng/mg)	N	26.34	<0.001	22.40	0.001	30.63	0.001	6.90	<0.05
	R	22.00;	<0.001	25.14	<0.001	39.07	<0.001	9.64	0.01
	NxR	24.89	<0.001	35.33	<0.001	39.95	<0.001	9.67	0.01
Cyanobacteria (µg chl*a*/cm²)	N	2.80	<0.05	0.81	n.s.	5.08	<0.05	0.12	n.s.
	R	6.45	<0.001	5.28	<0.05	33.67	<0.001	3.44	n.s.
	NxR	1.53	n.s.	0.09	n.s.	7.75	<0.01	0.55	n.s.
Diatoms (µg chl*a*/cm²)	N	0.98	n.s.	0.93	n.s.	1.75	n.s.	0.73	n.s.
	R	3.16	<0.001	4.39	<0.05	28.45	<0.001	3.44	n.s.
	NxR	1.76	n.s.	0.13	n.s.	12.00	<0.01	1.18	n.s.
Chlorophyll *a* (µg/cm²)	N	41.04	<0.001	0.28	n.s.	14.82	<0.01	110.16	<0.001
	R	13.74	<0.001	12.08	<0.01	6.34	<0.05	14.53	0.001
	NxR	13.74	<0.001	6.57	<0.05	3.74	n.s.	22.19	<0.001
Yeff	N	3.72	0.01	0.78	n.s.	4.59	n.s.	2.06	n.s.
	R	4.86	<0.001	0.30	n.s.	6.00	<0.05	5.24	<0.05
	NxR	2.23	<0.05	0.02	n.s.	0.99	n.s.	3.16	n.s.
PHO (µmol MUF/ cm²·h)	N	1.82	n.s.	0.17	n.s.	0.38	n.s.	11.17	<0.01
	R	0.73	n.s.	3.48	n.s.	4.96	<0.05	5.77	<0.05
	NxR	1.14	n.s.	1.22	n.s.	0.43	n.s.	3.29	n.s.

Figure 1_review

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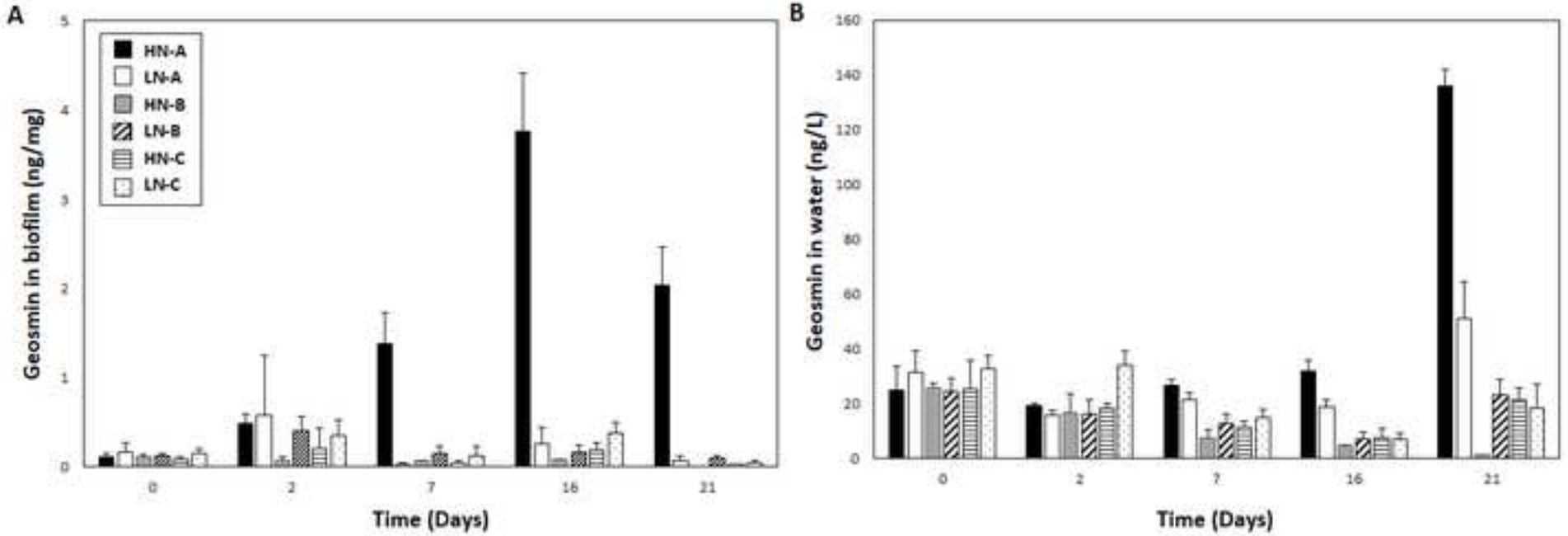
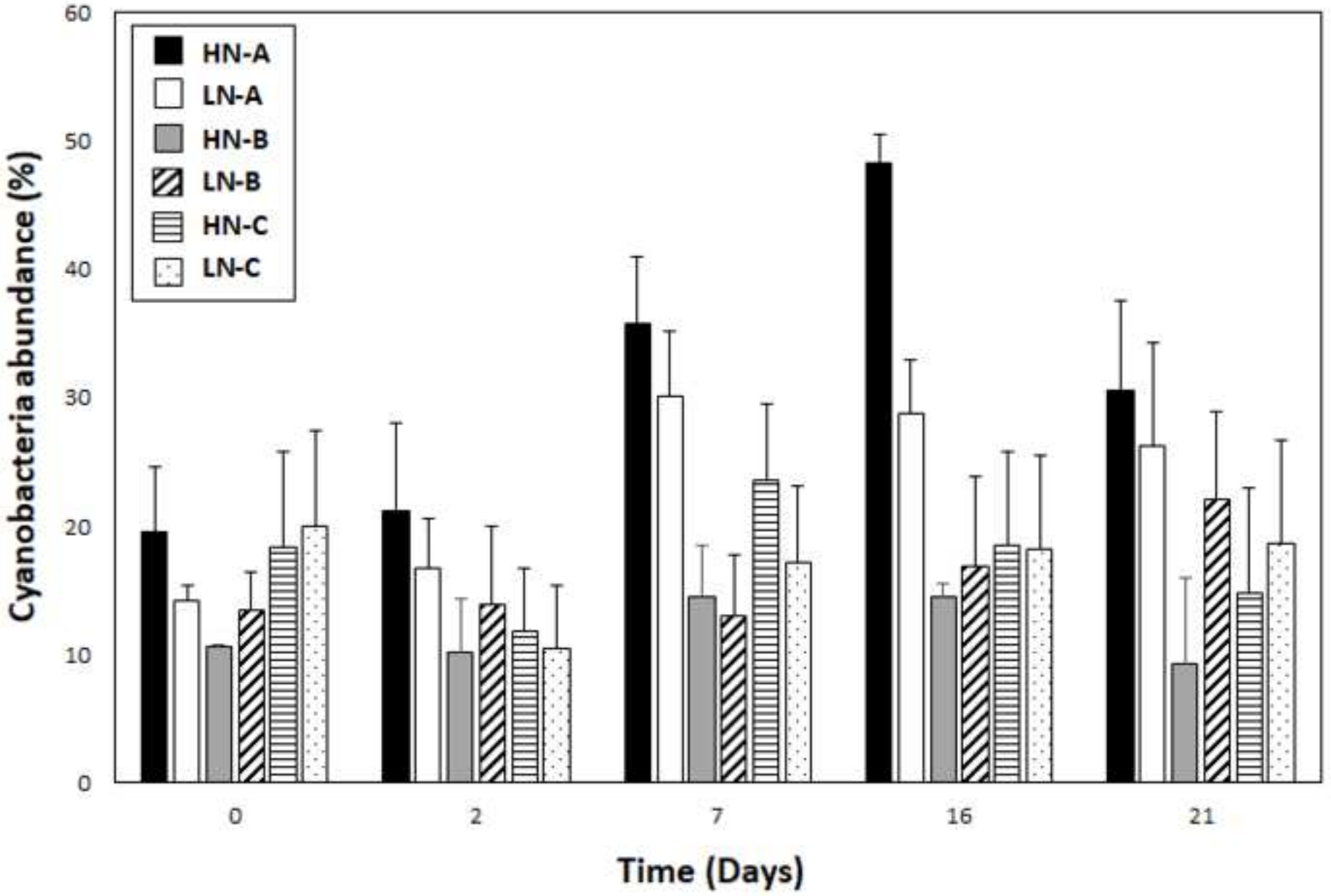
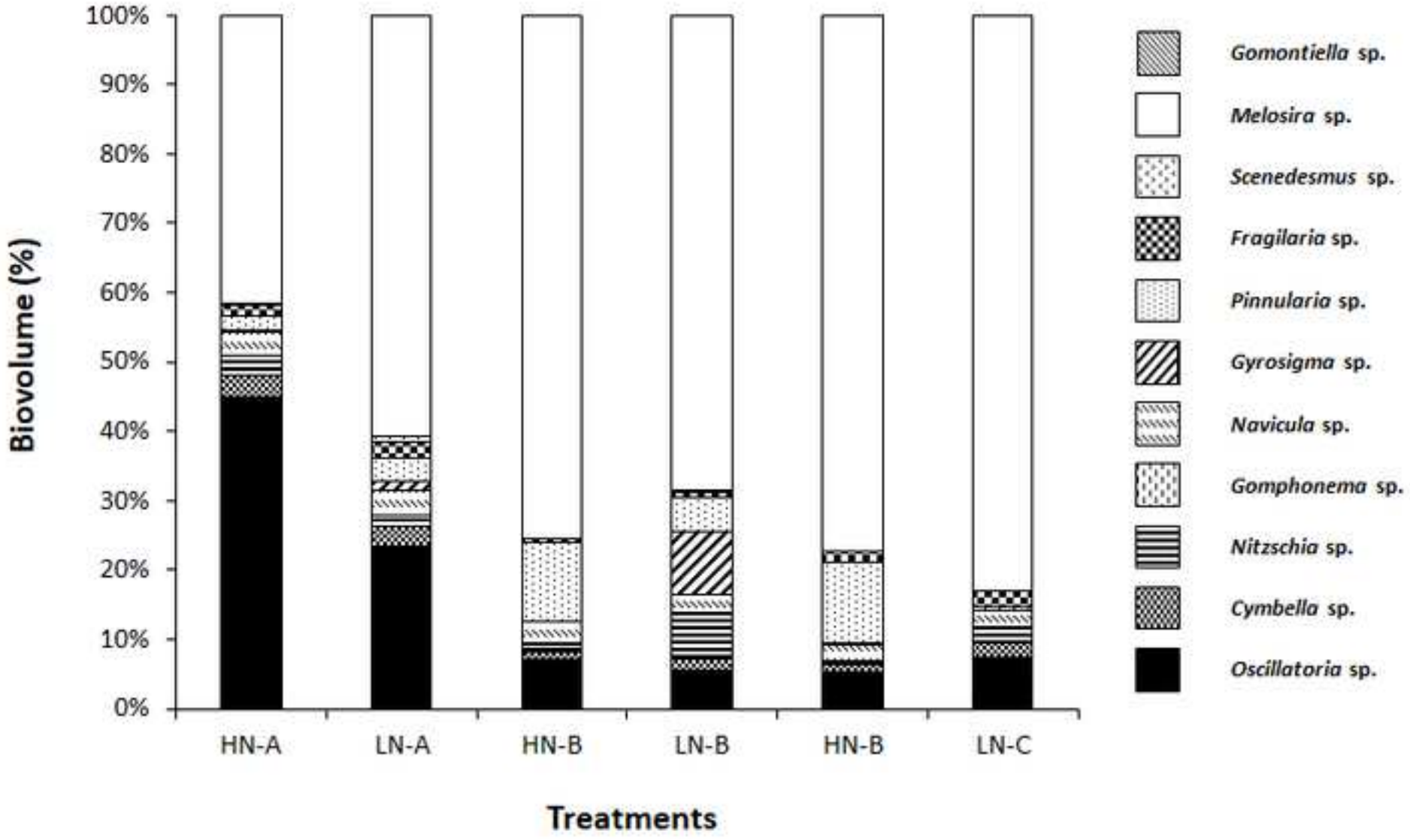


Figure 2_review

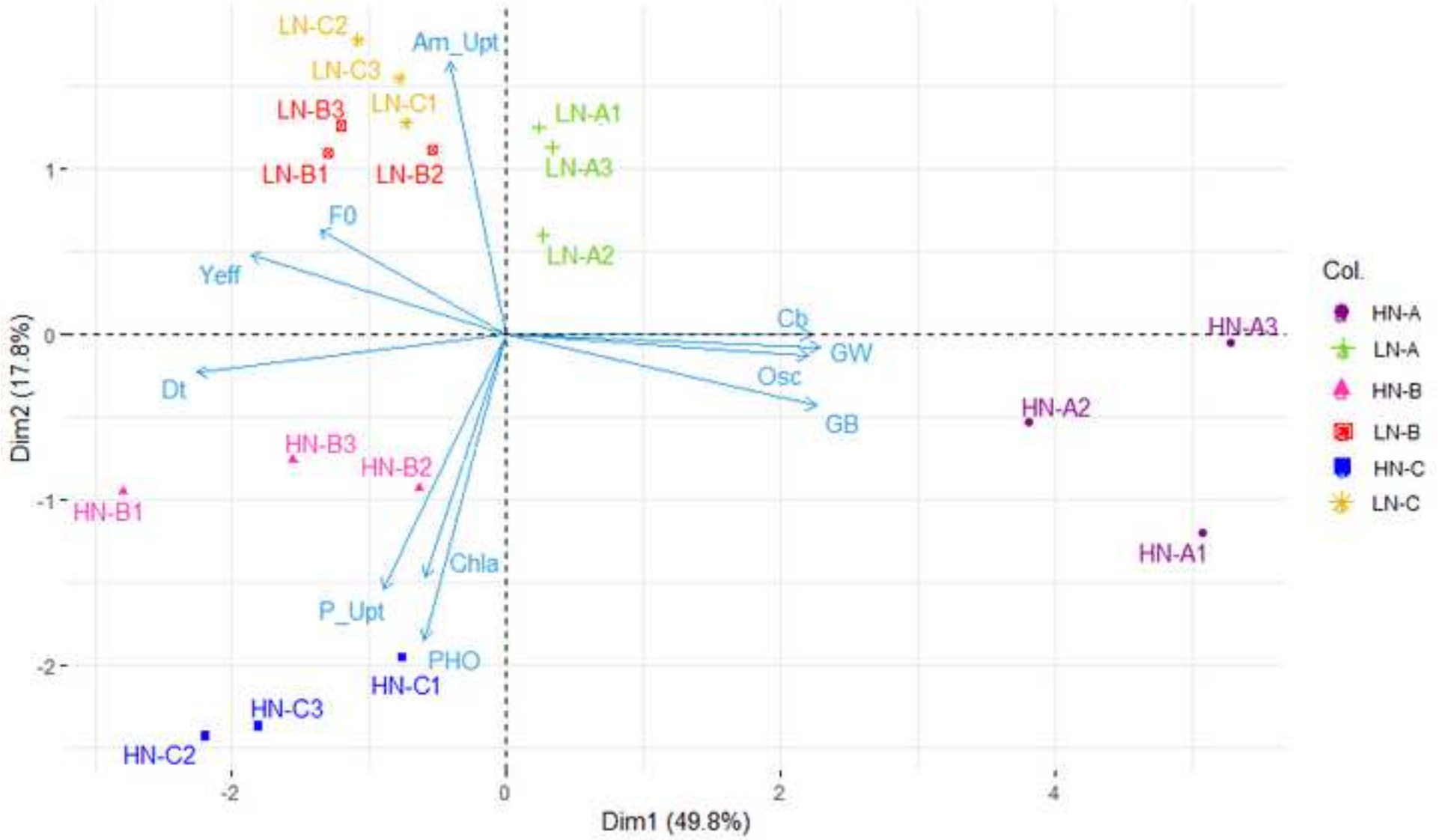
Click here to access/download;Figure;Figure 2_201029.tif





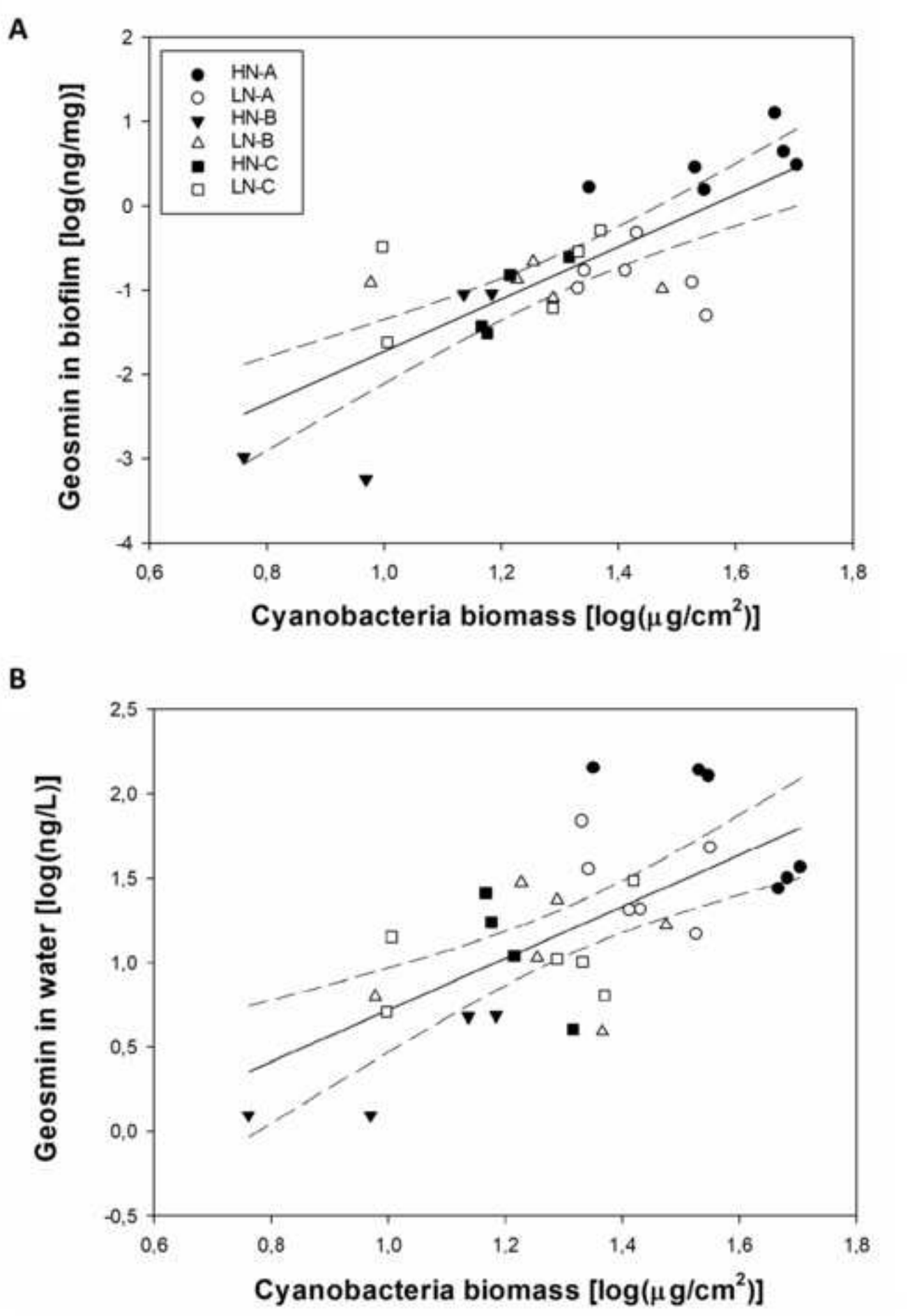
Figure

Click here to access/download;Figure;Figure 4.tiff



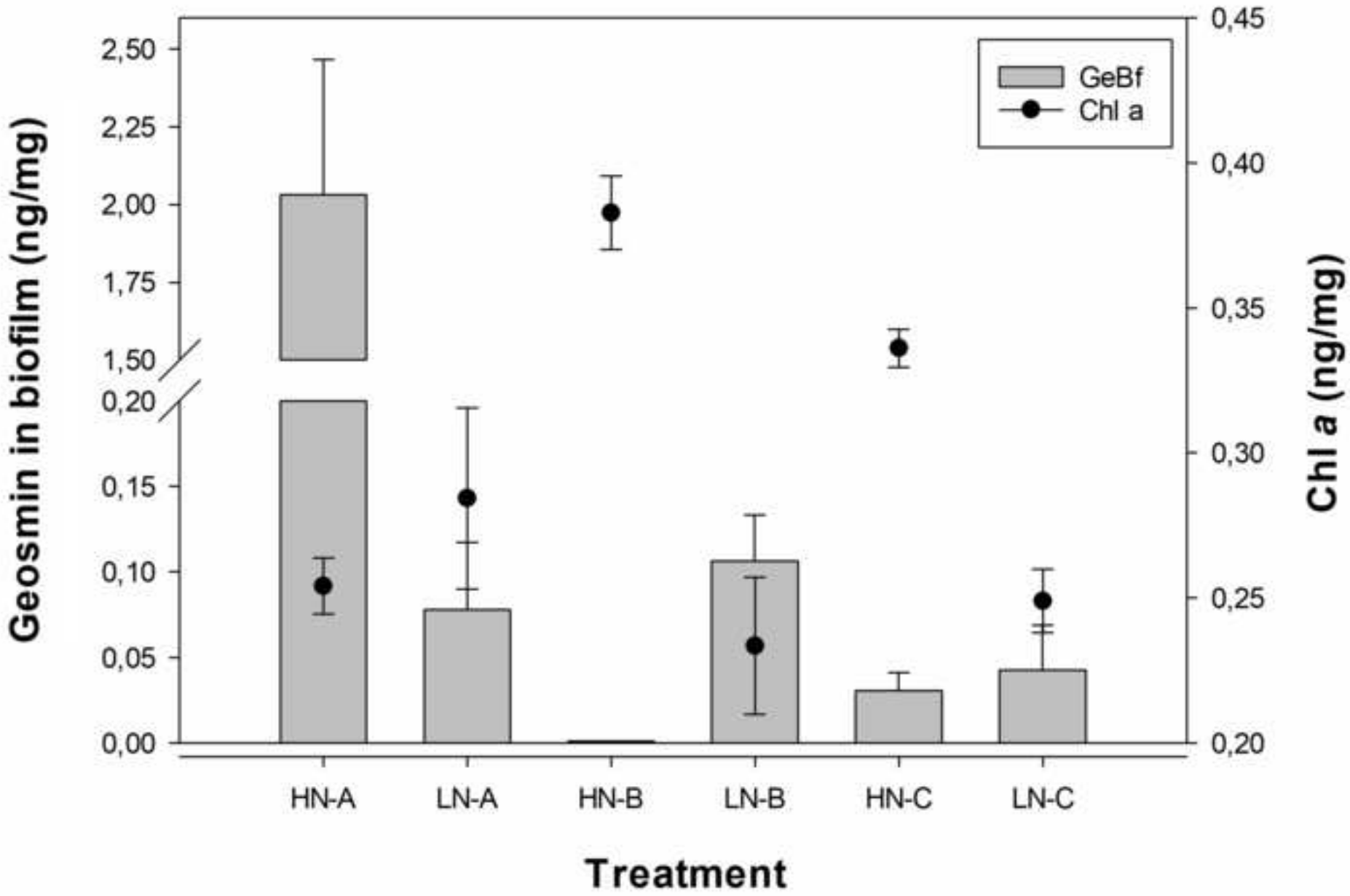
Figure

Click here to access/download;Figure;Figure 5.tif



Figure

Click here to access/download;Figure;Figure 6.tif



Credit Author Statement

CE: Conceptualization, Methodology, Writing-Original Draft Preparation, **MA**: Conceptualization, Investigation, **SP**: Supervision, **MR**: Investigation, Resources, **LV-P**: Investigation, Methodology, **MO**: Supervision, **LL**: Supervision, **LP**: Conceptualization, Investigation, Writing.

*Declaration of Interest Statement

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.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: