Phytoplankton communities in a coastal and offshore stations of the northern Adriatic Sea approached by network analysis and different statistical descriptors

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Francesca Neri: Conceptualization, Methodology, Software, Formal analysis, Writing- Original draft. Tiziana Romagnoli: Investigation. Stefano Accoroni: Conceptualization, Formal analysis, Visualization, Writing - Review & Editing. Marika Ubaldi: Investigation. Angela Garzia: Formal analysis, Andrea Pizzuti: Methodology, Software, Alessandra Campanelli: Investigation. Federica Grilli: Investigation. Mauro Marini: Supervision. Cecilia Totti: Supervision, Conceptualization, Writing - Review & Editing.

Phytoplankton communities in coastal and offshore stations of the northern Adriatic Sea approached by network analysis and different statistical descriptors

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Abstract

The Northern Adriatic Sea is one of the most productive areas of the Mediterranean Sea. Long-term series of phytoplankton are crucial to detect changes in the marine ecosystems, due to its high sensitiveness to environmental conditions.

In this study, we compared two long-term phytoplankton time series (1988-2019) related to a coastal and an offshore station located along the LTER Senigallia-Susak transect (Northern Adriatic Sea), using several statistical descriptors: diversity indices, multivariate statistical analyses (PCA, HCPC, NMDS), IndVal (indicator value analysis) and graph-network analysis. The coastal station was found to be more variable than the offshore one, being directly affected by the Western Adriatic Current and therefore by riverine inputs. The two stations appeared to be more different in winter and autumn, and more similar in summer when riverine waters spread offshore in stratified conditions. Due to its more oligotrophic condition, the offshore phytoplankton community showed a higher biodiversity than the coastal one, where phytoplankton blooms occurred frequently.

Graph-network analysis turned out to be a useful tool to study the phytoplankton community through the number of interactions occurring among phytoplankton taxa, that was higher at the offshore station.

This study highlighted that any evaluation of the Good Environmental Status (as required by the Marine Strategy Framework Directive) should consider the oceanographic differences between different areas, combining several statistical approaches.

Keywords

Phytoplankton; Long-term series; graph-network analysis; community ecology; Marine Strategy Framework Directive

1. Introduction

The Northern Adriatic Sea (NAS), the northernmost basin of the Mediterranean Sea, represents one of the most productive areas of the Mediterranean Sea and is characterized by a shallow depth, a high riverine input (mainly from the Po River), and by a dominant cyclonic circulation (Artegiani et al., 1997a; Campanelli et al., 2011; Degobbis et al., 2000; Grilli et al., 2020).

In the NAS, the trophic state and the seasonal rhythm of phytoplankton communities are directly influenced by the vertical structure of the water column (stratification vs mixing) (Neri et al., 2022) and the circulation regime: the Western Adriatic Current (WAC) conveys southwards the nutrient-rich waters from the northern subbasin (Artegiani et al., 1997b; Campanelli et al., 2011; Marini et al., 2008) and the Eastern Adriatic Current flows northwards along the eastern coast, bringing Ionian saltier, warmer and more oligotrophic waters (Poulain and Cushman-Roisin, 2001). In the recent decade, other forcings, such as an increasing anthropogenic pressure and significant meteoclimatic alterations superimposed, determining new tendencies in the seasonal trend of trophic status and of planktonic communities (Cibic et al., 2018; Grilli et al., 2020; Ninčević Gladan et al., 2010; Totti et al., 2019).

Data from Long-Term Ecological Research (LTER) are crucial to study potential tendencies and changes in the phytoplankton community (Cerino et al., 2019; Marić et al., 2012; Mozetič et al., 2010; Neri et al., 2022; Totti et al., 2019) and to disentangling its variability, basic structure, phenology and regularity (Longobardi et al., 2022; Vascotto et al., 2021; Winder and Cloern, 2010). Four LTER marine areas are present in the NAS (Gulf of Venice, Gulf of Trieste, Po River delta, and Senigallia-Susak Transect), where the interannual variability of physical parameters, trophic condition and phytoplankton variability have been intensively studied. Among these, the Senigallia-Susak Transect (SST) is located in the lower part of the NAS, where the WAC becomes more distinct (Russo and Artegiani, 1996). Since 1988, data of phytoplankton (abundance and biomass) and abiotic parameters have been collected in the SST, with a *ca.* monthly frequency. Previous studies on the SST have already highlighted that the annual cycle of phytoplankton differed between the coastal and offshore stations: in the coastal station the phytoplankton annual maximum occurs in winter months (Totti et al., 2019), while offshore in June-July (Neri et al., 2022).

Phytoplankton is a well-known ecosystem service provider, as it produces more than 50% of the world's oxygen, contributes to ocean carbon cycling, climate regulation and to higher trophic level/food production (Blanchard et al., 2012; Falkowski et al., 2004; Hays et al., 2005; Khatiwala et al., 2009; Martin et al., 2011; Richardson et al., 2000; Tweddle et al., 2018; Vallina and Simó, 2007). The fast turnover and the high sensitiveness to environmental conditions make the phytoplankton an optimal proxy reflecting the main changes in the marine ecosystems. Therefore, phytoplankton has been included in the Marine Strategy Framework Directive for the assessment of the Good Environmental Status (GES) of pelagic habitats (2008/56/EC). Although it is particularly accounted for diversity and food web descriptors, many authors suggested that phytoplankton should be even more considered in the pressure-related descriptors and the marine management processes (European Commission, 2010; European Commission, 2017; McQuatters-Gollop et al., 2015, 2019,2022; Murillas-Maza et al., 2020; Tweddle et al., 2018).

To describe the phytoplankton community structure, it is recommended to use a combination of aspects and metrics, e.g., abundances, biomass, intensity of blooms, composition, diversity indices (Cozzoli et al., 2017; Francé et al., 2021; Hering et al., 2010, Varkitzi et al., 2018). Functional diversity should also be considered as it takes into account the “role” of the organisms in the ecosystem, what they do and how they interact, influencing ecosystem processes and functioning (Díaz and Cabido, 2001; Lyashevska and Farnsworth, 2012; Petchey and Gaston, 2006). The use of graphs (a mathematical formalism useful for describing and representing a relationship on a finite and discrete set of elements, Harary, 1969), or networks, based on interactions, regardless the type, can give insights into the properties and dynamics of communities, as this method simplifies an ecological system representing the interactions among organisms in terms of nodes (taxa) and edges (relationships) (Costa et al., 2019; D'Alelio et al., 2016; Delmas et al., 2018). Graph-network analysis applied on phytoplankton data could provide information on interspecific interactions and functioning of this trophic level which is the basis of most food webs.

Another useful tool providing information on the "importance" of the taxa in the community is given by indicator value analysis (IndVal) which reveals key taxa of a certain grouping factor (e.g., season, environmental conditions), based on relative abundances and frequencies (Dufrêne and Legendre, 1997).

Most of the studies about testing and application of phytoplankton indicators in the Mediterranean Sea cover coastal areas, while the literature related to open water stations is much scarcer (Francé et al., 2021; Markogianni et al., 2017; Ninčević-Gladan et al., 2015; Varkitzi et al., 2018).

In this study, several statistical descriptors (diversity indices, multivariate analyses, IndVal and graph-network analyses) were used (i) to compare the phytoplankton community structure between two stations located in the SST, one coastal and one offshore, and thus differently affected by oceanographic conditions and (ii) to test the suitability of these descriptors in highlighting the main features of the two sites in terms of community composition (e.g. opportunistic/seasonal taxa), main forcings affecting the phytoplankton dynamics, and functioning in terms of species interactions.

2. Materials and Methods

2.1. Study area and sampling

The sampling stations are located along the Senigallia-Susak Transect which is included in the LTER Italian stations. Two stations were considered for the study: SG01 (bottom depth: 12 m) and SG05 (bottom depth: 55 m), located at 1.2 and 15 nM from the western NA coast, respectively (Figure 1).

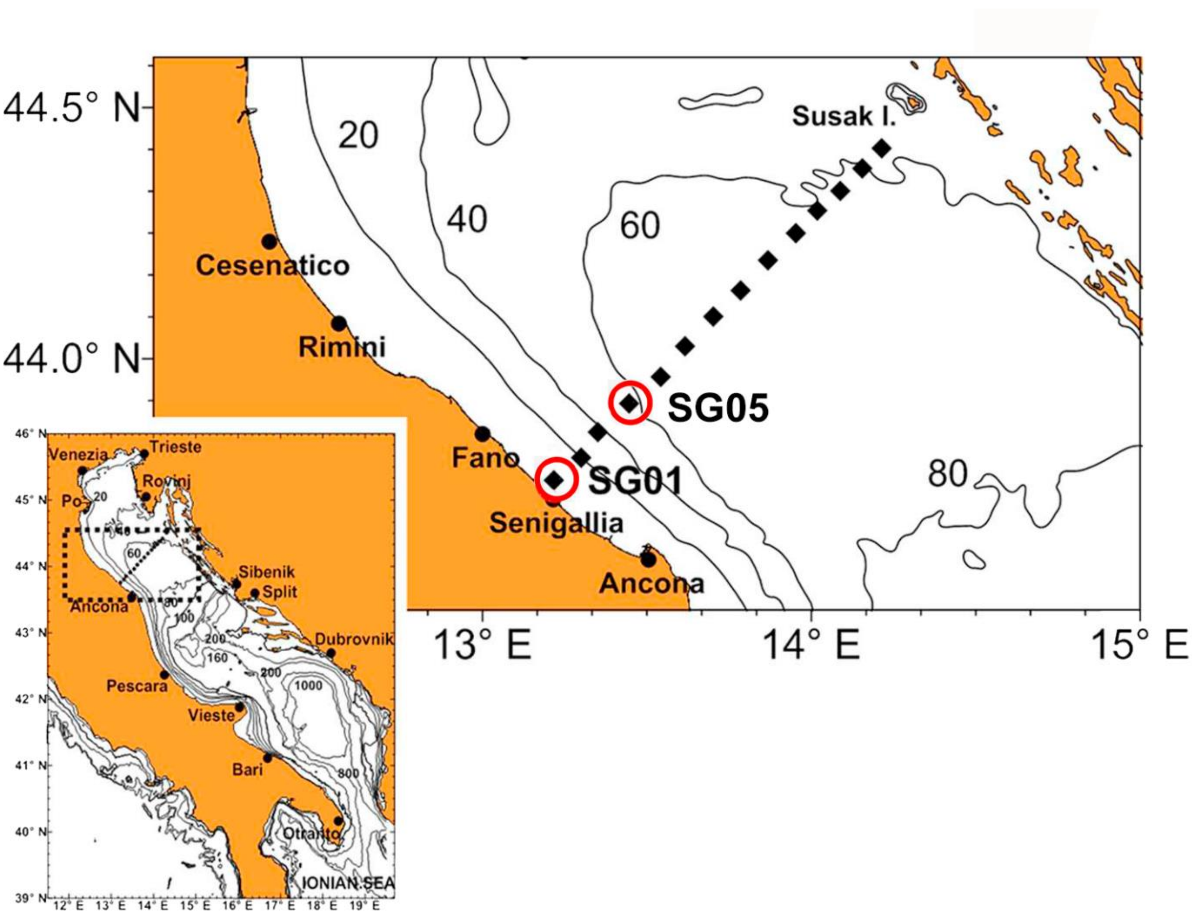

Fig. 1. Map of the study area. The Senigallia-Susak Transect (dotted line) and the sampling stations are shown. Coastal (SG01) and offshore (SG05) stations are highlighted by the red circles.

For both stations, data were collected from 1988 to 2019 on board of several oceanographic vessels (S. Lo Bianco, Tecnopesca 2, G. Dallaporta, Tethis, Copernaut Franca, Urania, Alliance, Minerva, Bannock, D'Ancona, Actea). Sampling was carried out with approximately a monthly frequency, although sometimes quarterly and with some periods of interruptions, particularly for the offshore station (the longest of which was between 2003 and 2012 for phytoplankton sampling), due to greater difficulties in sampling. Conductivity-Temperature-Depth (CTD) data were acquired by a Neil Brown Instrument System (NBIS) (from 1988 to 1991), and later by a SeaBird Electronic SBE 911plus (after 1992).

Niskin bottles or Rosette system were used to collect water samples for determination of dissolved inorganic nutrients (nitrite-NO_2, nitrate-NO_3, ammonia-NH_4, orthophosphate-PO_4 and orthosilicate-$Si(OH)_4$) and for phytoplankton analysis at the following depths: surface (1 m) and bottom (12 m) for SG01 and surface (1 m), base of mixed layer, maximum fluorescence depth (when present) and bottom for SG05. Samples for nutrient analysis were filtered (GF/F Whatman, 0.7 μm), and preserved at −22 °C in polyethylene Falcon until analysis. Phytoplankton samples were collected in 250 ml dark glass bottles and stored at 4°C until analysis after adding 0.8% prefiltered and neutralized formaldehyde (Throndsen, 1978).

2.2. Nutrient analysis

Perkin Elmer spectrophotometer 550A model (1988 to 1998), autoanalyzer TRAACS 800 BRAN+LUEBBE (1999 to 2005) and QUAATRO Technicon (after 2005) were used for nutrient analyses (Strickland and Parsons, 1972). Dissolved Inorganic Nitrogen (DIN) concentration is intended as the sum of NO_2, NO_3 and NH_4 concentrations.

2.3. Phytoplankton analysis

An inverted microscope (ZEISS Axiovert 135) equipped with phase contrast was used for the identification and counting of phytoplankton, following the Utermöhl method (Edler and Elbrachter, 2010). Counting was carried out at 400x magnification, along transects or in random visual fields, depending on cell abundance, to count a minimum of 200 cells. Moreover, a half of the Utermöhl chamber was analyzed at 200x magnification for a more precise estimation of less abundant microphytoplanktonic taxa.

Phytoplankton taxa were grouped into major groups (diatoms, dinoflagellates, coccolithophores, phytoflagellates and others), and abundances were expressed as cells l^{-1}. Dinoflagellates were considered as a taxonomical group and both autotrophic and heterotrophic species were included in counting. Phytoflagellates are an informal group that includes haptophytes (except coccolithophores), cryptophytes, chrysophytes, dictyochophytes, raphidophytes, chlorophytes and euglenophytes. Others include cyanophytes and *incertae sedis*, although this group was not considered for the study.

2.4. Data analysis

Only the surface (0.5 m) depth was considered for the data analysis, leading to a dataset of 7,482 samples of Temperature, Salinity, DIN, PO_4, $Si(OH)_4$ DIN:PO_4, $Si(OH)_4$:DIN and phytoplankton abundances. All the analyses were performed using the R software (R Core Team, 2021), version 4.1.1. Principal Component Analysis (PCA) and Hierarchical Clustering on Principal Components (HCPC) were performed on Temperature, Salinity, DIN, PO_4, $Si(OH)_4$, DIN/PO_4 and $Si(OH)_4$/DIN ratios. In order to allow comparability, data were scaled prior to analysis. PCA and HCPC functions from the FactoMineR package (Lê et al., 2008) were used. In the same way, PCA and HCPC were performed also using the main phytoplankton group abundances.

Phytoplankton groups were analysed through Non-Metric Multidimensional Scaling (NMDS), using the metaMDS function from the vegan package (Oksanen et al., 2022) and setting the autotransform as true. In order to have an insight on the seasonality, NMDS was performed on the different seasons, which were divided as follows: winter (January–March), spring (April–June), summer (July–September), autumn (October–December), as already done in other studies (Bernardi Aubry et al., 2006; Grilli et al., 2020, 2005). Permutational multivariate analysis of variance (PERMANOVA) was used to test for significant differences among the groups that were highlighted by the NMDS, using the adonis function in the vegan package (Oksanen et al., 2022). An analysis of multivariate homogeneity (PERMDISP) (Anderson, 2006) was performed using the betadisper function from the vegan package (Oksanen et al., 2022) as PERMANOVA is sensitive to data dispersion (Anderson, 2001).

For each season, Shannon diversity index (H') (Shannon, 1948) and Pielou's evenness (J') (Pielou, 1975) were used to study diversity and equitability, calculated using the diversity function available in the vegan package (Oksanen et al., 2022). To avoid the influence of the sample sizes to the diversity, rarefied richness was also measured using the rarefy function (vegan package). Margalef and Menhinick indices were calculated using the corresponding functions in the abdiv package (Bittinger, 2020). Two-sample Wilcoxon tests (also known as 'Mann-Whitney' test) was used to check for significant differences between the two stations, using the Wilcox.test function in the stats package (R Core Team, 2021). Pearson's correlations among indices were calculated using rcorr function from the Hmisc package (Harrell, 2022) to study the relationships among them and uniqueness of the information given by each index.

The interactions among the phytoplankton community in the different seasons of each station were investigated by graph-network analysis, where a graph is a mathematical formalism useful for describing and representing a relationship on a finite and discrete set of elements (Harary, 1969). The graph-network analysis was performed using the igraph package in R (Csardi and Nepusz, 2006). Species abundances were log transformed prior to the analysis and undetermined taxa were excluded for this analysis. Pearson's correlations between species were calculated using the stats package (R Core Team, 2021). Only the correlations with the statistical significance coefficient ($p \leq 0.05$) were selected for the construction of the graph. Positive and negative interactions were considered separately. In our study, we relied on the construction of undirected weighted graphs for station description, formally defined on a triple of sets as G = (V, E, W), in which V and E are respectively the set of vertices and edges, W is the set of weights associated to edges given by correlation values. The study makes use of both local and global measures to identify meaningful features of networks. In particular, we considered the closeness centrality measure (Freeman, 1979) and the degree (Harary, 1969) to study the capability of each taxon to interact with others in the system. The closeness parameter measures how "close" a node (species) is to all the others in the network and how quickly it communicates with the others (Mason and Verwoerd, 2007): the node with the highest closeness value would more likely influence the overall network (Costa et al., 2019; Estrada and Bodin, 2008). Instead, the degree is the number of connections and so the number of nodes another one is connected to, giving an idea of the involvement of a species in the network, without taking into account the weight, and so the strength, of the connection itself (Opsahl et al., 2010), therefore providing a different information on the community dynamics.

For small and disconnected networks (i.e., where a maximum of 5 vertices for each connected subgraph was present) only the degree was considered. Furthermore, we calculated the network betweenness, the diameter and the clustering coefficient (even if not discussed). The detailed methods and the equations used for the graph-network analysis are presented in Supplementary Materials.

The Indicator Value (IndVal), which combines the relative abundance and relative frequency of occurrence of a species in a given period (Dufrene and Legendre, 1997), was calculated for each station to identify the phytoplankton key species for each season in the networks that resulted from the graph-network analysis. The INDSPANA software (version 1.1) was used.

3. Results

3.1. Physical and chemical parameters

The PCA biplot of physical and chemical parameters for the two stations is shown in Figure 2A. The first two axes explained 59% of the total variability. Discrimination between the sampling stations was primarily driven by differences in DIN, which gave the major contribution to the ordination, followed by salinity, $Si(OH)_4$ and DIN/PO_4. SG01, located in the coastal area, appeared more variable and influenced by changes in the concentrations of the chemical parameters than SG05, the offshore station, which showed more stable conditions and higher salinity values (Figure 2A).

The HCPC analyses performed on the physico-chemical parameters clustered the sampling points in three main groups (Figure 2B). Two groups included several SG01 samples, one characterized by higher values of phosphorous and silicates, and the other with lower values. Almost all SG05 sampling points (and few SG01 ones) were grouped into a third cluster which was characterized by high salinity and low nutrient concentrations. A similar pattern was also observed for the HCPC performed on the main phytoplankton group abundances as the sampling points clustered in three groups (Figure S1B). One group was characterized by high values of coccolithophores and included only SG01 samples (with just one exception), while another group was characterized by high abundances of diatoms and phytoflagellates (almost all SG01 samples). Almost all the SG05 sampling points were included in a third group together with several SG01 sampling points.

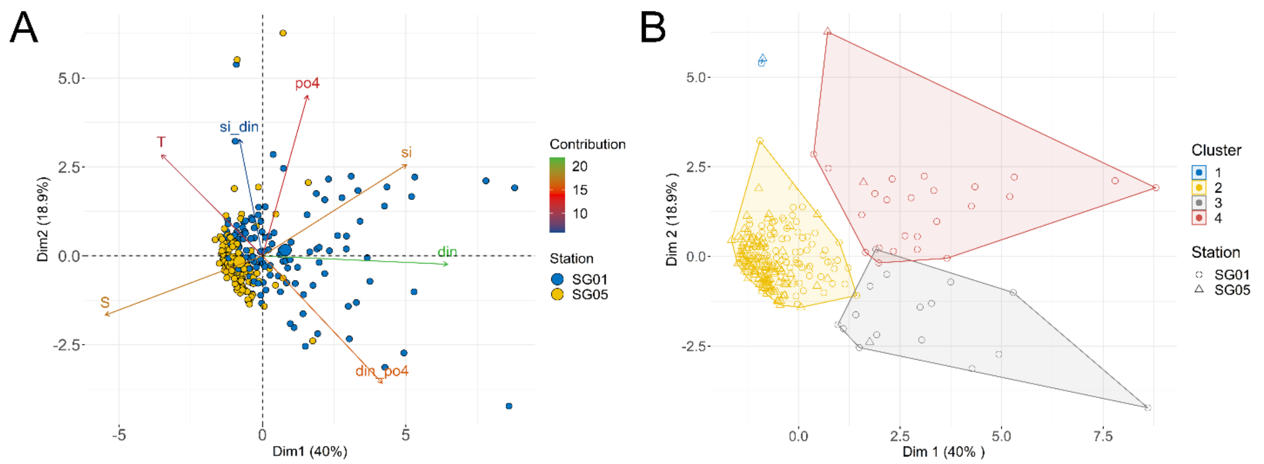

Fig. 2. Principal Component Analysis (PCA) (A) and Hierarchical Clustering on Principal Components (HCPC) (B) performed on physical-chemical parameters (Temperature (T), Salinity (S), DIN (din) , PO_4 (po4), $Si(OH)_4$ (si), DIN/PO_4 (din_po4) and $Si(OH)_4$/DIN (si_din)) in SG01 and SG05. In the PCA biplot the variables (vectors) are presented by their contributions to the principal components (gradient colours of vectors). In the HCPC plot the clusters are represented by a different colour.

3.2. Phytoplankton groups

The mean seasonal values of abundances of each phytoplankton group are shown in Table 1. In winter, significant higher values of diatoms (p<0.001), dinoflagellates (p<0.05) and phytoflagellates (p<0.001) were found in SG01 compared to SG05. On the contrary, coccolithophores showed significant higher values in SG05 than in SG01 (p<0.001).

In spring, summer and autumn, significant higher values of diatoms (p<0.01, p<0.01, p<0.05, respectively), dinoflagellates (p<0.001, p<0.05, p<0.01, respectively) and phytoflagellates (p<0.001) were found in SG01 than in SG05, while no significant difference between the two stations was observed for the coccolithophores (p>=0.05).

Table 1. Seasonal mean abundances (mean ± standard error, cells l^{-1}) of diatoms, dinoflagellates, coccolithophores and phytoflagellates for each station (SG01 and SG05) and season. Differences between the two stations are expressed as ns, not significant; *p < 0.05; **p < 0.01; ***p < 0.001.

		Mean ± std. error (cells l^{-1})			
Station	**Season**	**Diatoms**	**Dinoflagellates**	**Coccolithophores**	**Phytoflagellates**
SG01	Winter	6,466,862 ± 1,422,876	56,299 ± 7,682	19,194 ± 4,693	3,503,962 ± 545,124
SG05		110,792 ± 33,054	19,183 ± 2,890	26,497 ± 5,585	501,589 ± 43,029
p-Level		***	*	***	***
SG01	Spring	2,453,544 ± 637,242	155,958 ± 18,666	43,417 ± 9,709	2,631,848 ± 225,760
SG05		334,466 ± 94,910	55,529 ± 5,877	31,857 ± 11,370	922,234 ± 124,585
p-Level		**	***	ns	***
SG01	Summer	543,100 ± 94,769	84,709 ± 12,731	16,186 ± 3,456	2,016,555 ± 164,520
SG05		231,219 ± 77,968	43,780 ± 7,371	8,831 ± 2,364	484,784 ± 53,842
p-Level		**	*	ns	***
SG01	Autumn	1,269,870 ± 325,545	77,508 ± 9,986	120,190 ± 97,733	2,587,784 ± 281,576
SG05		203,113 ± 39,037	23,086 ± 2,549	16,242 ± 2,089	381,088 ± 31,244
p-Level		*	**	ns	***

The results of the Non-Metric Multidimensional Scaling (NMDS), performed for each season on the abundances of each phytoplankton group, are represented in Figure 3. A marked divergence between samples belonging to the two stations was observed in winter (Figure 3A), whilst no clear distinction was found in spring (Figure 3B) and summer (Figure 3C), and only a slight difference was observed in autumn (Figure 3D). Comparing the two stations in each season, the PERMANOVA analysis highlighted significant differences between SG01 and SG05 (p<0.001), supporting the ordination results. Significant values of dispersions were observed in winter and autumn (*p*<0.001 and *p*<0.001, respectively), while in spring and summer any significant dispersion was found (*p*>0.05).

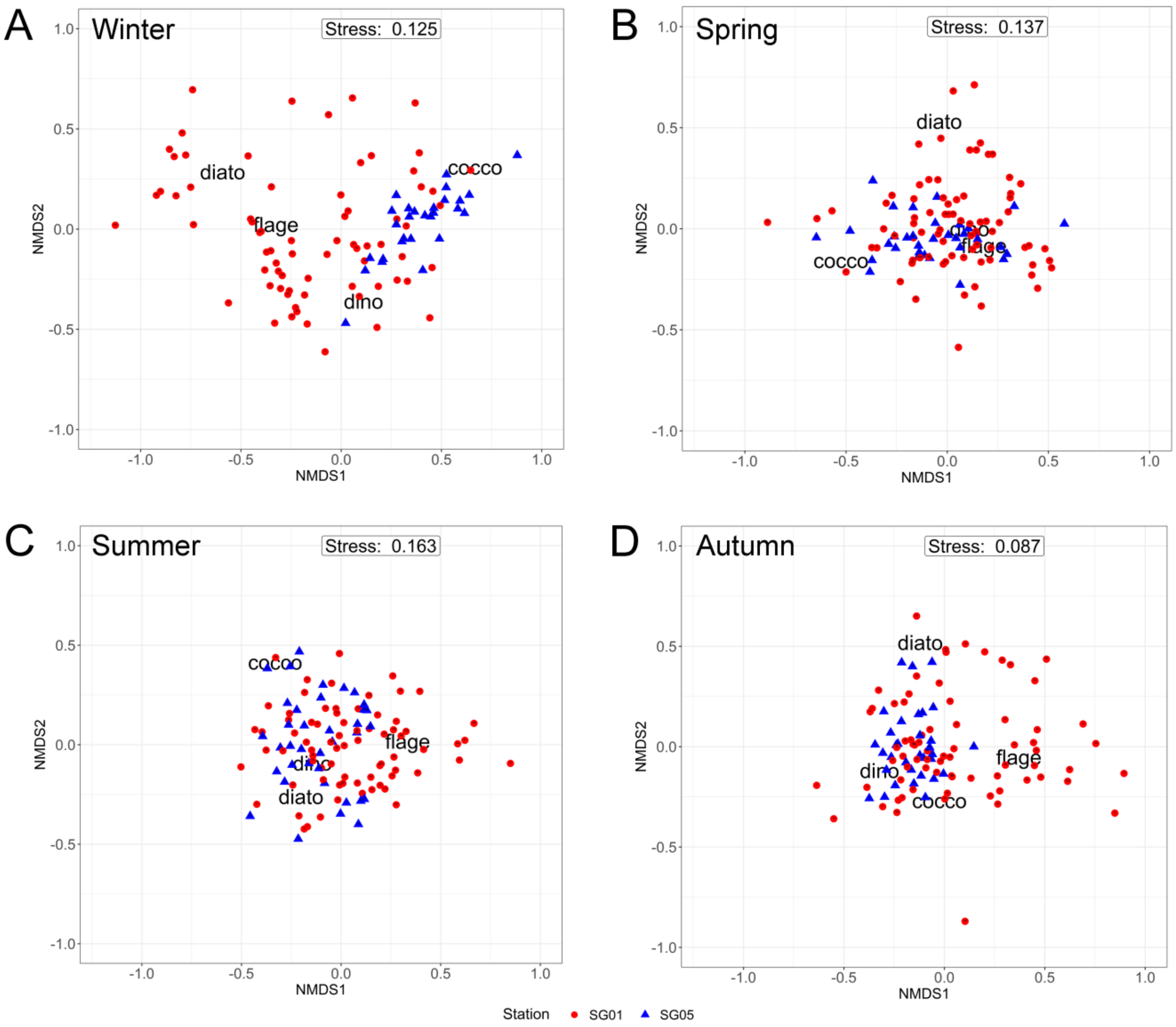

Fig. 3. NMDS performed on winter (A), spring (B), summer (C), autumn (D) on the abundances of each phytoplankton group in SG01 and SG05: diatoms (diato), dinoflagellates (dino), coccolithophores (cocco), phytoflagellates (flage).

3.3. Diversity

The total list of taxa (Table S1) included 174 Bacillariophyceae (diatoms), 136 Dinophyceae (dinoflagellates), 32 Prymnesiophyceae (of which 30 coccolithophores), 5 Cryptophyceae, 8 Chrysophyceae, 3 Raphidophyceae, 9 Dictyochophyceae, 6 Chlorophyceae, 4 Euglenophyceae, 1 cyanobacteria.

The diversity indices, performed for each season, are shown in Figure 4. All indices (Shannon diversity index (H), Pielou's evenness (J), Rarefied richness, Menhinick and Margalef indices) highlighted higher values in SG05 than in SG01 in winter (Wilcoxon test, $p<0.001$) and in autumn (Wilcoxon test, $p<0.01$ for rarefied richness and Shannon and Menhinick indices; $p<0.05$ for evenness and Margalef index). In spring, Shannon diversity index (Wilcoxon test, $p<0.05$), Pielou's evenness (Wilcoxon test, $p < 0.01$) and Menhinick index (Wilcoxon test, $p < 0.05$) were found to be higher in SG05, while no differences were observed in terms of rarefied richness and Margalef index (Wilcoxon test, $p > 0.05$). No significant differences between the two stations were found in summer (Wilcoxon test, $p > 0.05$).

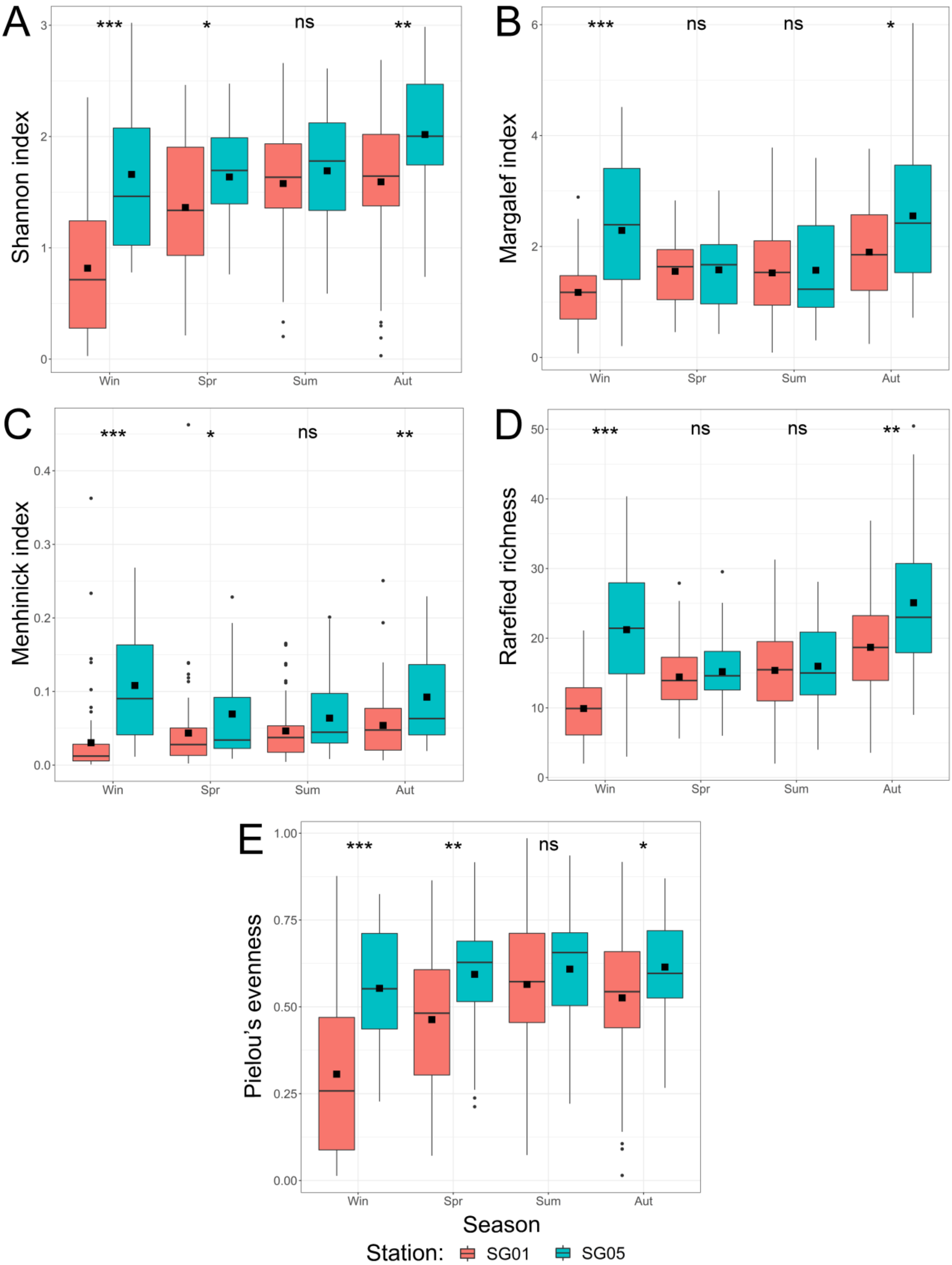
A
Shannon index
*** * ns **
Win Spr Sum Aut
B
Margalef index
*** ns ns *
C
Menhinick index
*** * ns **
D
Rarefied richness
*** ns ns **
E
Pielou's evenness
*** ** ns *
Season
Station: SG01 SG05

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Fig. 4. Shannon index (A), Margalef index (B), Menhinick index (C), Rarefied richness (D) and Pielou's evenness (E), calculated for each season, are shown for both stations. Box plots report the data distribution with the mean (□), the median (line), the interquartile range (box), the non-outlier range (vertical bars), the outliers (○). *** ($p<0.001$), ** ($p<0.01$), * ($p<0.05$), ns (not significant).

In both stations and in all the seasons, significant correlations were observed between most of the indices (Table 2), but with the following exceptions.

In winter, in SG01 station, no correlation was found for Pielou's Evenness vs Margalef index, while in SG05, for Pielou's Evenness vs Menhinick and vs Margalef indices and for Pielou's Evenness and Rarefied richness.

In spring, in SG01 station no correlation was found for Pielou's Evenness vs Margalef and vs Menhinick and for Menhinick vs Shannon index, while in SG05 for Pielou's Evenness vs Rarefied richness and vs Menhinick index and for Shannon index vs Margalef and vs Menhinick indices.

In summer, in both stations, no correlation was found for Pielou's Evenness vs Rarefied richness and vs Menhinick and for Shannon vs Menhinick. In SG05 Shannon index showed no correlation also vs Margalef index. In autumn, both in SG01 and SG05, Pielou's Evenness was not correlated with Rarefied richness, Menhinick and Margalef indices.

Table 2. Correlations between the different indices (Rarefied richness, Pielou's evenness, Shannon, Margalef, Menhinick indices) in SG01 (A,C,E,G) and SG05 (B,D,F,H) in winter (A,B), spring (C,D), summer (E,F) and autumn (G,H). Values indicated in italic are significant at $p<0.05$, those in bold italic are significant at $p<0.01$, those in bold italic and underlined are significant at $p<0.001$.

A	R.richness	Shannon	Evenness	Margalef	Menhinick
R.richness	1				
Shannon	<u>***0.76***</u>	1			
Evenness	<u>***0.62***</u>	<u>***0.95***</u>	1		
Margalef	<u>***0.72***</u>	<u>***0.39***</u>	0.2	1	
Menhinick	<u>***0.75***</u>	<u>***0.77***</u>	<u>***0.68***</u>	<u>***0.61***</u>	1

B	R.richness	Shannon	Evenness	Margalef	Menhinick
R.richness	1				
Shannon	<u>***0.68***</u>	1			
Evenness	0.03	<u>***0.6***</u>	1		
Margalef	<u>***0.98***</u>	<u>***0.58***</u>	-0.11	1	
Menhinick	<u>***0.83***</u>	<u>***0.65***</u>	0.12	<u>***0.82***</u>	1

C	R.richness	Shannon	Evenness	Margalef	Menhinick
R.richness	1				
Shannon	<u>***0.66***</u>	1			
Evenness	<u>***0.43***</u>	<u>***0.93***</u>	1		
Margalef	<u>***0.72***</u>	*0.23*	-0.07	1	
Menhinick	<u>***0.41***</u>	0.2	0.12	<u>***0.42***</u>	1

D	R.richness	Shannon	Evenness	Margalef	Menhinick
R.richness	1				
Shannon	***0.54***	1			
Evenness	-0.05	<u>***0.71***</u>	1		
Margalef	<u>***0.79***</u>	0.23	*-0.37*	1	
Menhinick	<u>***0.62***</u>	0.28	-0.04	<u>***0.69***</u>	1

E	R.richness	Shannon	Evenness	Margalef	Menhinick
R.richness	1				
Shannon	<u>***0.57***</u>	1			
Evenness	-0.06	<u>***0.7***</u>	1		
Margalef	<u>***0.89***</u>	<u>***0.32***</u>	***-0.31***	1	
Menhinick	<u>***0.48***</u>	0.15	-0.09	<u>***0.53***</u>	1

F	R.richness	Shannon	Evenness	Margalef	Menhinick
R.richness	1				
Shannon	***0.44***	1			
evenness	-0.18	<u>***0.76***</u>	1		
Margalef	<u>***0.95***</u>	0.25	*-0.37*	1	
Menhinick	<u>***0.68***</u>	0.19	-0.26	<u>***0.79***</u>	1

G	R.richness	Shannon	evenness	Margalef	Menhinick
R.richness	1				
Shannon	<u>***0.6***</u>	1			
Evenness	0.03	<u>***0.74***</u>	1		
Margalef	<u>***0.91***</u>	<u>***0.4***</u>	-0.19	1	
Menhinick	***0.32***	***0.34***	0.19	***0.33***	1

H	R.richness	Shannon	Evenness	Margalef	Menhinick
R.richness	1				
Shannon	<u>***0.65***</u>	1			
Evenness	0.02	<u>***0.74***</u>	1		
Margalef	<u>***0.94***</u>	<u>***0.54***</u>	-0.11	1	
Menhinick	<u>***0.76***</u>	<u>***0.56***</u>	0.14	<u>***0.81***</u>	1

3.4. Phytoplankton community composition: Indicator Value Analysis

The IndVal was calculated seasonally for each station (Table S2), highlighting the representative taxa for each station on a seasonal basis. In the SG01 station, in winter the representative taxa were *Skeletonema marinoi*, *Chaetoceros curvisetus*, *Thalassiosira rotula*, *Dictyocha fibula*, *Chaetoceros danicus*. In spring, the taxa with significant values were *Dactyliosolen fragilissimus*, *Prorocentrum micans*, *Nitzschia longissima*, *Cyclotella* spp., *Tripos fusus*, *Pseudo-nitzschia* cfr. *delicatissima*, *Chaetoceros tenuissimus*, *Pseudo-nitzschia* cfr. *delicatissima*, *Tripos fusus*, *Noctiluca scintillans*, *Prorocentrum cordatum*, *Alexandrium* spp., *Protoperidinium* cfr. *steinii*, *Dinophysis sacculus*, *Prorocentrum triestinum*, *Cyclotella* spp. In summer, the most representative taxa were *Proboscia alata*, *Guinardia flaccida*, *Nitzschia gobbii*, *Cerataulina pelagica*, *Pseudo-nitzschia* cfr.

pseudodelicatissima, *Calycomonas* sp., *Pleurosigma* spp., *Nitzschia gobbii*, *Calciosolenia brasiliensis*, *Hemiaulus hauckii*, *Pseudoscourfieldia marina*, *Prorocentrum compressum*. In autumn, significant taxa were *Lioloma pacificum*, *Chaetoceros danicus*, *Asterionellopsis glacialis*, *Eucampia cornuta*, *Chaetoceros affinis*, *Protoperidinium diabolus*.

In the SG05 station, in winter the most representative taxa were *Skeletonema marinoi*, *Emiliania huxleyi*, *Pseudosolenia calcar avis*, *Chaetoceros affinis* and *Diploneis* spp. In spring, significant taxa were *Dactyliosolen fragilissimus*, *Cyclotella* spp., *Prorocentrum micans*, *Euglena* spp. In summer, *Proboscia alata*, *Cerataulina pelagica*, *Pseudoscourfielda marina* and *Calycomonas* sp. were found to have significant values. In autumn, most significant taxa were *Guinardia striata*, *Lioloma pacificum*, *Cylindrotheca closterium*, *Asterionellopsis glacialis*, *Pseudo-nitzschia* cfr. *delicatissima*, *Pleurosigma* spp.

3.5. Graph-network analysis

The results of the graph-network analysis, performed on each season, showed that the networks based on positive interactions (Figure 5) were characterized by a higher number of vertices (taxa) and edges (interactions) than the ones based on negative interactions (Figure S2), which often showed many disconnected subgraphs. Network betweenness, diameter, clustering coefficient (not discussed in this study) are shown in Table 3.

The winter networks based on positive interactions were characterized by 47 taxa and 200 interactions in the coastal station SG01 (Figure 6A) and 54 taxa (of which 2 disconnected, i.e., *Chaetoceros compressus* and *Pseudosolenia calcar avis*), and 178 interactions in the offshore station SG05 (Figure 6B). The two stations showed similarities in terms of closeness ranges (0.007–0.020 and 0.009–0.020 in SG01 and SG05, respectively). The species with the highest value of closeness was *Leptocylindrus danicus* in SG01 (closeness = 0.020), and *Chaetoceros lorenzianus* in SG05 (closeness = 0.020). *Chaetoceros curvisetus* was the one with the highest number of total interactions in both stations (degree = 22 and 19, in SG01 and SG05, respectively). As regards the networks based on negative interactions (Figure S2 A,B), in both stations, *Emiliania huxleyi* was the species with the highest values of closeness and degree. Considering both types of winter networks, stronger interactions were found in SG05 than in SG01: SG05 showed the 85% and 68% of the positive and negative correlations with r≥0.4, while in SG01 station most of the correlations (94% and 81% for positive and negative interactions, respectively) were weak (r<0.4).

The spring networks built on positive interactions were characterized by 50 taxa and 171 interactions in the SG01 station (Figure 6C) and 49 taxa (of which 2 disconnected, i.e., *Chaetoceros compressus*, *Leptocylindrus minimus*), and 110 interactions in the SG05 station (Figure 6D). The two stations showed similarities in terms of closeness ranges (0.007–0.018 in SG01 and 0.007–0.019 in SG05). In SG01 *Thalassionema nitzschioides* was the species with the highest value of closeness (0.018), although *Cerataulina pelagica* had the highest degree value (18). Offshore, *Chaetoceros tortissimus* showed both the maximum of closeness (0.019) and degree (11). Instead, in the negative networks, in the coastal station (Figure S2C) *Pseudokephyrion* spp. was the species with the maximum of closeness (0.04), while *Pseudoscourfieldia marina* showed the highest value of degree (6). The offshore network (Figure S2D) was characterized by several disconnected subgraphs. *Calycomonas* sp., *Emiliania huxleyi* and *Dactyliosolen fragilissimus* were the species showing the higher number of negative interactions. In the SG05 station, the networks showed a 68% and 11% of strong positive and negative correlations (r≥0.4), respectively. No interactions with r<0.3 was found in both graphs. On the contrary, in the SG01 station, most of the correlations were very weak as the 51% and the 83% of positive and negative interactions, respectively, showed r<0.3.

Summer networks based on positive correlations were characterized by 48 nodes (taxa) and 154 edges (interactions) in SG01 (Figure 6E) and 54 taxa (of which two disconnected, i.e. *Chaetoceros costatus*, *Dactyliosolen fragilissimus*), and 129 interactions in SG05 (Figure 6F). The coastal station showed higher closeness ranges (0.007–0.017) than the offshore one (0.005–0.015). In the first case, *Chaetoceros lorenzianus* was the species with the highest value of closeness, although *Chaetoceros curvisetus* showed the maximum of degree (14). In the second case, *Syracosphaera pulchra* showed the highest value of closeness (0.015) and of degree (14). As regards the networks based on negative interactions (Figure S2E,F), in both stations *Pseudoscourfieldia marina* was the species with the maximum of closeness (0.05 and 0.10 in SG01 and SG05, respectively) and degree (13 and 9 in SG01 and SG05, respectively). The networks of the SG01 station were characterized by weak interactions among taxa, as only the 16% and 5% of positive and negative correlations, respectively, showed $r \geq 0.4$. In the SG05 networks, about half of the correlations were found with $r \geq 0.4$ (50% and 44% for positive and negative correlations, respectively).

Autumn networks based on positive interactions were characterized by 51 vertices (taxa) and 278 edges (interactions) were found at the SG01 station (Figure 6G), while 56 vertices and 225 edges were found at SG05 (Figure 6H). The two stations showed similar closeness ranges (0.007–0.020 and 0.006–0.019 in SG01 and SG05, respectively). In SG01, the maximum of closeness was observed for *Cerataulina pelagica* (0.020), which was also the species with the highest number of interactions, together with *Dactyliosolen fragilissimus* (degree = 25). In SG05, *Chaetoceros curvisetus* was the species with the maximum closeness (0.019) and degree (22). The negative networks were both strongly disconnected graphs. In SG01, *Tripos muelleri*, *Calciosolenia murrayi* and *Pseudo-nitzschia* cfr. *pseudodelicatissima* were the species showing the higher number of negative interactions (Figure S2G), while in SG05 *Calycomonas* sp., and *Emiliania huxleyi* showed the highest degree value (Figure S2H). Stronger interactions were found for SG05 than for SG01. The latter was characterized by weak positive (73% with $r \leq 0.4$, of which 42% with $r < 0.3$) and negative (100% with $r < 0.4$, of which 92% with $r < 0.3$) interactions. Instead, in SG05 67% and 42% were the percentages of positive and negative correlations with $r \geq 0.4$.

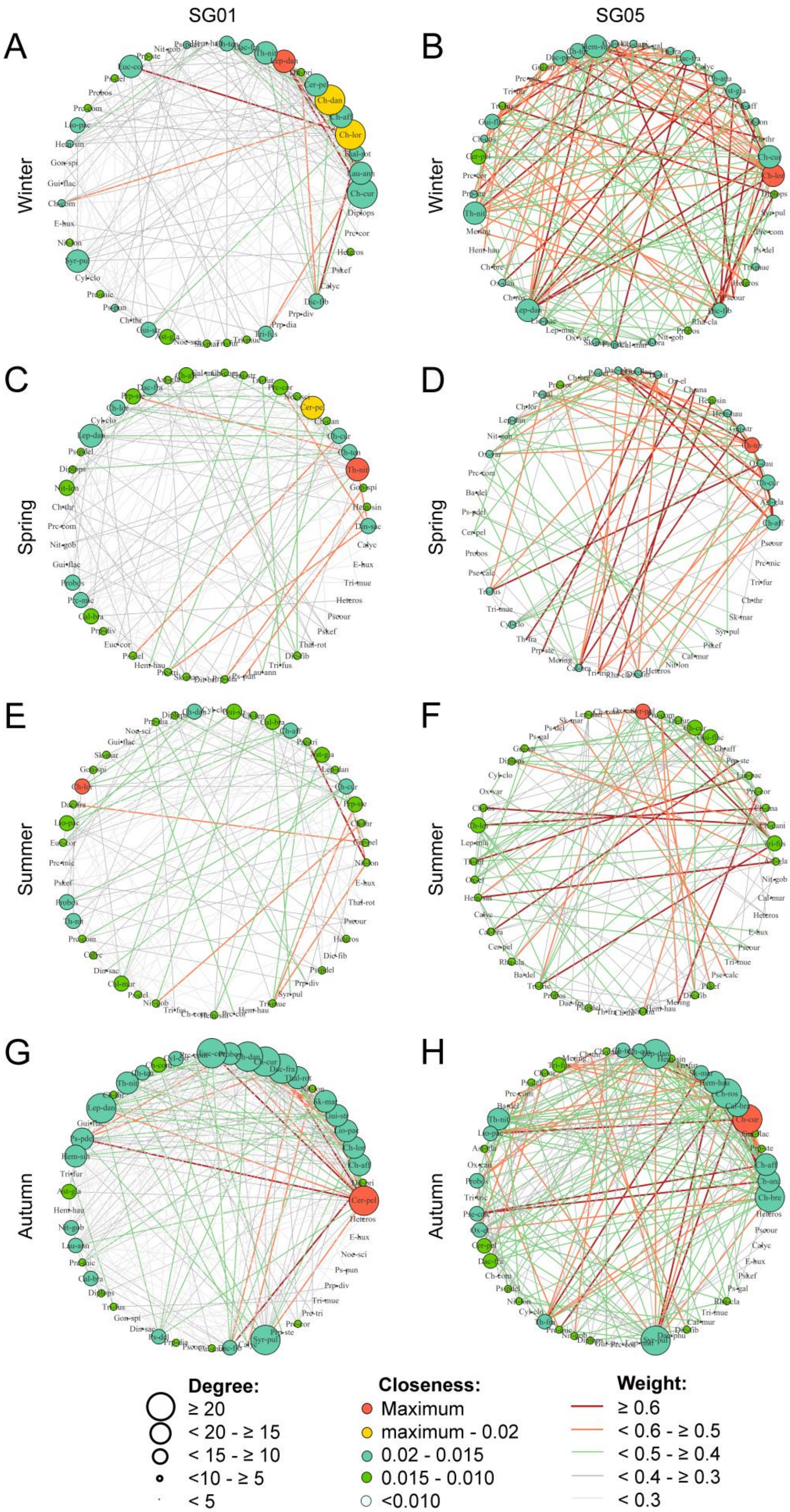
SG01
SG05
A
B
Winter
Winter
C
D
Spring
Spring
E
F
Summer
Summer
G
H
Autumn
Autumn
Degree:
≥ 20
< 20 - ≥ 15
< 15 - ≥ 10
<10 - ≥ 5
< 5
Closeness:
Maximum
maximum - 0.02
0.02 - 0.015
0.015 - 0.010
<0.010
Weight:
≥ 0.6
< 0.6 - ≥ 0.5
< 0.5 - ≥ 0.4
< 0.4 - ≥ 0.3
< 0.3

Fig. 5. Networks based on winter (A,B), spring (C,D), summer (E,F), autumn (G,H) positive correlations in SG01 (A,C,E,G) and SG05 (B,D,F,H). The dimension of the circle is proportional to the degree and the different colours are related to closeness. Line colours and thickness depend on the strength of the interaction. The names of the taxa are abbreviated as shown in Supplementary Table 3.

Table 3

Network parameters calculated on the seasonal network of both stations.

		Attributes		
		Network betweenness	**Diameter**	**Clustering coefficient**
SG01	**Winter**	0.15	4.09	0.49
SG01	**Spring**	0.13	4.77	0.35
SG01	**Summer**	0.11	4.19	0.38
SG01	**Autumn**	0.10	4.00	0.59
SG05	**Winter**	0.14	3.75	0.49
SG05	**Spring**	0.14	3.54	0.34
SG05	**Summer**	0.16	5.8	0.31
SG05	**Autumn**	0.09	4.57	0.48

4. Discussion

In this study we highlighted the key role of the oceanographic conditions in discriminating the two stations along the Senigallia-Susak Transect and the ability of the used indices and descriptors in underlying the differences in the phytoplankton communities. In both stations a clear seasonal rhythm in the annual cycle of phytoplankton was highlighted, but this rhythm markedly differed between the two stations, reflecting the different oceanographic regimes. Indeed, offshore (55 m depth) the main seasonal driver is the vertical structure of the water column (i.e., mixing vs stratification) which influences the conditions for the phytoplankton at the surface and in the whole mixed layer, in terms of light and nutrient availability. On the contrary, in the shallower coastal station (10 m depth), where the water column is mixed almost throughout the year, the main constrain is the outflow of riverine waters (itself related to the rain regime) carried by the WAC.

The statistical comparison of the offshore and coastal stations in terms of physico-chemical parameters highlighted that the offshore station, located beyond the WAC, is more stable than the coastal one, as the latter is more affected by changes in the meteorological conditions. Indeed, the western Adriatic coast is directly influenced by the continental waters (Totti and Artegiani, 2001), among which the Po River stands out, characterized by a much higher concentration of nitrogen, compared to phosphorous (Grilli et al., 2020). These waters conveyed by the WAC reduce salinity and increase nitrate and silicate concentrations (Artegiani et al., 1997b; Campanelli et al., 2011; Marini et al., 2008), which are those factors mostly discriminating the coastal sampling points by the PCA. The offshore station is not directly affected by freshwater inputs, except when the riverine waters expand eastward during stratification (Neri et al., 2022). Consequently, the environmental conditions of the offshore station result more stable throughout the year.

The effects of the different environmental conditions and oceanographic circulation along the SST are also evident considering the diversity of the phytoplankton community and the seasonal variability of each phytoplankton group, as well as the structure of the communities in terms of interspecific interactions. In this regard, the two stations differed mainly in winter and autumn as suggested by the diversity indices and NMDS analysis. Indeed, Campanelli et al. (2011) observed that the riverine input in the NAS affected nutrient concentrations and phytoplankton growth particularly in those seasons. On the contrary, the two stations appeared to be more similar in summer, as shown by the more grouped samples and by comparable diversity indices, due to the spreading south-eastward of the floods of the Po River in stratified conditions (Neri et al., 2022).

This variability among seasons was also highlighted both by the HCPC and NMDS performed on the phytoplankton abundances and by the significant dispersion of the samples.

As the effect of the riverine input is more evident along the coast, higher phytoplankton abundances were found always in the coastal station. Only in winter coccolithophores were found to have higher mean abundances offshore, in correspondence of high salinity values (Neri et al., 2022; Totti et al., 2019), as commonly reported worldwide (Baumann et al., 2005; Menschel et al., 2016).

Diversity indices have been suggested and used to analyse the phytoplankton diversity for the GES assessment in the Mediterranean Sea, as they were found to be able to distinguish different environments and different anthropogenic impacted areas (mainly coastal) (Francé et al., 2021; Rombouts et al., 2019; Varkitzi et al., 2018). In this study, all the tested diversity indices agreed in highlighting a higher similarity in summer between the two stations and a higher diversity in the offshore station. This higher diversity in the offshore station can be related to the oligotrophic conditions of waters beyond the WAC, and it was observed even in other Adriatic and Mediterranean oligotrophic areas (Bužančić et al., 2016; Francé et al., 2021; Varkitzi et al., 2020), but it could be partly influenced by the methodological approach of the Utermöhl method. In fact, the sample volume settled in the cylinder/chamber for analysis is normally higher in offshore stations (40–100 ml) than in coastal ones (5–25) due to different cell concentrations, increasing the probability to find rarer species. Comparing the diversity indices, as expected, significant relationships were found among them for most combinations. However, the correlations and the significances changed among the seasons and between the stations, suggesting that a combination of indices is more appropriate to describe the phytoplankton diversity, and to better estimate its variation in time and space. In both stations and in all the seasons, Pielou's evenness showed no significant correlations with other indices, as already found by Rombouts et al. (2019), suggesting that this index could give not redundant information when combined with others. In some cases, also the Shannon index showed no significant correlations, suggesting a usefulness of this indicator.

The IndVal analyses highlighted taxa that are key elements of a certain season based on their abundance and frequency of occurrence (Dufrene and Legendre, 1997), showing some differences and similarities between the two stations, as reported in previous studies in the same area (Neri et al., 2022; Totti et al., 2019). Regardless to any environmental variability, some phytoplankton species expressed a marked seasonal behavior (see also Longobardi et al., 2022), as for example *Skeletonema marinoi* that is a significant indicator of the winter community. In other cases, the timing of species could be directly affected by changes in the trophic state, mainly linked to the riverine inputs, which could lead to increase/decrease in abundances, also favoring opportunistic taxa to the detriment of others. Indicator species are related to the specific environmental conditions of a certain period (stratification/mixing regime, photoperiod etc.), while non-indicator taxa are not apparently linked with certain environmental factors and are more homogeneously distributed throughout the year. As a consequence, the latter show more interactions with the overall phytoplankton community, as noticeable from the graph-network analysis, through which we represented the interactions among organisms in a "simplified" way (in terms of nodes, i.e. taxa, and edges, i.e. relationships). In our study, the highest values of closeness (which takes into account the number of interactions and their strength) were found for species identified as non-indicator and therefore for ones not considered as key species of a certain season (e.g., *Leptocylindrus danicus*, *Thalassionema nitzschioides*, *Chaetoceros lorenzianus*, *Cerataulina pelagica* in the coastal station and *Chaetoceros lorenzianus*, *C. tortissimus*, *C. curvisetus*, *Leptocylindrus danicus* and *Syracosphaera pulchra* offshore). Few exceptions were found in the coastal station, for some widespread taxa (i.e., *Chaetoceros danicus* and *Proboscia alata*) that showed high values of closeness even in those seasons for which they are indicators. Considering the degree (which does not consider the weight, and so the strength, of the connection itself), some differences were observed between the two stations. Offshore, key species (following the IndVal analysis) were found to have a low number of total interactions (e.g., *Skeletonema marinoi* and *Pseudosolenia* *calcar avis* in winter, *Prorocentrum micans* in spring, *Pseudoscourfieldia marina* and *Cerataulina pelagica* in summer and *Cylindrotheca closterium* in autumn). On the contrary, the coastal station did not show a clear pattern, underlying the role of the marked seasonality related to direct riverine inputs. Indeed, here some indicator taxa were found to have low degree values (e.g., *Skeletonema marinoi* in winter, *Tripos fusus* in spring and *Guinardia flaccida* in summer), while others showed high degree values (e.g., *Chaetoceros danicus* and *C. curvisetus* in winter, *Prorocentrum micans* in spring, *Proboscia alata* in summer).

As expected, the networks based on negative correlations were made of a lower number of vertices and edges than the ones with positive correlations and were often disconnected (and thus difficult to be interpreted). Without a prey-predator context, the negative interactions among phytoplankton taxa can underly competition for the same resources (nutrients, light, etc.) but also temporal changes of single taxa, that produce a negative correlation, as the case of *Emiliania huxleyi* (the species with the highest values of closeness and degree in the negative networks, in both stations), whose decrease has been reported by previous studies (Neri et al., 2022; Totti et al., 2019).

The offshore station was found to have stronger relationships both in the network based on positive and negative correlations, due to its higher stability and to the different seasonal behavior of the oceanographic conditions respect to the coastal station, allowing the establishment of stronger connections. In the offshore station, the weaker relationships were found in summer, the season where a major similarity between the two stations was also highlighted by the NMDS analysis, underlying again the role of water stratification in affecting phytoplankton community (Neri et al., 2022).

Metrics based on network approaches (such as the Ecological Network Analysis indices) have been proposed as food web indicators, as they cross multiple trophic levels and can give a holistic view on the trophic web (Fath et al., 2019; McQuatters-Gollop et al., 2022; Safi et al., 2019; Tomczak et al., 2013). In our study, graph-network analysis based on positive and negative interactions was able to detect differences in phytoplankton community/functionality of the two stations and changes in the phytoplankton abundances.

5. Conclusions

Ecosystem comparison of long-term data series allows a better understanding of the uniqueness of the environments, the key aspects controlling biodiversity and productivity, as well as the processes adopted to respond to natural and human-induced pressures (Acri et al., 2020; Kröncke et al., 2019; Megrey et al., 2009; Murawski et al., 2010), which is fundamental for an effective marine ecosystem management. In our study we combined different approaches to compare coastal and offshore areas located along the Senigallia-Susak Transect in the NAS, both in terms of physico-chemical parameters and phytoplankton community, and to gain insights in the functioning of the phytoplankton community and the main forcings affecting it.

The multivariate statistical analyses showed that the coastal station is more variable and directly affected by riverine inputs than the offshore one, which is located beyond the WAC, leading to differences in both phytoplankton abundances and community structure. These differences should be taken in consideration when any evaluation of Good Environmental Status is carried on. The two stations were more different in winter and autumn when the water masses are clearly separated, and more similar in summer, when coastal waters spread offshore during the stratification regime.

Higher abundances of all groups except coccolithophores were found in the coastal station, while the offshore station was characterized by higher diversity than the coastal one. All the used diversity indices were able to discriminate between stations and highlighted the effect of the different environmental/oceanographic conditions on the phytoplankton diversity. Pielou’s evenness could give non-redundant information as low, and in some case not significant, correlations were found with other indices.

The graph-network analysis, which can be used to study the interactions inside a community, revealed a phytoplankton community structure that is highly influenced by oceanographic conditions and confirmed that non-indicator species have more interactions because are more present throughout the year. The networks were able to detect the differences between the two stations in the terms of phytoplankton communities and dynamics, referable to different environmental and oceanographic conditions, and long-term variations in the abundances. Therefore, graph-network analysis could be a useful tool, not only for the food web descriptors, but also to highlight structure and differences in the phytoplankton communities, which are the basis of most food webs and provide many ecosystem services. Instead, the IndVal analysis is useful to find out the key taxa of a certain season. In this way, the combination of the two analyses can give different information, in some way complementary and non-redundant, on the phytoplankton community structure and composition.

In conclusion, the combination of different methods, such as statistical analysis, biodiversity indices, Indicator Value analysis and graph-network analysis, allows insights in the "functioning" of different areas and the forcings affecting phytoplankton communities, necessary information to assess the environmental status of different areas.

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