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1 **Nitrogen as the main driver of benthic diatom composition and diversity in oligotrophic**  
2 **coastal systems**

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24 *Cocconeis*

25 **Abstract**

26

27 Phytoplankton is the main indicator group for eutrophication in coastal ecosystems,  
28 however its high dispersal potential does not enable the assessment of localized effects of  
29 coastal nutrient enrichment. Benthic diatoms are sessile microalgae associated with sandy  
30 substrates and have the potential to reflect more localized pollution impacts. Although  
31 benthic diatoms are widely used bioindicators in freshwater systems, they have rarely been  
32 used for assessing the eutrophication status of oligotrophic environments such as the  
33 eastern Mediterranean Sea. In the present study, we assess the efficiency of benthic  
34 diatoms as bioindicators of nutrient enrichment in oligotrophic coastal systems, by  
35 investigating the effect of different physicochemical conditions and nutrient concentrations  
36 on the assemblage composition, diversity and individual species populations. To do this, we  
37 sampled along a eutrophication gradient formed by anthropogenic nutrient inputs from a  
38 metropolitan area. The main driver of assemblage composition, diversity and biomass of  
39 diatoms was nitrogen concentration and its temporal and spatial changes. Nitrogen loadings  
40 were positively correlated with increased biomass of *Cocconeis* spp. and negatively  
41 correlated with *Mastogloia* spp.. Our findings suggest that in coastal ecosystems of  
42 oligotrophic marine ecoregions, benthic diatom assemblage structure and specific  
43 taxonomic groups can be reliable predictors of coastal eutrophication offering higher spatial  
44 resolution compared to phytoplankton.

45

46 **1. Introduction**

47

48 Coastal ecosystems play an important ecological role, hosting a high number of species and  
49 largely contributing to global productivity (Cloern et al. 2013). At the same time, they are  
50 important from a socio-economic point of view, since they provide multiple goods and  
51 services (e.g. fisheries, recreation, tourism). However, anthropogenic activities such as  
52 urbanization and intensive agriculture often lead to increased nutrient runoff, which can  
53 affect ecosystem health due to eutrophication and harmful algal blooms. Eutrophication  
54 poses a greater risk on oligotrophic systems, such as the ultra-oligotrophic Eastern  
55 Mediterranean (Tsirtsis et al. 2008). In these systems, species composition of primary  
56 producers such as microalgae reflects low nutrient concentrations that are prevalent during  
57 most of the year. However, increased nutrient loads could change the nutrient  
58 stoichiometry, causing shifts in the composition and productivity of microalgal assemblages  
59 with significant impacts on higher trophic levels and ecosystem goods and services  
60 (Hillebrand & Kahlert 2001). Due to the susceptibility of these oligotrophic systems to  
61 episodic events of nutrient enrichment (Spatharis et al. 2007, 2008) it is imperative to  
62 establish the causal mechanisms as well as to reveal bioindicator species, sensitive to detect  
63 such community shifts.

64

65 Phytoplankton is an efficient indicator group for coastal eutrophication (e.g. Spatharis &  
66 Tsirtsis 2010) and is routinely being used during the Water Framework Directive  
67 (2000/60/EC) assessments of coastal water quality (EC, 2000). However, because it can be  
68 passively dispersed via currents, it is not able to reflect finer scale coastal eutrophication hot  
69 spots that are due to point sources of pollution or hydrodynamic isolation. On the other

70 hand, benthic microalgae, in their majority diatoms, should be able to reflect impacts of  
71 point source coastal pollution at a higher spatial resolution compared to their planktic  
72 counterparts due to their limited dispersal potential. Due to their short generation times,  
73 they can reflect changes in nutrient levels through shifts in their species composition,  
74 abundance and diversity (Morin et al. 2016). Although benthic diatoms are extensively used  
75 in freshwater ecosystems, as efficient indicators of nutrient loading and ecological water  
76 quality during water monitoring programs (Prygiel & Coste 1996, Kelly et al. 1995, 1998),  
77 very little is known on their effectiveness as an indicator group in oligotrophic coastal  
78 ecosystems. By increasing our knowledge on ecological preferences of the different species,  
79 we could help establish the use of benthic diatoms in Water Framework Directive  
80 assessments of coastal waters.

81

82 Despite the importance of marine benthic diatoms in the functioning of coastal ecosystems  
83 (Van den Hoek et al. 1979, MacIntyre et al. 1996, Cahoon 1999), little is known on  
84 environmental factors driving their structure and composition (e.g. Cibic et al. 2007, 2011,  
85 2012). The focus of previous studies was mainly on ecosystem characterized of tidal flats  
86 (e.g. Agatz et al. 1999), estuaries and brackish waters such as Venice lagoon (Facca & Sfriso  
87 2007) or on extreme environments (e.g. gas and thermal vents in the Aeolian Islands-  
88 Rogelja et al. 2016). In such nutrient-rich systems, salinity and light can play a significant role  
89 in driving benthic diatom assemblage structure (e.g. Admiraal 1984, Underwood 1994,  
90 Underwood et al. 1998, Hillebrand & Sommer 1997, Weckstrom & Juggins 2005, Borja &  
91 Dauer 2008, Ulanova et al. 2009, Du et al. 2017). However, primary producers at mid-  
92 latitudes tend to be less limited by light and more by the availability of nutrients (Moore et  
93 al. 2013). In this context, drivers of change in benthic diatom assemblages, as well as the

94 autecology of the benthic diatom species along gradients of nutrient enrichment, have been  
95 largely overlooked (Cibic et al. 2011, Desrosiers et al. 2013). Addressing this question can  
96 help establish marine benthic diatoms as an efficient tool for water quality assessment in  
97 coastal ecosystems.

98

99 This study aims to assess the potential of benthic diatoms to detect small scale spatial  
100 differences in water quality due to eutrophication, in an oligotrophic ecoregion (i.e. the  
101 Eastern Mediterranean). Towards this aim, we sampled along a eutrophication gradient  
102 within a semi-enclosed gulf and investigated the response of benthic diatom assemblages  
103 and individual species to environmental covariates, including physical seawater properties  
104 and nutrient loadings. Considering diatom response at the assemblage level, we  
105 investigated the response of assemblage characteristics such as frustule abundance, species  
106 richness and evenness as well as assemblage composition. With respect to the sensitivity of  
107 individual species' populations in detecting shifts in environmental conditions, we  
108 investigated the response of the abundance of each diatom species to changes in the  
109 environmental covariates. This analysis enabled us to identify the main drivers of benthic  
110 diatom structural changes as well as specific species sensitive to shifts in nutrient  
111 concentrations.

112

## 113 **2. Methods**

114

### 115 ***2.1. Study sites and sampling design***

116 Saronikos gulf falls within the typical oligotrophic marine system of the Eastern  
117 Mediterranean ecoregion. It is a semi-enclosed gulf, located in the central-western part of

118 Aegean Sea, with an area of about 1117 Km<sup>2</sup>, and circa 450 m maximum depth (Griggs et al.  
119 1978, Krasakopoulou & Karageorgis 2005, Friligos 1982). Although there are no major  
120 riverine inputs, Saronikos waters are influenced by the Athens metropolitan area due to  
121 point discharges of sewage (Simboura & Zenetos 2005, Scoulos et al. 2007). The gulf has  
122 been the focus of numerous studies assessing water quality and eutrophication, all  
123 reporting a eutrophic status in the inner gulf and a mesotrophic in the outer part of the gulf  
124 (e.g. Ignatiades et al 1992, Tsirtsis et al 2008).

125

126 Six sites were sampled along the northern coastline of the gulf (Fig. 1). The selection of sites  
127 was based on land use of the surrounding area, comparable substrate and low wind and  
128 wave action, and was further established by pilot samplings. Station S1 (37°39'20.N, 24°  
129 0'28.E) is located at the east entrance of the gulf with no obvious anthropogenic impacts.  
130 Station S2 (37°49'2.N, 23°45'58.E) is about 40 kilometers west of the entrance of the gulf  
131 where the adjacent coastal areas are sparsely populated, therefore it is affected by limited  
132 urban runoffs. Station S3 (38° 0'58.62N, 23°35'51.E) is located in the most enclosed part of  
133 the gulf (Elefsis bay) and it is directly influenced by sewage and runoff from the Athens  
134 metropolitan area and the commercial port of Piraeus. Station S4 (37°57'16.N, 23°11'40.E)  
135 and station S5 (37°58'39.N, 23°21'7.E) are located further off the gulf and are exposed  
136 mainly to mild agricultural and urban runoff. Lastly, station S6 (37°52'44.N, 22°59'32.E) is  
137 located at the inner west part of the gulf and is influenced by limited urban runoff.

138

139 Sampling of benthic diatoms was carried out on a monthly basis, from June 2006 to June  
140 2007. At each sampling site, triplicate core samples of virtually undisturbed sediment were  
141 collected approximately 20-25 meters from the coast and at a maximum depth of 4 meters.

142 Samples were collected during intervals of limited wind and wave action to avoid turbulence  
143 of the sea bottom. Based on the observation that diatom assemblage require approximately  
144 14 days to respond to changes in nutrient concentrations (Sundback & Snoeijns 1991, Agatz  
145 et al. 1999), we used environmental data that were collected 14 days before the benthic  
146 diatom collection (although environmental data from the same day were also available). Our  
147 sample size thus consisted of 216 samples corresponding to 6 sites sampled at 12 months,  
148 with 3 replicates per site.

149

## 150 **2.2. Environmental variables**

151 At each replicate position physicochemical parameters were measured in situ. Temperature  
152 and Dissolved Oxygen (DO) were measured using a 58 YSI dissolved oxygen meter, salinity  
153 and conductivity were measured using a model 33 YSI S-C \_T meter, pH was measured using  
154 a Consort P501 portable meter. PAR was also recorded at each station in situ using a LI Cor  
155 185B photometer, however due to the small depth and the low turbidity, over 99% of the  
156 surface PAR was available at the bottom during samplings all year round, and thus all sites  
157 had the same light availability at each sampling. Therefore, light was not considered as a  
158 driver of assemblage change. For the determination of substrate type, sediment samples  
159 were taken at each site, they were sorted by dry sieving and grain size percentages were  
160 recorded. All sites were classified as sand according to ISO 14688-1:2017 with 95% of grain  
161 size over 90 $\mu$ m, therefore no difference on species composition based on substrate was  
162 expected (Table S1). Nutrients were analyzed following the protocols of Parsons et al. (1984)  
163 and Standard Methods (1980). Seawater samples of 1 liter, in triplicate, were collected from  
164 each site just above the sea bottom, from where diatom samples were collected and were  
165 filtered in the lab using Millipore HA filters of 0.45  $\mu$ m pore size. The filtrate was used to



166 determine dissolved inorganic concentrations of phosphate ( $\text{PO}_4^{3-}$   $\mu\text{g L}^{-1}$ ), silicate ( $\text{SiO}_2$   $\mu\text{g L}^{-1}$ ) and Dissolved Inorganic Nitrogen (DIN  $\mu\text{g L}^{-1}$ ), as the sum of nitrite ( $\text{NO}_2^-$   $\mu\text{g L}^{-1}$ ), nitrate  
167 ( $\text{NO}_3^-$   $\mu\text{g L}^{-1}$ ) and ammonium ( $\text{NH}_4^+$   $\mu\text{g L}^{-1}$ ). Chlorophyll a and c ( $\text{mg m}^{-2}$ ) were estimated  
168 according to Parsons et al. (1984) using seawater samples extracted by the sediment cores  
169 through filtering with Millipore HA filters of 0.45  $\mu\text{m}$  pore size.  
170

171

### 172 **2.3. Species identification**

173 Sediment cores were collected from each sampling location on a monthly basis to be used  
174 for taxonomic identification and quantitative analysis (abundance estimation). The cores  
175 were collected with a Plexiglas tube of 3.3 cm diameter. We didn't expect important bias  
176 due to the presence of dead frustules (e.g. Gillett et al. 2009); however, to ensure that most  
177 of the frustules will be alive before acid treatment, the sediment cores were allowed to  
178 stand in the lab for 24 hours under light, to attract diatoms to the surface. Moreover,  
179 samples from the selected sites were examined prior to the treatment with acid, while  
180 diatoms were still alive in order to determine the percentage of dead to living diatoms. The  
181 results showed that the number of dead frustules was negligible (less than 1% of the total),  
182 as. The first centimeter of each core was cut and 20 ml of distilled water were added. As  
183 both epipelagic and epipsammic diatoms in the sediment were aimed to be collected, a two-  
184 step process was applied, following Agatz 1999. First, the sample was vigorously shaken in  
185 order to extract the diatoms from the sediment in the overlaying water (i.e. epipelagic  
186 diatoms, Round et al 1990) and the supernatant was collected. Second, 20 ml of water were  
187 added to the remaining sediment and the sample was placed in an ultrasonic vibration bath  
188 for 2 minutes to loosen any epipsammic attached diatoms, however the number of diatoms  
189 found after this treatment was not significant. The two subsamples were then merged and

190 brought to the same volume for all samples. The extracted diatoms were then treated with  
191 concentrated acids using the classic protocols of Hasle & Fryxell (1970) and Schrader (1973).  
192 A total of 216 samples (12 months x 6 sites x 3 replicates) were used for taxonomic  
193 identification and estimation of abundance. For each sample three replicates slides of 250  
194  $\mu\text{L}$  were mounted using Naphrax<sup>®</sup> resin and 250 fields were counted in each slide. The  
195 quantitative method used ensured the estimation of total abundance. Frustule counts were  
196 carried out using a Carl Zeiss photonic microscope at x1000 magnification and pictures were  
197 taken with a Canon G7 digital camera. Taxonomic identification to species level was carried  
198 out using reference floras (Hustedt 1930, 1964, Hendeby, 1964, Round et al 1990, Witkowski  
199 et al. 2000, Lange-Bertalot 2001, Weckstrom & Juggins 2006, Bukhtiyarova 2006, Levkov  
200 2009, Louvrou et al 2012).

201

#### 202 **2.4. Data analysis**

203 Benthic diatom species composition (i.e. the entire information of species and abundances  
204 across samples) was used to express changes in space and time, as well as more synoptic  
205 information on assemblage characteristics such as frustule abundance (number of frustules  
206  $\text{cm}^{-3}$ ), chlorophyll a ( $\text{mg m}^{-2}$ ), species richness and evenness, the latter expressed with  
207 Pielou's index (Pielou 1975, Heip et al. 1998). To test whether environmental covariates  
208 ( $\text{SiO}_2$ ,  $\text{PO}_4$ , DIN, pH, DO, temperature, salinity) and the assemblage characteristics presented  
209 spatial (i.e. between sites) and temporal (i.e. between seasons) variation, we performed  
210 ANOVA with site, season and their interaction as explanatory variables. In each season we  
211 incorporated the three corresponding months, starting from the first sampling in summer  
212 (June-August, Table S2).

213

214 To test the effect of environmental conditions on diatom assemblage characteristics we  
215 used linear mixed effects models. Our experimental design was nested because all of the six  
216 sites were sampled within each one of twelve consecutive months. To account for  
217 dependencies in the residuals due to the clustering effect of month and site, we included  
218 those factors as a nested random effect (1|Month|Site). In order to retain the most  
219 significant environmental covariates, we carried out a model selection procedure using the  
220  $\Delta$ AIC criterion (Burnham and Anderson 1998) based on the log likelihood approach. This  
221 analysis was performed using the full dataset of 216 samples in R Studio (v.2.4.1) using the  
222 software packages lme4 v.1.1-12 (Bates, et al., 2015), MuMIn v.1.15.6 (Barton 2016) and  
223 faraway v.1.0.7 (Faraway 2016). Our models were the following:

224

225 Assemblage characteristic= Si + PO4 + DIN + pH + DO + Temperature + Salinity +  
226 (1|Month|Site)

227

228 To test the effect of environmental covariates on assemblage composition we used  
229 Canonical Correspondence Analysis (CCA). This analysis was performed in R Studio using the  
230 vegan package v.2.5-2 (Oksanen et al 2018) for multivariate analysis after normalisation of  
231 the environmental variables and with no transformation of species abundances.  
232 Normalisation was made to ensure that all variables used were at the same scale and  
233 transformation on species abundances was not necessary as abundance data were not  
234 characterised by dominance. Model selection was performed by minimising the AIC criterion  
235 (Burnham and Anderson 1998) using permutational analysis (999 maximum permutations)  
236 on the following full model:

237

238 Species composition data = Month+Si + PO4 + DIN + pH + DO

239 The CCA plot was generated using the default function plot, scaled for species, with no Hill  
240 scaling and with site scores as weighted averages. Species were not presented in the plot for  
241 clarity, due to their high number.

242

243 Finally, we checked which diatom species are particularly sensitive to changes in  
244 environmental variables and especially nutrient concentrations. To address this objective,  
245 we first detected those diatom species that were more dominant and/or characteristic in  
246 the assemblages of each of our 6 sites and tested how each one of our environmental  
247 covariates was affecting their variation in frustule abundance, using Spearman correlation  
248 analysis. To find the species that characterized the assemblages of the six sites, we  
249 performed indicator species analysis (De Caceres & Legendre 2009). Based on that, we were  
250 able to detect indicator species for each site as well as to define their positive predictive  
251 value (i.e. the probability that a sample containing this species belongs to this site) and their  
252 sensitivity (i.e. the probability of finding the species in the samples belonging to this site). A  
253 positive predictive value of 1 indicates that the species belongs to samples of this site only,  
254 whereas a sensitivity of 1 indicates that the species appears in all samples belonging to this  
255 site. Indicator species analysis was performed in R Studio (v.2.4.1) using function multipatt  
256 from package indicpecies (De Caceres & Legendre 2009).

257

### 258 **3. Results**

259

#### 260 ***3.1. Description of environmental conditions***

261 All environmental covariates presented seasonal variation (see Table S3) that was more  
262 important than the spatial variation among sites (Fig. S1). An exception to this was DIN  
263 whereby the effect of site was stronger than the effect of season with site S3 presenting  
264 significantly higher values irrespective of season (Fig. S1). Silicates and pH also showed  
265 differences among the six sites, however the directionality of these differences depended  
266 on the season (i.e. significant interaction between site and season, Table S3). Phosphates  
267 did not show variation between sites and the effect of season was much weaker than the  
268 other environmental covariates. The N:P ratio was around the threshold of 22 (Martiny et al  
269 2014), with some extreme values in cases of very low P concentrations, resulting to shifts in  
270 nutrient limitation (Fig. S1). As expected, season had the main effect on both salinity and  
271 temperature (Table S3).

272

### 273 **3.2. Description of benthic assemblages**

274 A total number of 448 different taxa of benthic diatoms were identified to the level of  
275 species across our 216 samples. Most taxa were rare and only 284 contributed with more  
276 than 1% to the total frustule abundance. Assemblage characteristics were strongly affected  
277 primarily by site and secondarily by season (Fig. S2) and the variation between sites always  
278 depended on season (i.e. significant interaction between site and season, Table S3). The two  
279 measures of assemblage biomass, chl a and frustules abundance were positively correlated  
280 (Spearman correlation coefficient 0.36,  $p < 0.001$ ). Both of them showed significant spatial  
281 variability whereby sites S1, S3 (and S5 in the case of chl a) presented the higher values  
282 consistently across seasons. Species richness was also variable between stations; however,  
283 the variability was heavily depended on season. Evenness was not highly variable between  
284 sites with the exception of site S3 which presented significantly lower values.

285

### 286 **3.3. Effect of environmental conditions on assemblage characteristics**

287 All assemblage characteristics seemed to be sensitive to the variation of at least one  
288 environmental covariate with the exception of evenness, which was only affected by pH  
289 (Table 1). Dissolved Inorganic Nitrogen (DIN) had a statistically significant effect on chl a,  
290 species richness and frustule abundance ( $p < 0.05$ ) and ranked first in importance within the  
291 model regarding frustule abundance. The effect of DIN on frustule abundance (N) and  
292 species richness (SR) was pronounced during Winter (SR:  $r^2 = 62.2\%$ ,  $p < 0.001$ , N:  $r^2 = 71.5\%$ ,  
293  $p < 0.001$ ) and Spring (SR: 8.5,  $p < 0.05$ , N:  $r^2 = 46.4\%$ ,  $p < 0.001$ ) but was weak or not significant  
294 during Summer (SR:  $r^2 = 5.5\%$ ,  $p > 0.05$ , N:  $r^2 = 14.7\%$ ,  $p < 0.05$ ) and Autumn (SR:  $r^2 = 5.4\%$ ,  $p > 0.05$ ,  
295 N:  $r^2 = 0.5$ ,  $p > 0.05$ ) (Fig. 2). The other two most important covariates affecting assemblage  
296 characteristics were dissolved oxygen (DO) and pH (Table 1) and their effect depended on  
297 season. Specifically, DO and pH had a negative effect on frustule abundance, which was only  
298 significant during spring (DO: slope = -63.301,  $r^2 = 8.9\%$ ,  $p < 0.05$ , pH: slope = -179.759,  $r^2 = 18.3\%$ ,  
299  $p < 0.01$ ). Regarding species richness, pH did not have a significant effect within seasons,  
300 whereas DO only had a significant negative effect during winter (slope = -8.95,  $r^2 = 8.5\%$ ,  $p < 0.05$ )  
301 and spring (slope = -8.2,  $r^2 = 9.0\%$ ,  $p < 0.05$ ). Silicates and phosphates did not have an effect on  
302 any assemblage characteristic (Table 1).

303

### 304 **3.4. Effect of environmental conditions on assemblage composition**

305 Each of the six sites presented a characteristic diatom composition. The samples, based on  
306 non-transformed species-abundance data, were grouped primarily based on the sampling  
307 site rather than the month of sampling (see same colour points in Fig. 3), indicating that  
308 space is a more important driver of assemblage composition than time. The environmental

309 covariates with the greatest and most significant impact on this assemblage pattern was  
310 Dissolved Inorganic Nitrogen (CCA, F-ratio=2.09,  $p<0.01$ ), silicates (CCA, F-ratio=1.74,  
311  $p<0.05$ ), and salinity (CCA, F-ratio=1.44,  $p<0.05$ ).

312

### 313 ***3.5. Sensitivity of specific species and genera to environmental conditions***

314

315 Assemblage composition is primarily driven by DIN as seen in Fig. 3 and this pattern is  
316 mainly due to positive correlations of diatom species abundances with DIN (Table 2).  
317 Specifically, 37 species showed significant positive correlations with DIN concentration, of  
318 which the genera of *Cocconeis* and *Tryblionella* were represented with 6 and 4 species  
319 respectively, whereas two *Mastogloia* species presented negative correlations with DIN. The  
320 species that presented correlations with DIN were indicator species of site 3, the site with  
321 the highest nitrogen input. Furthermore, the two species of the genus *Mastogloia* that  
322 presented negative correlations were only found in this site during summer and autumn  
323 when nitrogen loads were low. During winter and spring, when nitrogen inputs increased,  
324 they went extinct. These results were also validated by indicator species analysis, which  
325 tested for the species characterising each of the sites along the gradient affected by  
326 different nutrient loads. For instance, *Mastogloia* species characterized the less impacted  
327 site 1, whereas *Cocconeis* species characterized the most severely impacted site 3 (Table  
328 S4).

329

330

## 331 **4. Discussion**

332

333 Our findings indicate a gradient of eutrophication based on nitrogen inputs in Saronikos gulf  
334 that shapes benthic diatom assemblages by increasing both biomass and species richness  
335 and changing species composition. Despite the fact that other environmental conditions  
336 also had a considerable variation in time (e.g. silicates, DO, pH, temperature), nitrogen  
337 concentration showed a clear significant effect on different aspects of assemblage structure  
338 of benthic diatom assemblages. Nitrogen loads were also strongly correlated with particular  
339 diatom genera (especially of the genus *Cocconeis*), suggesting the potential of benthic  
340 diatoms as bioindicators of marine eutrophication in oligotrophic ecoregions.

341

342 Nitrogen and its compounds is a common source of eutrophication both in coastal and  
343 freshwater ecosystems impacted by urban sewage or agricultural activities (e.g. Agatz et al.  
344 1999, Tornés et al. 2018, Spatharis et al. 2007a,b). Nitrogen inputs caused by anthropogenic  
345 activities could change nutrient stoichiometry, especially the N:P ratio, and cause shifts in  
346 phytoplankton assemblage composition (Philippart et al. 2000). In our study, nitrogen  
347 inputs, especially in site 3, increased the N:P ratio above 22 and thus changed nutrient  
348 limitation from nitrogen to phosphorus, causing a shift of the assemblages from species  
349 proliferating in nitrogen-poor to species thriving in nitrogen-rich environments.

350 Species that were favoured by the increased nitrogen concentrations belonged to the  
351 *Cocconeis* and *Tryblionella* genera. The genus *Tryblionella* has been also linked to  
352 eutrophication in previous studies for Baltic sea (Agatz et al. 1999) whereas *Cocconeis* in  
353 fresh waters has been found in nutrient rich conditions (Bellinger & Sigee 2010). The  
354 increased abundances of *Cocconeis* spp. during nutrient enrichment events could be linked  
355 to their ability for efficient packing in limited space within biofilms, avoiding growth  
356 limitation due to density dependence phenomena (Sullivan 2019), especially in the



357 restricted size of a sand grain. Other species that presented high positive correlation with  
358 DIN were *Opephora mutabilis* and *Bacillaria paxillifera*. *Opephora mutabilis* has shown  
359 decline with increased anthropogenic disturbance in Chesapeake Bay (Cooper, 1995,  
360 1995a), while the species presented an increase with eutrophication in Roskilde Fjord  
361 (Clarke et al. 2003). This apparent discrepancy could be attributed to the ambiguity  
362 regarding the genus taxonomy in earlier years (Sabbe & Vyverman 1995). *Bacillaria*  
363 *paxillifera* is known to thrive in environments impacted with eutrophication such as urban  
364 areas in Helsinki (Häyrén 1921). However, in our study *Cocconeis* and *Tryblionella* species  
365 showed the strongest positive signal with nutrient enrichment and are thus suggested as  
366 good bioindicators of eutrophication and impacted water quality in oligotrophic marine  
367 coastal systems. Furthermore, the consistent behaviour of the species belonging to the  
368 above genera suggest that these diatoms could be used as indicators of eutrophication even  
369 at the genera level.

370

371 On the other hand, species belonging to the genus *Mastogloia* showed a negative signal  
372 with nutrient enrichment. *Mastogloia* species have been previously associated with  
373 oligotrophic conditions in the North Pacific Ocean where they have been reported to co-  
374 occur and bloom with microalgae species known to host nitrogen-fixing bacteria as  
375 *Hemialus* spp and *Rhizosolenia* spp (Venrick 1974, Villareal et al. 2012). Furthermore,  
376 *Mastogloia* species are common in the oligotrophic Caribbean coast (Gaiser et al. 2010), and  
377 they have been found to co-exist near estuaries with *Rhopalodia* spp which also hosts  
378 nitrogen-fixing bacteria, (Smeti, unpublished data). Their presence in nitrogen poor  
379 environments indicates their ability to withstand nitrogen limitation and thus explain the  
380 negative correlation with increased nitrogen in our study. For this reason, we recommend

381 that *Mastogloia* species could be used as efficient indicators of nitrogen-poor systems in  
382 oligotrophic marine coastal systems.

383

384 In our study, nitrogen had a positive effect on biomass and diversity. This effect was more  
385 pronounced in winter and spring, when nitrogen inputs were increased. This is in agreement  
386 with similar studies on phytoplankton assemblages in oligotrophic marine systems which  
387 suggested species richness as an efficient index of eutrophication (Spatharis & Tsirtsis 2010).

388 The reason for this increase has been attributed to the fact that increase in nitrogen leads to  
389 the increase in abundance of rare species, making them detectable in samples and  
390 increasing the species richness of the sample (Tsirtsis et al. 2008). In freshwater systems,  
391 species richness is not used as a reliable indicator of water quality, since it usually presents a  
392 hump-shaped relationship, with low species richness in both pristine and highly impacted  
393 condition (e.g. Pandey et al. 2017). However, water quality reflects much more  
394 environmental conditions than nutrient enrichment and it is possible that in freshwater  
395 ecosystems other factors such as the alkalinity of water can also play an important role.  
396 Furthermore, the linear trend observed in our study could indicate that nitrogen inputs  
397 were not high enough to allow homogenization and dominance of very few species tolerant  
398 to increased eutrophication, and thus the observation of a hump shape. Therefore, although  
399 species richness demonstrates the desired qualities for a nutrient enrichment index being  
400 both monotonic and linear (Spatharis & Tsirtsis 2010), its use is not advised due to potential  
401 sample size biases. On the other hand, benthic diatom biomass expressed as frustule  
402 abundance seems to be a robust proxy of eutrophication also in agreement with  
403 corresponding phytoplankton studies (Spatharis & Tsirtsis 2010).

404

405 Although nitrogen and phosphorus inflows into coastal marine ecosystems have been  
406 increasing over the past few decades due to anthropogenic activities, mainly agriculture and  
407 urban growth (Jickells 1998), silicate concentrations have remained relative stable in coastal  
408 waters. This is possibly due to the fact that anthropogenic activities with Si sources are  
409 relatively scarce and have remained stable over the past decades (Admiraal et al. 1990,  
410 Conley 1997, Aure et al. 1998, Gilpin et al. 2004). In many marine diatoms the ratio N:Si is  
411 found to be relatively balanced, around 1 within their biomass (Redfield et al. 1963,  
412 Brzezinski 1985). Even though other studies have demonstrated a co-limitation by Si and  
413 PO<sub>4</sub> in the microphytobenthos (Cibic et al. 2007), phosphates were not important in our  
414 study. On the other hand, silicate concentrations seemed to drive assemblage composition  
415 in our study and we also observed a sensitivity of the genera *Cocconeis* and *Tryblionella* also  
416 for silicate together with nitrogen. However, Si presented a higher variation with time and it  
417 could not be used efficiently as an indicator of anthropogenic nutrient inputs in coastal  
418 ecosystems. The same seemed to be the case with salinity, which affected assemblage  
419 composition and selected genera, but presented a higher variation in time, following rainfall  
420 events. Thus, it cannot be used as an indicator of anthropogenic activities.

421

422 Previous studies assessing the eutrophication status of Saronikos gulf based on  
423 phytoplankton, have separated between two coarse ecological levels and namely the inner  
424 eutrophic and outer mesotrophic Saronikos (Pagou, 2005, Tsirtsis et al 2008). Our study  
425 demonstrates the potential of benthic diatoms to detect point sources of eutrophication, as  
426 within inner Saronikos, coastal sites covered a range of conditions from pristine to  
427 impacted. These findings suggest that using data from both phytoplankton and

428 phytoplankton in a complementary way could lead to a more complete and informative  
429 assessment of the ecological quality of coastal waters.

430

## 431 **5. Conclusions**

432 Overall, our study suggests that both species composition as well as biomass and diversity of  
433 benthic diatom assemblages are sensitive to temporal fluctuations and fine scale spatial  
434 variability of nitrogen concentrations caused by anthropogenic inputs. Therefore, a focus on  
435 benthic diatom communities could provide finer spatial resolution of coastal eutrophication  
436 compared to the traditional approaches relying on phytoplankton biomass and diversity. We  
437 specifically recommend the use of total frustule abundance as well as the abundances of  
438 genera *Cocconeis* and *Tryblionella* as reliable indices of nutrient enrichment in oligotrophic  
439 nutrient-limited ecoregions. The genus *Mastogloia* can be used as indicators of nutrient-  
440 poor and more pristine conditions. As our study is the first to suggest the importance of  
441 these genera in detecting localized hotspots of nutrient enrichment and pristine conditions  
442 in marine coastal systems, future studies could focus on their special autecological  
443 characteristics, such as their need for specific macronutrients. The development of a  
444 multiparametric index could further consider diversity indices that are independent of  
445 sampling effort such as the Menhinick diversity index (Spatharis & Tsirtsis 2010) or  
446 taxonomic distinctness index (Clarke & Warwick 2003).

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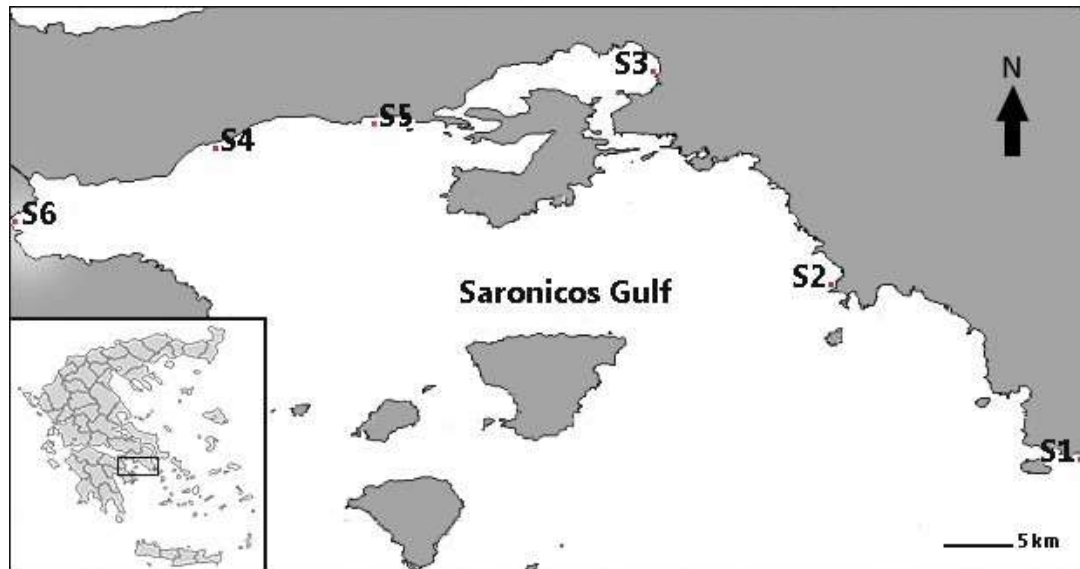


715 **Figures**

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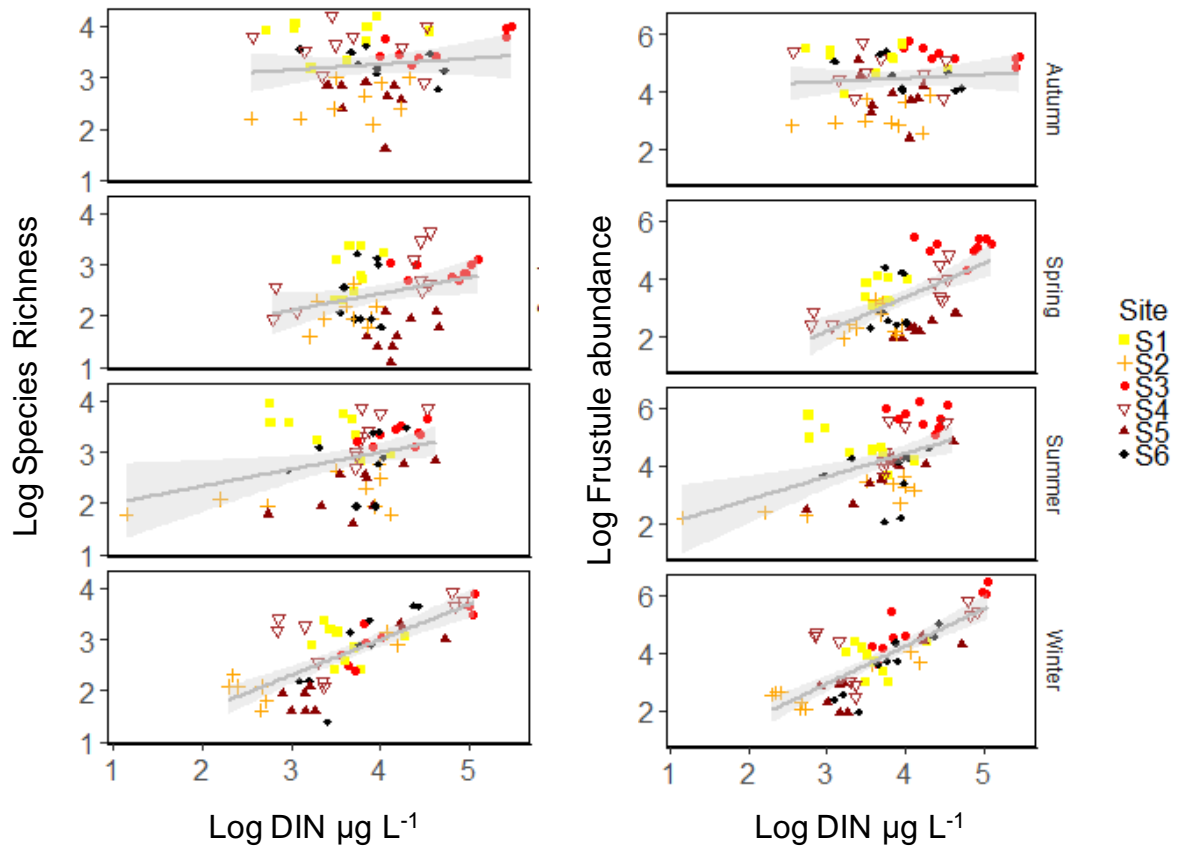
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721 Figure 1. Map of Saronikos Gulf, Greece showing the locations of the six sampling sites along  
722 the northern coast with station 1 being the closest to the open Aegean Sea and station 3  
723 being the most enclosed, situated within the heavily impacted Elefsina bay.

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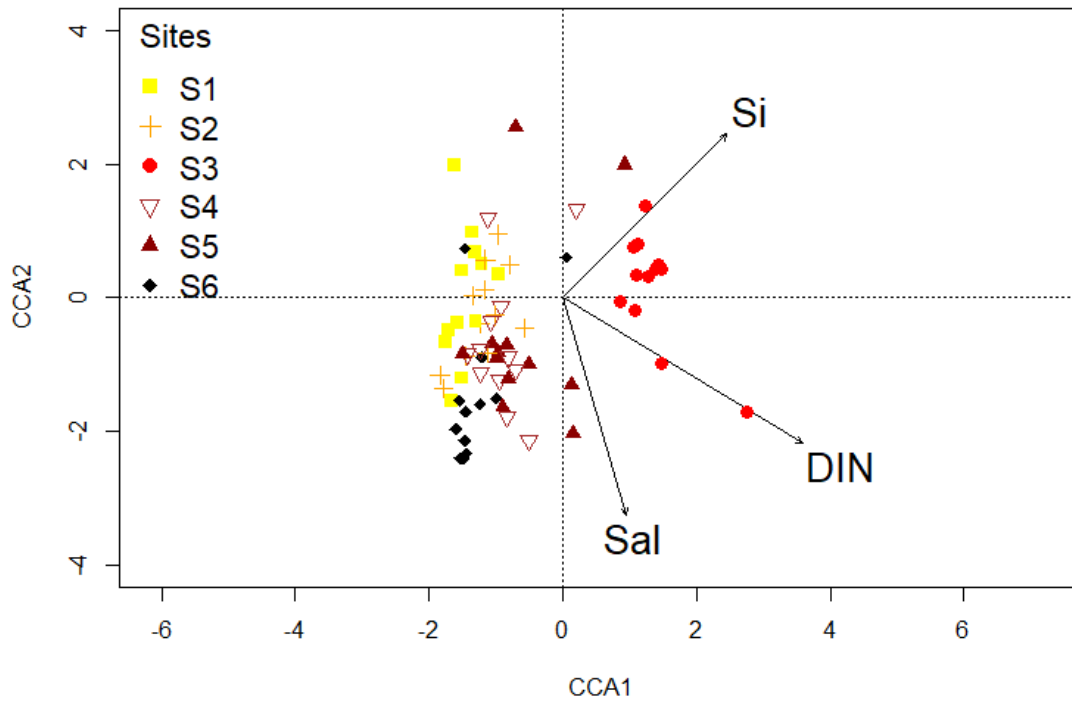
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728 Figure 2. Effect of dissolved inorganic nitrogen (DIN) on frustule abundance and species  
 729 richness of benthic diatom assemblages according to season. Each point represents the  
 730 average of three replicates per site and colors indicate the different sites. Lines indicate the  
 731 regression between DIN and the corresponding assemblage characteristic with 95%  
 732 standard error funnel.

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735 Figure 3. Canonical Correspondence Analysis (CCA) showing the grouping of 72 samples (6  
 736 sites x 12 months) according to the site (colored circles) and the three covariates (arrows)  
 737 that drive the observed similarities between the samples, namely the Dissolved Inorganic  
 738 Nitrogen (DIN), Silicates (Si) and Salinity (Sal). The analysis was based on non-transformed  
 739 species abundance data.

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743 Table 1. Slope coefficients, corresponding confidence levels (\* indicates the 95%, \*\*  
 744 indicates the 99% and \*\*\* indicates the 99.9% confidence level) and rank order of  
 745 importance in the model (in brackets) testing the effect that each environmental covariate  
 746 (DIN, SiO<sub>2</sub>, PO<sub>4</sub>, pH, Salinity, Temperature, DO) had on an assemblage characteristic (chl<sub>a</sub>,  
 747 frustule abundance, species richness, evenness). Values are not shown for covariates that  
 748 did not have a significant effect on an assemblage characteristic.

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<b>Environmental covariate</b>	<b>Chlorophyll a</b>	<b>Frustule abundance</b>	<b>Species richness</b>	<b>Evenness</b>
DIN	0.20741*(6/7)	0.911***(1/7)	0.089**(3/7)	-
SiO <sub>2</sub>	-	-	-	-
PO <sub>4</sub>	-	-	-	-
pH	(2/7)	(3/7)	(1/7)	(1/7)
Salinity	(3/7)	-	-	-
Temperature	-	7.576*(4/7)	-	-
DO	(1/7)	(2/7)	-7.303*(2/7)	-

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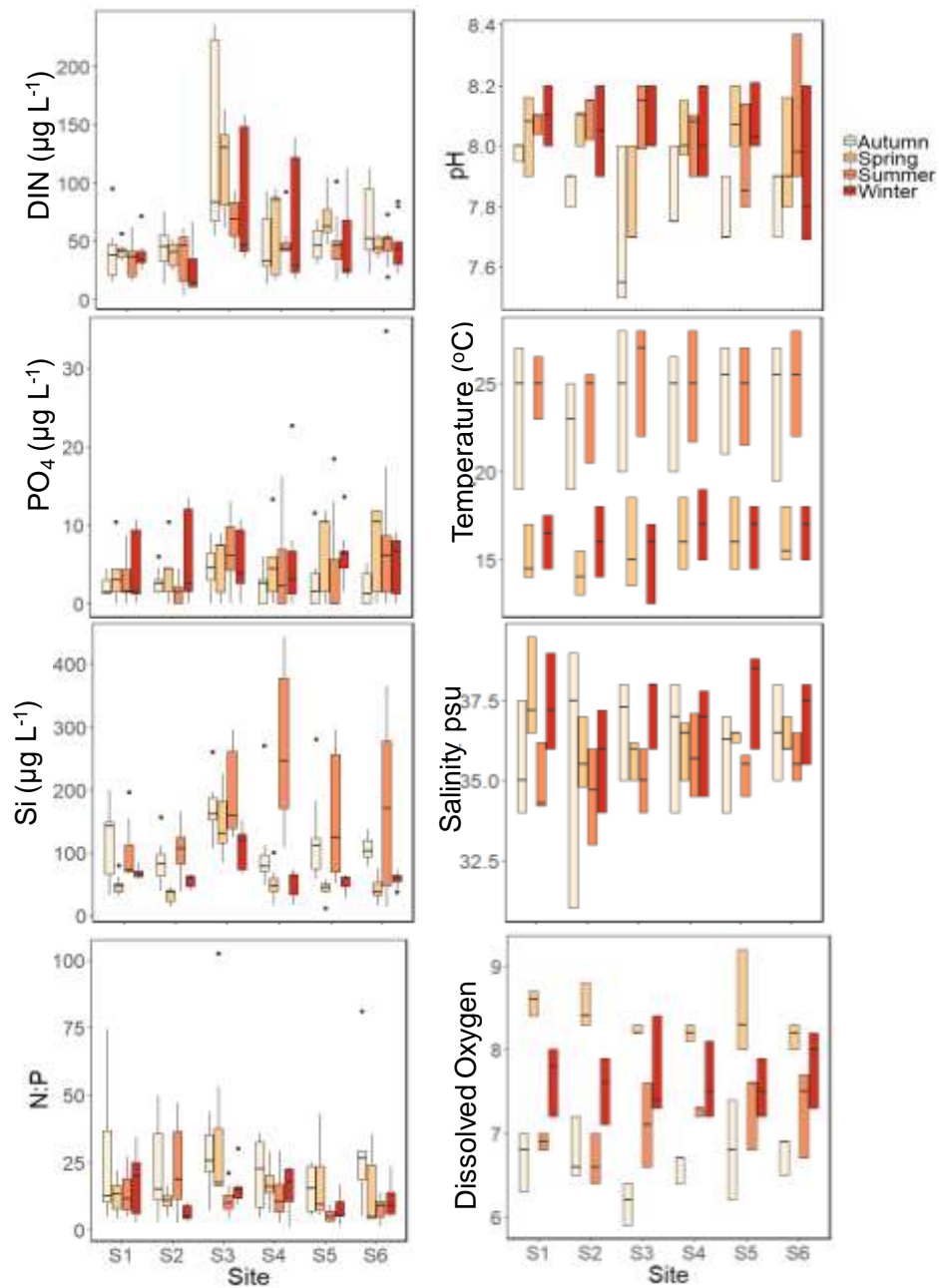
754 Table 2. Spearman rank correlation coefficients between the frustule abundance of a  
 755 species and DIN concentration. Yellow, red and dark red indicate significant positive  
 756 correlation at the 95%, 99%, and 99.9% confidence level, whereas light blue indicates  
 757 significant negative correlation at the 95% confidence level. Analysis was based on 72  
 758 samples (6 sites x 12 months), replicates were averaged.  
 759

Species	C.C	Significance level
<i>Tryblionella compressa</i> , Poulin, M., Bérard-Therriault, L., Cardinal, A. & Hamilton, P.B. (1990)	0.51	***
<i>Cocconeis stauroneiformis</i> , Okuno, H. (1957)	0.48	***
<i>Cocconeis hoffmanni</i> , Simonsen, R. (1959)	0.44	***
<i>Cocconeis guttata</i> , Hustedt, F. & Aleem, A.A. (1951)	0.43	***
<i>Cocconeis</i> sp1	0.42	***
<i>Campylodiscus clypeus bicostata</i> , Hustedt, F. (1930)	0.39	***
<i>Opephora mutabilis</i> , Sabbe, K. & Vyverman, W. (1995)	0.36	***
<i>Fallacia forcipata</i> , Round, F.E., Crawford, R.M. & Mann, D.G. (1990)	0.35	**
<i>Pleurosigma elongatum</i> , Smith, W. (1852)	0.35	**
<i>Surirella scalaris</i> , Giffen, M.H. (1967)	0.35	**
<i>Tryblionella hungarica</i> , Frenguelli, J. (1942)	0.35	**
<i>Bacillaria paxillifera</i> , Marsson, T. (1901)	0.33	**
<i>Navicula borneoensis</i> , Hustedt (1964)	0.33	**
<i>Nitzschia liebetruthii</i> , Rabenhorst, L. (1864)	0.32	**
<i>Halamphora acutiuscula</i> , Levkov, Z. (2009)	0.31	**
<i>Amphora marina</i> , Smith, W. (1857)	0.31	**
<i>Ardissonea crystallina</i> , Cleve, P. T. & Grunow, A. (1880)	0.31	**
<i>Psammodictyon constrictum</i> , Round, F.E., Crawford, R.M. & Mann, D.G. (1990)	0.31	**
<i>Tryblionella lanceola</i> , Cleve, P.T. (1878)	0.31	*
<i>Halamphora coffeaformis</i> , Levkov, Z. (2009)	0.30	*
<i>Fallacia minima</i> , Witkowski et al. (2000)	0.30	*
<i>Fallacia</i> sp1	0.30	*
<i>Karayevia amoena</i> , Bukhtiyarova, L.N. (2006)	0.30	*
<i>Catenula adhaerens</i> , Mereschkowsky, C. (1903)	0.29	*
<i>Cocconeis peltoides</i> , Hustedt, F. (1939)	0.29	*
<i>Auricula</i> sp	0.28	*
<i>Navicula pavillardii</i> , Hustedt, F. (1939)	0.28	*
<i>Cocconeis distans</i> , Gregory, W. (1855)	0.27	*
<i>Navicula carinifera</i> , Schmidt, A.[W.F.] (1874)	0.26	*
<i>Tryblionella apiculata</i> , Gregory, W. (1857)	0.26	*
<i>Achnanthes brockmannii</i> , Hustedt (1959)	0.25	*
<i>Dimeregramma minor nana</i> , Van Heurck, H. (1896)	0.25	*
<i>Lyrella abrupta</i> , Round, F.E., Crawford, R.M. & Mann, D.G. (1990)	0.25	*
<i>Lyrella</i> sp1	0.25	*
<i>Surirella brebissonii</i> , Krammer, K. & Lange-Bertalot, H. (1987)	0.25	*
<i>Parlibellus adnatus</i> , Witkowski, A., Lange-Bertalot, H. & Metzeltin, D. (2000)	0.24	*
<i>Petrodyction gemma</i> , Round, F.E., Crawford, R.M. & Mann, D.G. (1990)	0.24	*
<i>Caloneis linearis</i> , Boyer, C.S. (1927)	-0.25	*
<i>Mastogloia peragalli</i> , Cleve, P.T. (1892)	-0.25	*
<i>Mastogloia macdonaldi</i> , Hustedt, F. 1931–1959	-0.26	*
<i>Meloneis gorgis</i> , Louvrou, I., D.B.Danielidis & A.Economou-Amilli (2012)	-0.31	*

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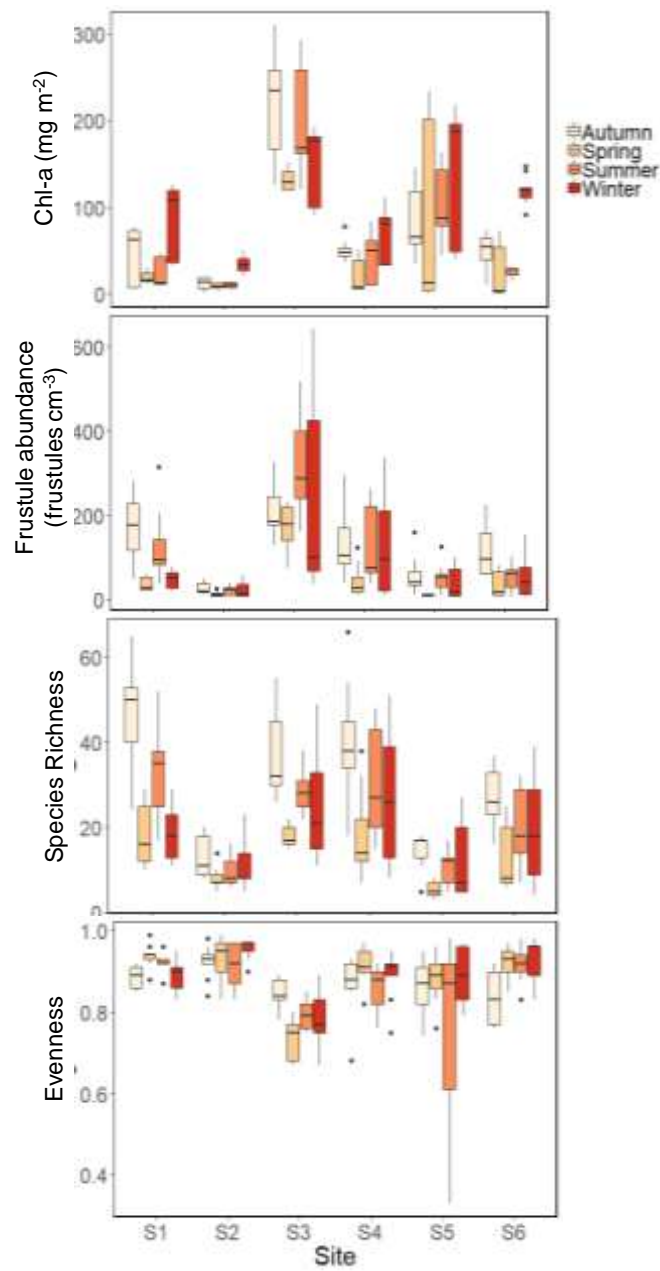
766 Figure S1. Box plots showing the variability of DIN,  $\text{PO}_4$ ,  $\text{SiO}_2$ , N:P, pH, salinity and DO across  
 767 the six sampling sites and four seasons. This analysis is based on total sample size of 216.

768 Each box includes 9 values (3 replicates x3 months per season).

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773 Figure S2. Boxplot showing the variability of different assemblage characteristics including  
774 chlorophyll a (mg/m<sup>2</sup>), frustule abundance (number of frustules/cm<sup>3</sup>), species richness and  
775 evenness index J, across the six different sites depending on the season. This analysis is  
776 based on total sample size of 216 and each point represents the average of 24 values (3  
777 replicates x 3 months per season).

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779 Table S1. Depth and grain size (%) of the sampling sites. In bold are the three dominant grain size  
 780 classes in each site. The categorization to gravel, sand, silt is based on ISO 14688-1:2017

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		S1	S2	S3	S4	S5	S6	
Depth (m)		3.6	1.8	2.0	2.1	2.5	2.3	
Grain size class (%)	Gravel	4 mm	2.48	0.00	0.32	0.13	1.37	0.00
		2.8mm	1.14	0.01	0.58	0.19	1.40	0.00
	Sand	2 mm	2.15	0.10	2.15	0.32	1.32	0.00
		1.4 mm	6.64	0.24	8.25	0.91	3.13	0.02
		1 mm	<b>17.00</b>	0.33	15.62	1.14	5.00	0.01
		710 µm	<b>38.97</b>	0.58	<b>22.78</b>	2.51	9.40	0.12
		500 µm	<b>28.06</b>	0.92	<b>24.32</b>	10.71	<b>16.40</b>	0.28
		350 µm	3.34	1.96	<b>11.49</b>	<b>30.48</b>	<b>19.28</b>	0.98
		250 µm	0.10	8.15	4.21	<b>35.29</b>	<b>17.91</b>	11.31
		180 µm	0.03	<b>18.75</b>	2.01	<b>12.99</b>	11.02	<b>34.27</b>
		125 µm	0.04	<b>44.45</b>	1.54	4.15	8.34	<b>42.95</b>
		90 µm	0.03	<b>22.21</b>	2.15	0.78	3.45	<b>8.22</b>
	63 µm	0.03	2.21	2.59	0.18	1.27	1.58	
	Silt	<63 µm	0.00	0.09	2.00	0.23	0.70	0.23

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784 Table S2. Sampling tables and their assignment to months and season in the present study. All sites  
785 were sampled the same day.

<b>Sampling dates</b>	<b>Month</b>	<b>Season</b>
27/6/2006	June	Summer
10/7/2006	July	Summer
29/8/2006	August	Summer
14/9/2006	September	Autumn
3/10/2006	October	Autumn
7/11/2006	November	Autumn
19/12/2006	December	Winter
23/1/2007	January	Winter
22/2/2007	February	Winter
29/3/2007	March	Spring
19/4/2007	April	Spring
3/5/2007	May	Spring

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789 Table S3. The effect of season, site and their interaction (site x season) on the variation of 8  
790 environmental covariates and 4 assemblage characteristics. Values indicate the F-ratio test  
791 result and asterisks the corresponding confidence levels (\* indicates the 95%, \*\* indicates  
792 the 99% and \*\*\* indicates the 99.9% confidence level).

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<b>Covariate</b>	<b>Site</b>	<b>Season</b>	<b>Site x season</b>
<b>DIN</b>	19.016***	3.716*	-
<b>PO4</b>	-	3.536*	-
<b>SiO2</b>	11.689***	41.589***	3.190***
<b>DO</b>	2.822*	280.1562***	3.9156***
<b>pH</b>	5.774***	36.6627***	4.032***
<b>Salinity</b>	2.298*	15.494***	1.759*
<b>Temperature</b>	-	236.793***	-
<b>Species richness</b>	32.584***	29.608***	2.231**
<b>Frustule abundance</b>	40.315***	9.976***	1.979*
<b>Chl a</b>	18.510***	77.675***	3.795***
<b>Evenness</b>	20.209***	-	3.409***

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807 Table S4. Indicator species for each of the six sites. These samples represent the 12 different  
 808 months that each site was sampled over an annual cycle. When detected, only species with  
 809 indicator value  $\geq 0.8$  are included, otherwise species with indicator value  $> 0.5$ . In brackets is  
 810 the total number of indicator species for each site. All species associations to the sites are  
 811 significant at the 99.9% confidence level. Host species that are found only in samples of  
 812 each site have a positive predictive value of 1 and species found in all samples of this site  
 813 have sensitivity of 1 (eg for site 3, this species is *Cocconeis hoffmanni*).  
 814

		Positive predictive value	Sensitivity	Indicator Value
<b>Site 1</b> <b>(28 species)</b>	<i>Achnanthes reichardtiana</i>	0.950	0.917	0.933
	<i>Mastogloia crucicula</i>	0.894	0.833	0.863
	<i>Mastogloia corsicana</i>	0.880	0.833	0.856
<b>Site 2</b> <b>(4 species)</b>	<i>Carinasigma rectum</i>	0.694	0.667	0.680
	<i>Toxonidea insignis</i>	0.750	0.583	0.661
<b>Site 3</b> <b>(25 species)</b>	<i>Cocconeis hoffmanni</i>	1.000	1.000	1.000
	<i>Tryblionella compressa</i>	0.947	1.000	0.973
	<i>Cocconeis stauroneiformis</i>	0.995	0.917	0.955
	<i>Cocconeis sp1</i>	0.905	1.000	0.951
	<i>Cocconeis guttata</i>	0.886	1.000	0.941
	<i>Amphora coffeaformis</i>	0.803	1.000	0.896
	<i>Campylodiscus clypeus var. bicostata</i>	0.961	0.667	0.800
<b>Site 4</b> <b>(27 species)</b>	<i>Perissonea cruciata</i>	1.000	1.000	1.000
	<i>Planothidium quarnerensis</i>	0.926	1.000	0.962
	<i>Meloneis akytos</i>	0.978	0.750	0.856
	<i>Cocconeis californica</i>	0.915	0.750	0.828
	<i>Psammodiscus nitidus</i>	0.855	0.750	0.801
	<i>Cocconeopsis patrickae</i>	0.853	0.750	0.800
<b>Site 5</b> <b>(1 species)</b>	<i>Dickieia subinflatooides</i>	0.627	0.833	0.723
<b>Site 6</b> <b>(11 species)</b>	<i>Delphineis sp2</i>	0.950	0.917	0.933
	<i>Actinoptychus minutus</i>	0.909	0.833	0.870
	<i>Delphineis surirella australis</i>	0.950	0.750	0.844
	<i>Delphineis livingstonii</i>	0.921	0.750	0.831

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