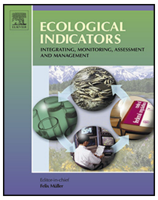
Ecological Indicators 56 (2015) 106–115

Contents lists available at ScienceDirect

Ecological Indicators

journal homepage: www.elsevier.com/locate/ecolind



The response of phytoplankton community to anthropogenic pressure gradient in the coastal waters of the eastern Adriatic Sea

Živana Ninčević-Gladan*, Mia Bužančić, Grozdan Kušpilić, Branka Grbec, Slavica Matijević, Sanda Skejić, Ivona Marasović, Mira Morović

Institute of Oceanography and Fisheries, Split, Croatia

ARTICLE INFO

Article history:
Received 4 August 2014
Received in revised form 29 January 2015
Accepted 10 March 2015

Keywords:
Water quality assessment
Chlorophyll *a*
Phytoplankton
Diversity
WFD
MSFD

ABSTRACT

In order to test the response of phytoplankton to anthropogenic pressure, data of chlorophyll *a* concentration, phytoplankton abundance, and composition are analyzed in relation to anthropogenic pressure gradient and environmental variables such as temperature, salinity and nutrients. Investigated sites encompassed wide tropic range according to a preliminary determination of anthropogenic pressure, quantified through the LUSI index. Statistical analyses indicated nitrates and silicates as proxies of freshwater influence, and phytoplankton single metrics such as concentrations of chlorophyll *a* and abundances as indicators of anthropogenic pressure. Boundary values for different water quality classes for coastal waters under indirect freshwater influence (Type II) are obtained according to gradient between concentration of chlorophyll a and pressure index (LUSI), which empirically fit to exponential equation. The response of phytoplankton diversity was not linear, as the highest diversity was observed in the area with intermediate disturbance level. CCA analysis identified *Skeletonema marinoii*, *Scrippsiella trochoidea*, *Guinardia flaccida*, *Leptocylindrus* spp., *Prorocentrum* spp., *Proboscia alata*, *Eutreptiella* spp., and *Pseudonitzschia* spp. as local eutrophication indicators, whose abundances increased with nutrients loads.

1. Introduction

Eutrophication as one of the oldest and major threats in coastal zones around the globe is well documented in scientific literature (Marasović et al., 2005; Smith et al., 2006; Garmendia et al., 2012; Sebilo et al., 2013). Within the European marine environment, eutrophication related processes are recognized as a problem to be monitored and managed by European directives, which established the framework for the protection of inland surface, transitional, coastal, and ground waters. Due to differences in operative indicators and assessment methodologies, it is often difficult to compare eutrophication status among regional marine water bodies. So far, trophic status assessment in the Adriatic Sea was mostly based on chlorophyll *a* concentration (Weinbauer et al., 1993; Zavatarelli et al., 2000) and TRIX index (Penna et al., 2004; Mozetič et al., 2008). There are also rare studies on trophic status assessment based on phytoplankton density and biovolume (Viličić, 1989). All these assessments were done without distinct criteria for classification of different water types and they encompassed both naturally and anthropogenically induced eutrophication in the Adriatic Sea. However, the implementation of the European directives requires the development of ecologically-based classification systems for anthropogenically-induced pressures in all types of water bodies.

Due to its importance as primary producer in marine food webs, pivotal role in marine ecosystem processes and fast response to the changes in nutrient loads and environmental conditions, phytoplankton is one of crucial biological elements considered within the Water Framework Directive (WFD). In accordance with the WFD, phytoplankton parameter should be expressed through phytoplankton biomass, composition, abundance, and bloom frequency. These indices are also to be used in good environmental status (GES) assessment within 4 out of 11 qualitative descriptors contained in Marine Strategy Framework Directive (MSFD): Biodiversity (D1), Non-indigenous species (D2), Marine food web (D4), and Eutrophication (D5).

Technical and scientific work for the purposes of the Water Framework Directive (WFD) has been carried out in the coastal and transitional waters across the European Union. The coastal and transitional waters intercalibration exercise was carried out within four Geographical Intercalibration Groups (GIGs) – Baltic, Black Sea, Mediterranean, and North East Atlantic. According to the

---

* Corresponding author at: IOF, Šetalište I. Meštrovića 63, 21000 Split, Croatia. Tel.: +385 21 408015; fax: +385 21 358650.
E-mail address: nincevic@izor.hr (Ž. Ninčević-Gladan).

http://dx.doi.org/10.1016/j.ecolind.2015.03.018

report of Mediterranean intercalibration group (MED GIG) for phytoplankton, water typology has been defined through the salinity classes (WFD, Intercalibration technical report, 2009). Relationship between phytoplankton components and salinity has been well documented, and shown to be important in many cases (Levandowsky, 1972; Ahel et al., 1996; Marshall et al., 2006).

The aim of this study is to (1) contribute to the implementation of European directives in Croatian waters, (2) test the response of suggested phytoplankton indices to environmental disturbance, (3) select the most appropriate metrics for phytoplankton community that clearly signals the anthropogenic pressure, (4) describe the phytoplankton classification metrics for the assessment of ecological status of Croatian coastal water type II.

In the present study, the methodology of water quality assessment has been developed on 7 years dataset collected at sites which are not directly affected by freshwater inputs with annual salinity mean between 34.5 and 37.5 and belong to Type II according to report of MED GIG phytoplankton group.

2. Material and methods

2.1. Study area

The study area encompasses coastal waters under indirect freshwater influence, which constitute the major part of coastal waters in Croatia. Sampling was performed in three bays with different hydrophysiological characteristic along the Croatian eastern Adriatic coast (Fig. 1).

Station SB 203 (Fig. 1A) is located outside the Šibenik Bay and is under the influence of Krka River. Station's depth is 13 m and distance from the nearest land is 760 m. Since vertical distribution of density is more influenced by temperature then salinity, the highest stability of water column is observed during the summer when seasonal thermocline appears. In the winter period, a negative thermocline can occur due to freshwater inflow in the surface layer.

Stations ST101 and ST103 are located in the Kaštela Bay, distanced from the nearest land by 1500 and 550 m, respectively, while station CJ007 is located out of the Bay with the distance from the nearest land of 5300 m (Fig. 1B). Kaštela Bay is a semi-enclosed bay (area 61 km^2), which is under considerable anthropogenic influence due to agricultural areas extending along its northern coast and municipal and industrial effluents that enter the Bay. The Bay communicates with the adjacent sea through a relatively wide (1.8 km) and deep mouth (mean depth about 40 m). The most important influx of fresh water to this Bay is the river Jadro.

Station PL105 is located in the inner part of Mali Ston Bay with the distance from the land of 300 m (Fig. 1C). The Bay is located at the end of the Neretva Channel. The outer part is under the influence of River Neretva, while this influence diminishes toward the inner part of the Bay (Vukadin, 1981). A special feature of the Bay is related to its complex hydrology, characterized by strong groundwater springs in the inner part of the Bay and the large fresh water inflow of Neretva River in the outer part of the Bay. Owing to the hydrographical features and favorable primary production, the Bay is a suitable area for cultivating shellfish, and shellfish farms are historically located there. Today it is one of the most important locations for shellfish farming in Croatia.

2.2. Physical and chemical conditions

Sampling of all analyzed physical, chemical and biological parameters was performed simultaneously within the frame of national monitoring program "Jadran" in the 2001–2007 period. Sampling was performed on monthly basis, with at least seven sampling occasions per year (all seasons included). Temperature and salinity were measured with an IDRONAUT 316 CTD probe (during 2001–2004) and after that period measurements were performed with a Seabird-25 CTD probe. The data obtained during all cruises were averaged for each meter of depth following the manufacturer's recommended procedure (Seabird Manual).

Dissolved inorganic nutrient concentrations (nitrates, nitrites, ammonia, orthophosphates, and silicates) were determined colorimetrically with an AutoAnalyzer-3, according to Grasshoff (Grasshoff, 1976).

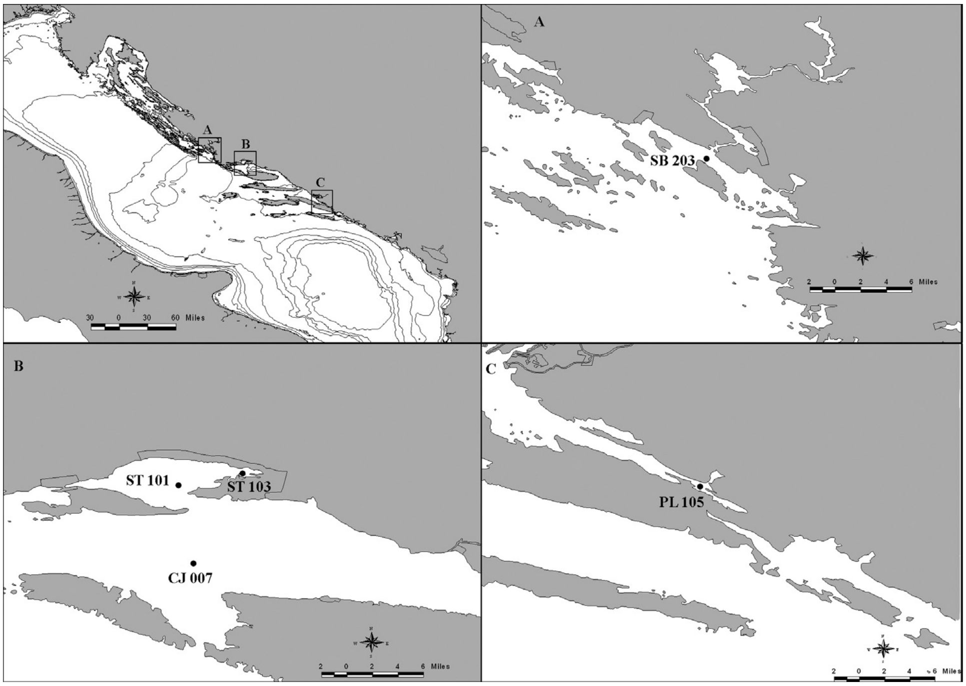


Fig. 1. Investigated area with sampling stations.

Table 1
Land uses simplified index scoring system according to Flo et al., 2011 slightly modify. Index quantifying potential pressures according to percent of land used in various anthropogenic activity.

Urban (%)	Agricultural (%)	Industrial (%)	Harbor (%)	Score
<3	<10	<10		0
3–33	10–40	>10	1–10	1
33–66	>40	>30	>10	2
>66		>60		3

2.3. Phytoplankton data

Phytoplankton data including concentration of chlorophyll *a* and phytoplankton community composition were collected at investigated stations in the period from 2001 to 2007 simultaneously with nutrients and CTD sampling. A total of 270 samples were analyzed. Chlorophyll *a* concentrations were determined fluorometrically from 90% acetone extracts (Strickland and Parsons, 1972). Phytoplankton abundance and community composition have been determined according to the Utermöhl method (Üthermöhl, 1958). Water samples (250 mL) were collected with Nansen bottles and were immediately preserved with formaldehyde, to a final concentration of 2% formaldehyde–seawater solution. Subsamples of 25 mL were settled in counting chambers for at least 12 h. Counting was performed for one transect of the sedimentation chamber using an inverted microscope with magnifications of 100×, 200×, and 400× for different taxa, depending on their respective sizes. In the case of blooms or high abundances of some species, counting was done in several randomly selected fields. Whenever possible, the identification was to the species level, although in some cases identification has been to genus or family level.

2.4. Anthropogenic pressure

Since the water quality assessment should be done in relation to anthropogenic pressure, preliminary evaluations of known pressures that could possibly affect the water quality within the study area were done using the land use pressures according to Corine Land Cover information system 2000–2006. Land uses simplified index (LUSI) was calculated according to Flo et al., 2011a. Assessment of anthropogenic pressure on coastal zone by calculating the LUSI index using the publicly available data is described in UNEP/MAP, 2011. The scoring system was slightly modified and presented in Table 1. Harbor areas (code 123) due to their direct influence on water quality were scored separately from other industrial influences. We analyzed the area within 5 km radius from the sampling sites to assure the precision of land pressure information for stations, which are more distant from the coast.

2.5. Statistical analysis

In order to enable comparisons between stations and the interpretation of results, differences arising from variable number of data measurements due to different depths of stations were avoided by analyzing only the values from the surface layer. Since data of Chl *a* concentrations have not shown normal distribution, they were log transformed prior to ANOVA analysis. Kolmogorov–Smirnov and Lilliefors tests were used for testing the data normality. One-way ANOVA test was used for determination of differences between stations in relation to various nutrients. *Post hoc* testing using the least significant difference (LSD) test was performed to pinpoint the origin of observed differences.

In addition to anthropogenic pressure information, environmental and biological data were subjected to principal component analysis (PCA), in order to identify groups with similar variability. Only those principal components showing Eigenvalues greater than 1 (Kaiser–Guttman criterion) were used. In order to obtain a better insight into the behavior of the output loadings, the orthogonal varimax rotation of extracted PC components was used. This method simplifies the interpretation because after a varimax rotation each original variable tends to be associated with one (or a small number) of orthogonal factors, and each of them represents only a small number of variables.

Non-parametric Spearman rank correlations were used for the analysis of untransformed data.

Relationship between calculated Menhinick index (M) and abundance data (N) was established introducing logarithmic equation of the form $M = a + b \log N$ to the field data. This relationship was found to be statistically significant ($R^2 = 0.389$; $P < 0.01$). Squares estimation of parameters a and b and standard error are obtained as $a = (0.113 \pm 0.008)$ and $b = (-0.016 \pm 0.002)$. The logarithmic equation also points to the maximum values of Menhinick index in the case when abundance tends toward its minimum values (M_{inf}). Maximum values of M index were obtained from the field data as an arithmetic mean of at least 4 maximum values ($M_{\mathrm{inf}} = 0.079$).

Relationship between the abundance of various phytoplankton species and environmental conditions were analyzed using Canonical correspondence analysis (CCA). Environmental variables due to different scale of measurements were standardized as follows: standardized value = (original value − mean)/standard deviation. In order to avoid colinearity of explanatory variables, oxygen, and nitrates are omitted due to strong linear relationship with salinity and silicates, respectively. Species abundances were fourth root transformed, because of the non-normal distribution and outliers observations, which can cause positively related variances of count variables and their means. Dixon test for outliers was applied on original data matrix. In order to reduce the large number of zeros due to the occurrences of rare species, the data matrix was reduced to most frequent species and several species of the same genus were pooled to genus level.

3. Results

3.1. Physical and chemical conditions

Annual mean salinity of surface water in the period from 2000 to 2007 at investigated stations was in range from 35.6 to 36.9, designating these waters as Type II according to the report of MED GIG phytoplankton group. More specifically, a water Type II is defined as coastal water, which is not directly affected by freshwater inputs and with annual salinity mean between 34.5 and 37.5. Temperature (T) and salinity (S) ranges at investigated stations measured in the water column are shown in Table 2. Absolute T range was from 6.4 to 28.7 °C due to seasonal variations and differences in depths among the sites. Vertical T distribution follows seasonal pattern, where warming starts in April and the thermocline is developed at approximately 5–10 m in June. Temperature maximum generally occurs in the surface layer in August. Deepening of the thermocline generally continues until October, after which the thermocline disappears. The exception was noted at station PL105 where, due to its shallowness, temperatures in the water column are equalized in August. Vertically homogenous T distribution is present throughout the December–March period in the entire water column and minimal values are measured. Absolute salinity range was between 24.8 and 38.7 (Table 2), with higher ranges at SB203 and PL105 stations influenced by strong Krka and Neretva rivers respective inflows, as well as at ST101 and ST103 located in the Kaštela Bay, which are influenced by River Jadro. Salinity range at CJ007 station placed in the Split Channel is mostly related to seasonal pattern of precipitation. Dissolved total

Table 2
Depth (m) of sampling stations and ranges of temperature (°C), salinity, oxygen saturation (%), nutrients (mmol m^{-3}), and secchi (m) in the water column at investigated stations during period 2001–2007, and type of anthropogenic pressure (%) including urban, industrial and agriculture influence and harbor presence within radius of 5 km from station according to Corine Land Cover 2006.

	PL105	SB203	ST103	ST101	CJ007
Depth	8	13	18	37	52
Temp	8.23–27.68	11.11–25.69	9.92–26.93	6.37–26.21	10.72–28.69
Salinity	31.32–38.00	24.79–38.56	29.19–38.28	29.60–38.33	34.56–38.75
Oxygen	88.10–118.33	88.29–122.72	83.48–144.64	90.52–123.65	87.47–119.75
Nitrate	0.04–8.42	0.03–16.12	0.002–12.95	0.05–8.57	0.01–3.56
Nitrite	0.002–1.017	0.010–0.328	0.012–0.850	0.001–0.546	0.001–0.340
Ammon.	0.05–6.00	0.20–4.27	0.09–11.82	0.08–7.27	0.13–3.68
Phosphate	0.012–1.875	0.011–0.245	0.025–2.150	0.012–0.363	0.002–0.201
Silicate	0.15–18.28	0.21–24.37	0.22–12.32	0.04 –5.72	0.10–9.58
Secchi	3–8	4–13	1–9	3–18	6–34
Urban	1	8	40	42	0
Industrial	0	3	13	3	0
Agriculture	12	15	12	26	0
Harbor	0	0	2	3	0

nitrogen concentrations (sum of nitrates, nitrites and ammonia; TIN) ranged between 0.17 and 24.90 mmol m^{-3}, with maximum values for each station in winter–spring period as a consequence of nutrient freshwater inputs and precipitation (Table 2). Orthophosphate (HPO_4^{2-}) concentration range was 0.002–2.149 mmol m^{-3}, with higher values in winter and spring. Orthosilicate concentrations (range: 0.03–24.38 mmol m^{-3}) were also in accordance with precipitation and river input of SiO_4^{4-} identified as increased concentrations in surface layer. In the warm period of the year when the water column is well stratified, increase in TIN, HPO_4^{2-}, and SiO_4^{4-} concentrations are found in the bottom layer of the water column due to dissolution of the nutrients from sediments.

3.2. *Factor analysis*

Factor analysis among environmental variables, anthropogenic pressure and phytoplankton indices (Chl *a* concentration and abundance) extracted four significant factors (eigenvalues >1) that explained 69% of variance. Factor 1 was defined by salinity, nitrate and silicate and explained 26% of variance (Fig. 2). Factor 2 was defined by transparency (Secchi), LUSI, Chl *a* and abundance and explained 20% of variance. Factor 3 was defined by ammonium and phosphate and factor 4 by temperature, explaining 13% and 10% of variance, respectively. Factor analysis showed that Chl *a*, phytoplankton abundance and transparency have similar variability as the LUSI index, suggesting that these variables represent good indicators of anthropogenic influence.

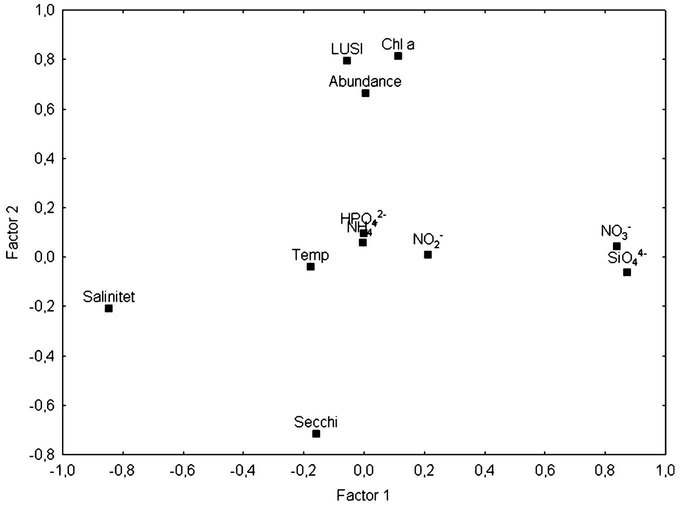


Fig. 2. Principal component analysis (PCA) of environmental variables (temperature, salinity, transparency, nitrate, nitrite, ammonium, phosphate, and silicate concentrations), anthropogenic pressure indicator (LUSI), and phytoplankton indices (phytoplankton abundance and chlorophyll *a* concentrations).

3.3. *Spearman rank correlations*

Spearman rank order correlations (Table 3) confirmed significant correlations between phytoplankton indices and both, anthropogenic indicators and freshwater influence. Concentrations of Chl *a* significantly correlated with nitrate and silicate concentrations while phytoplankton abundance correlated with phosphate (Table 3). Transparency showed significant relation with all analyzed variables. Negative correlation between temperature and silicate, nitrate and nitrite was recorded.

3.4. *Anthropogenic pressure*

Preliminary quantification of potential pressure revealed a wide range of anthropogenic pressure among investigated stations. The highest recorded LUSI index was 5 at station ST 103, followed by 3 at station ST 101, 2 at station SB 203, 1 at station PL 105, and 0 at station CJ 007. The highest LUSI value at station ST 103 is attributed to various influences. Urban areas cover 40% of the area in the analyzed radius of this station, 13% is industrial area, 12% agriculture areas, and cargo port occupied 2% of surrounding area (Table 2). About 40% of industrial area is mining activities. Station ST 101 is under the same sources of anthropogenic pressure as station ST 103, but it is more distant from these sources. Urban areas cover 42% of the area in the analyzed radius of this station, 3% is industrial area and 26% agricultural areas. Anthropogenic pressures at station SB 203 calculated through LUSI index are attributable to agriculture (15%) and urban influence (8%). According to Croatian national organization for water management (Hrvatske vode), station PL 105 is not under any point source pollution, and subjected only to a slight diffuse pollution due to soil infiltration and surface runoff. Low LUSI value at station PL105 is attributed to various agriculture areas (12%) and it is slightly above the threshold indicated in Table 1. Almost 60% of these surfaces are agricultural areas with significant share of natural vegetation and the rest is olive groves. There is no arable land influence. According to prior preliminary anthropogenic pressure assessment, station PL 105 was selected as a referent station due to its low anthropogenic pressure and salinity range that correspond to a prototype of Type II coastal water. Although the lowest LUSI index is calculated for station CJ007, station PL105 is selected as a referent station due to stronger freshwater influence, land vicinity and very weak anthropogenic impact, making this station a representative of the area

Table 3
Spearman rank order correlations between environmental variable (nutrients, salinity), antrophogenic pressure (LUSI), concentrations of chlorophyll *a* and phytoplankton abundance.

	LUSI	Secchi	Temp	Salinity	NO_3^-	NO_2^-	NH_4^-	HPO_4^{2-}	SiO_4^{4-}	Chl *a*
Secchi	−0.610*									
Temp.	−0.001	0.125*								
Salinity	−0.268*	0.309*	−0.004							
NO_3^-	0.158*	0.159*	−0.375*	−0.298*						
NO_2^-	0.059	−0.193*	−0.291*	−0.064	0.331*					
NH_4^+	0.096	−0.176*	−0.105	−0.013	0.157*	0.198*				
HPO_4^{2-}	0.203*	−0.189*	0.058	0.026	−0.084	−0.012	0.125*			
SiO_4^{4-}	0.058	−0.318*	−0.372*	−0.212*	0.450*	0.329*	0.093	−0.079		
Chl *a*	0.600*	−0.646*	−0.206*	−0.321*	0.233*	0.105	0.025	0.022	0.163*	
Abund.	0.522*	−0.319*	0.096	−0.361*	−0.030	−0.017	0.009	0.159*	−0.107	0.423*

* $p < 0.05$.

whose ecological status we would like to assess. The lowest LUSI index at station CJ 007 is a result of its distance from the land. The station is under some influence of municipal wastewater discharges from the surrounding land, including both, the mainland and the islands. According to one-way ANOVA, there was significant difference between stations in nutrient concentrations (Fig. 3). Concentrations of NO_3^- and SiO_4^{4-} were significantly higher at stations ŠI 203, PL105 and ST 103, confirming a stronger freshwater

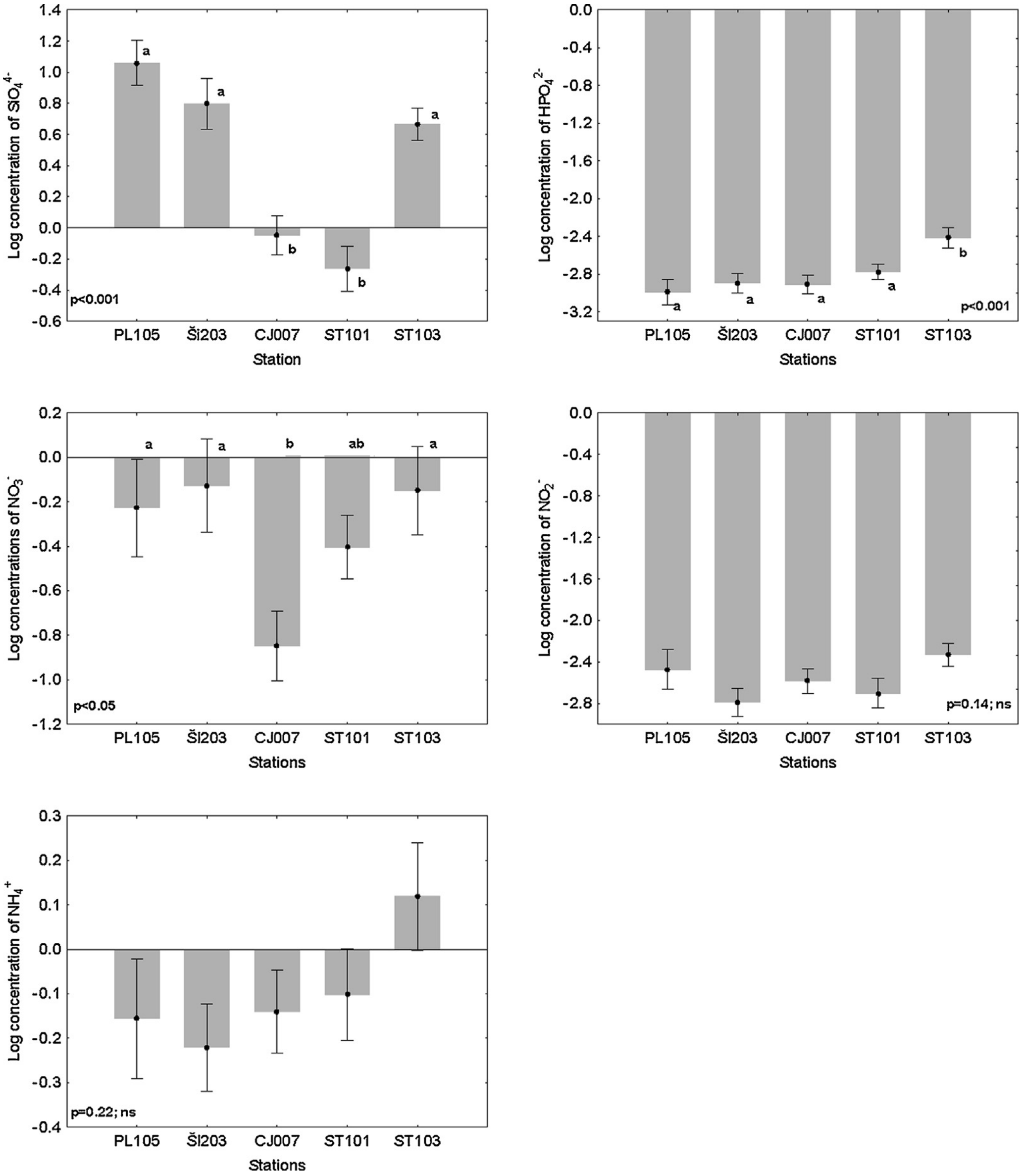


Fig. 3. Difference between stations in relation to nutrients concentrations obtained using the one-way ANOVA analysis. Results of *post hoc* analysis in cases of significant results in ANOVA test are showed by letters (a and b) labeled on the bars. Stations that do not have significantly different means are labeled with the same letter. Stations, which are not significantly different from either of the other two are labeled with both letters (a and b).

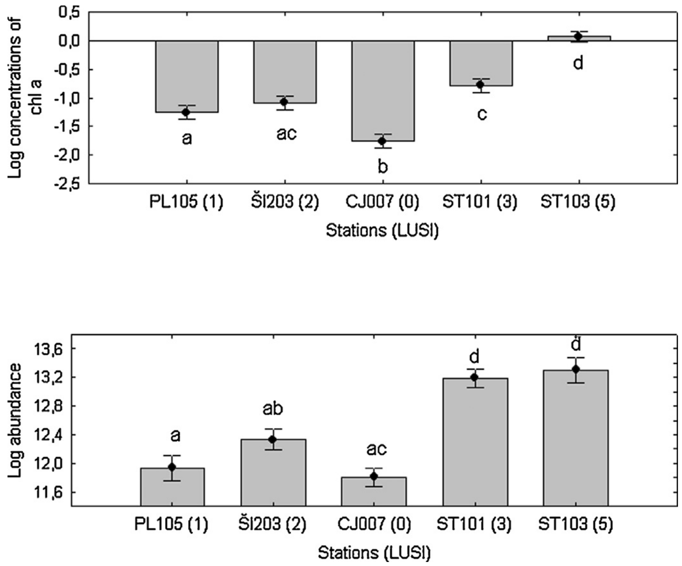


Fig. 4. Difference between stations in relation to phytoplankton abundance and chlorophyll *a* concentrations obtained using the one-way ANOVA analysis. Results of *post hoc* analysis in cases of significant results in ANOVA test are showed by letters (a and b) labeled on the bars. Stations that do not have significantly different means are labeled with the same letter. Stations, which are not significantly different from either of the other two are labeled with both letters (a and b).

influence at these stations in comparison to stations ST 101 and CJ 007. The highest concentrations of HPO_4^{2-} at ST 103 significantly differentiated this station from other investigated sites, due to the highest urban influence at this station. There was no significant correlation between stations in relation to NH_4^+ and NO_2^- concentrations. Nevertheless, a significant difference between station ST 103 with the highest concentrations of NH_4^+ and NO_2^- and station ŠI 203 with the lowest concentration of these nutrients was recorded ($p < 0.05$). According to nutrients, the highest eutrophication influence including both freshwater and urban influence was at station ST 103.

3.5. Concentrations of chlorophyll a (Chl a)

Significant difference in Chl *a* concentrations between stations with variable anthropogenic influences according to LUSI is revealed by ANOVA analysis (Fig. 4). Chlorophyll *a* concentrations were calculated as the 90th percentiles, which represents a recognized statistical approach designed to encompass the spread of data and omit highly skewed values which could be recorded during the bloom period (Clarke and Warwick, 1994). Chl *a* data from investigated stations calculated as 90th percentiles were plotted against its risk assessment. Since station PL 105 was selected as the referent station according to its physical and chemical characteristics and due to weak anthropogenic pressure, the 90th percentile of Chl *a* values recorded at this station was used as referent value. The 90th percentile at station PL 105 was 0.95 mg m^{-3}. Significant exponential relationship between the concentrations of Chl *a* (90th percentile) and LUSI index was described by equation $y = 0.642 * \exp(0.3542 * x)$ (y = the 90th percentile of Chl *a*; x = LUSI index) ($p < 0.001$).

Boundary values for high (H)/good (G), good (G)/moderate (M), and moderate (M)/poor (P) classes were calculated applying this equation for LUSI values of 2.5, 4, and 5.5, respectively. These LUSI thresholds were chosen using two criteria: equidistance in LUSI values and significant difference in Chl *a* concentration between stations with different LUSI values (Fig. 4). Obtained boundary values for the 90th percentile of Chl *a* for H/G, G/M, M/P classes were 1.56, 2.65, and 4.51, respectively. Ecological quality ratio (EQR) is the relationship between observed and reference condition value, with numerical values between 0 and 1. EQR values obtained through this study for boundary values for H/G, G/M, and M/P classes are 0.61, 0.36, and 0.21, respectively.

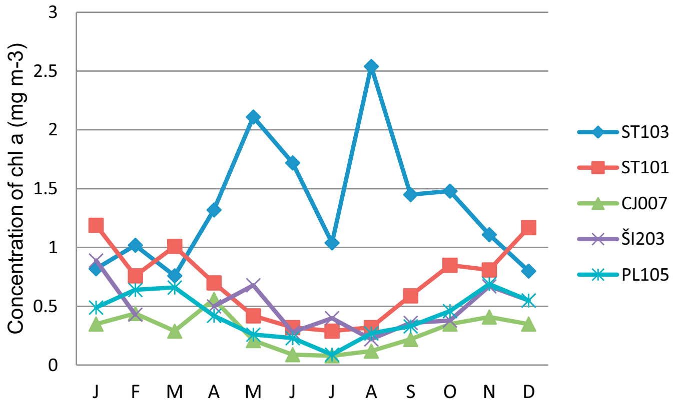


Fig. 5. Seasonal distribution of chlorophyll *a* concentrations at investigated stations during analyzed period from 2001 to 2007.

Changes in seasonal cycle were recorded at station ST103, which is under the strongest anthropogenic pressure (Fig. 5). Slight changes at station ST101 were visible through the increase of biomass in September during stratification conditions. At all other analyzed stations autumn–winter and spring maxima followed by summer stagnation were recorded.

3.6. Phytoplankton abundance and community composition

Significant differences among risk assigned stations in phytoplankton abundance were recorded (Fig. 4). Conversely to Chl *a*, phytoplankton abundance did not significantly differentiate between stations ST103 and ST101, nor separate the most land-distant station CJ007. Abundances were similar at referent station PL105 and station CJ007, which is in the area subjected to the same sources of anthropogenic pressure as stations ST101 and ST103, but it is more distant from the source of pressure. Abundances (90th percentile) at investigated stations were as follows: at PL105 it was 4.2×10^5 cells L^{-1}, at station CJ007 4.6×10^5 cells L^{-1}, at station ŠI203 6.9×10^5 cells L^{-1}, at station ST101 1.6×10^6, and station ST103 3.4×10^6 cells L^{-1}.

Spearman rank correlations between phytoplankton abundance and calculated diversity indices revealed Menhinick index (M) as the most significant indicator of elevated phytoplankton counts (Table 4). Relation between the abundance and Menhinick index revealed low diversity during high phytoplankton abundances, while both, low and high diversities were recorded during lower phytoplankton abundances (Fig. 6). ANOVA and *post hoc* analysis of Menhinick index values obtained at investigated stations showed significant difference ($p < 0.05$) between stations, separating the stations ŠI203 and CJ007 with higher diversity from stations PL105, ST101, and ST103 with lower diversity. Referent station PL105 was significantly different only from station ŠI203, characterized with the highest recorded diversity and medium anthropogenic pressure determined as LUSI 2.

Results of CCA analysis showed that abundances of specific taxa were determined by environmental variables (Fig. 7). Permutation test confirmed linear relationship between species' abundances and environmental variables ($p < 0.0001$). The first two axes explained 61.49% of variance. The increase in abundance with increasing concentrations of phosphate and anthropogenic impacts estimated through LUSI index was shown by *Scrippsiella trochoidea*

Table 4
Spearman rank order correlations between phytoplankton abundance and different diversity and evenness indices..

Diversity index	Spearman Rank
Margalef	
$d=(S-1)/\ln N$	**0.51**[*]
Pielou's evenness	
$E1=H'/\ln S$	−0.34
Simpson	
$D=\sum si=1Ni/(Ni-1)/N(N-1)$	0.10
Hill *N*1	−0.02
$N1=\exp(H')$	
Hill *N*2	0.10
$N2=1/\text{Simpson's}$	
Menhinick	**−0.80**[*]
$D=S/\sqrt{N}$	
Species no.	**0.62**[*]
Shannon	−0.02
$H'=-\sum si=1=Ni/N\ln Ni/N$	

[*] Bold values are significant at $p < 0.05$.

(Stein), *Guinardia flaccida* (Castracane) H. Peragallo, *Leptocylindrus* spp, *Prorocentrum* spp., *Proboscia alata* (Brightwell) Sündstrom followed by *Eutreptiella* spp. and *Pseudonitzschia* spp. Strong negative relationship with salinity was recorded for diatom *Skeletonema marinoii*. Taxa *Eutreptiella* spp., *Prorocentrum* spp., *Proboscia alata*, *Leptocylindrus* spp., and *Scrippsiella trochoidea* were positively related to temperature while *Chrysophyceae* group favored lower temperature and increased nitrite concentration.

Positive relation with ammonium was recorded for *Cyclotella* sp., *Scrippsiella trochoidea*, and *Guinardia flaccida*. Other analyzed taxa were close to the origin of the ordination plot and did not show distinct relation with used explanatory variables.

4. Discussion

In order to contribute to the implementation of European directives in Croatian waters, environmental research study aiming to understand how the phytoplankton community responds to anthropogenic pressure was conducted. Research was conducted according to the requirements of water-related European directives (Directive 2000/60/EC and Directive 2008/56/EC), known respectively as WFD and MSFD, which were developed to facilitate management and protection of the marine ecosystem. Ecological status assessment is the first step in the ecosystem-based management. Since quality assessment should be water type specific, suggested boundary conditions concern coastal waters, which are not directly affected by the freshwater inputs (Type II) with annual mean salinity in surface layer between 34.5 and 37.5 (WFD, Intercalibration technical report, 2009). In order to obtain a clear response of phytoplankton indices to the intensity of anthropogenic pressure in Croatian waters, sampling stations encompassing a wide range of pressure degree were selected. Investigated stations are under urban wastewaters, agricultural and industrial influences and according to previous assessments (Krstulović et al., 1997; Marasović et al., 1991; Viličić, 1989) cover a wide eutrophication gradient. This was also confirmed in the course of our study through quantification of anthropogenic pressure level using the LUSI index to ensure the sensitivity of indices. The first problem aroused while selecting the referent station based on LUSI, because the lowest LUSI value was calculated for station (CJ007) which is more distant from land than other stations and has a comparatively lower residence time than stations closer to the coast which are under the same anthropogenic sources influence (Tudor and Beg Paklar, 2014). Consequently, the station CJ007 is characterized by higher salinity value, which is close to the boundary limit for this water type. Because of these reasons station CJ007 was excluded as a referent station. Furthermore, the referent status was allocated to station PL105 based on its low preliminary assessed anthropogenic pressure, despite the fact that this station is the nearest to the coast and located in a somewhat sheltered area (Mali Ston Bay). Station PL105 is under a slight influence of agricultural activities from the surrounding landscape characterized with significant grounds covered by natural vegetation. The selection of referent station thus revealed the weakness of LUSI index for waters, which are further away from the land. Secondly, in the course of this study we recorded another problem related to EQR values, which could attain values higher than 1 at stations which are further away from the land (e.g., station CJ007), but still belong to water type II according to salinity means. According to

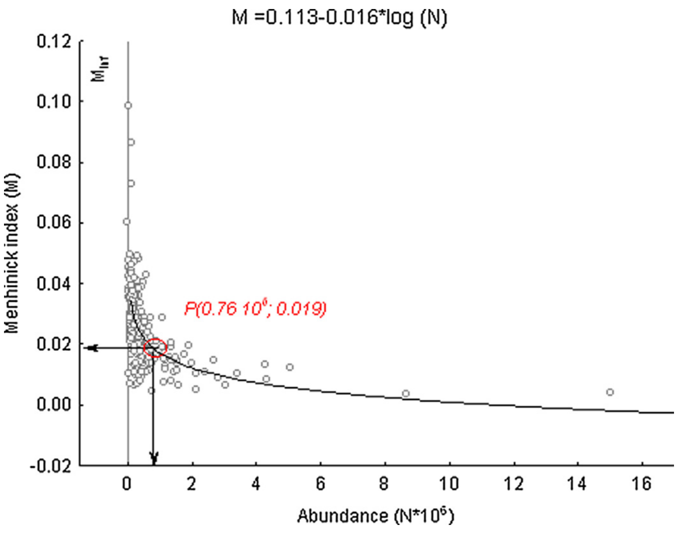


Fig. 6. Phytoplankton abundance and Menhinick diversity index relation obtained for the investigated data set.

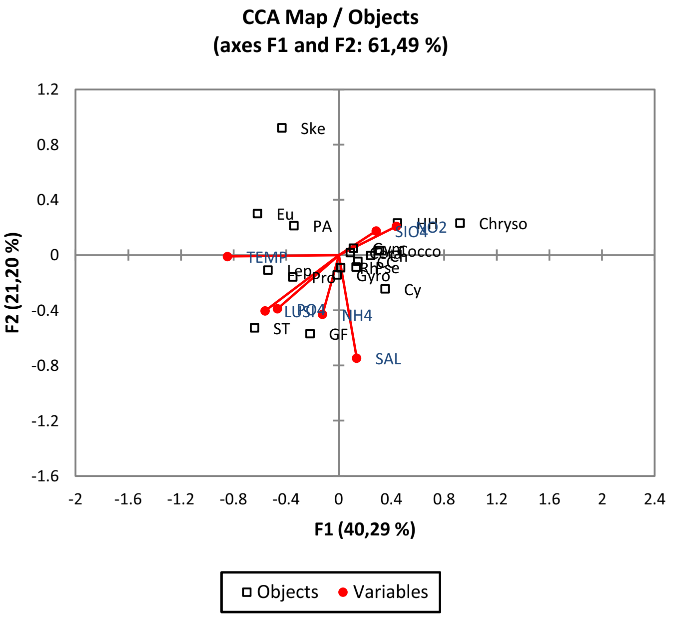


Fig. 7. Canonical correspondence analysis (CCA) between environmental explanatory variables and the most frequent phytoplankton taxa. The explanatory variables are represented by lines. Taxa abbreviations: Cera, *Cerataulina* spp.; Ch, *Chaetoceros* spp.; Cy, *Cyclotella* spp.; GF, *Guinardia flacida*; HH, *Hemiaulus hauckii*; Lep, *Leptocylindrus* spp.; CC, *Cylindrotheca closterium*; *Pse*, Pseudonitzschia spp.; PA, *Proboscia alata*; Rh, *Rhizosolenia* spp.; Ske, *Skeletonema marinoii*.; Gym, *Gymnodinium* spp.; Gyro, *Gyrodinium* spp.; Pro, *Prorocentrum* spp.; ST, *Scripsiella trochoidea*; Chryso, *Chrysophyceae* spp.; Cocco, *Coccolithophoridae* spp.; Eu, *Eutreptiella* spp.

WFD CIS Guidance Document No. 5, EQR values higher than 1 are acceptable for phytoplankton. This discrepancy is caused by the weakening of the anthropogenic and freshwater influence with distance from the land and should be taken into consideration in coastal water management and water quality assessments. Physical, chemical and biological characteristics, as well as land influence are changed significantly with distance from the coast, as it is well documented in recent studies (Flo et al., 2011b; Sebastiá et al., 2012). Freshwater tidal estuarine zones often support dense phytoplankton communities, with higher chlorophyll a concentrations than those found downstream (Muylaert et al., 2000). Freshwater influence on the investigated stations is reflected through the nutrients composition, with increasing concentrations of nitrates and silicates at more diluted stations (Figs. 3 and 4). Significant differences across regions and between specific coastal ecosystems within regions in the response of phytoplankton biomass to changing nutrients regime was also reported (Carstensen et al., 2011). In order to allow comparison of results considering the water quality assessment according to phytoplankton indices, it should be considered in relation to water specific type and within certain distance from the coast.

Advanced statistical analyses were applied in order to test the response of suggested phytoplankton indices to environmental disturbance. According to factor analysis and ANOVA test, there is significant relation between concentration of chlorophyll *a* and both, anthropogenic and freshwater influences. Clear gradient between concentration of chlorophyll a and pressure index (LUSI), which empirically fits to exponential equation is obtained. Exponential relationship between chlorophyll *a* and transparency, which was used as trophic measure was reported back in 1977 (Carlson, 1977) and was explained with additional production of chlorophyll a in response to subdued light intensity due to algal biomass augmentation. Nevertheless, relationship between concentration of chlorophyll *a* and nutrients is expected to flatten out because of self-shading and allelopathy.

Among eight tested indices related to diversity and evenness, Menhinick index (M), Margalef index (d), and species number (S) were found to be significantly correlated to abundance. In this study, phytoplankton biodiversity was assessed using the Menhinick index due to its strong and significant correlation with abundance. Effectiveness of Menhinick diversity due to its consistent and linear change through the trophic gradient has been documented (Spatharis and Tsirtsis, 2010).

Phytoplankton biodiversity was the highest at station (SB203) with only a moderate increase of anthropogenic pressure in comparison to referent station PL105. The lowest diversities were recorded at both, referent station (PL105) and stations under stronger anthropogenic pressures (ST101, ST103). The obtained results support the intermediate disturbance level hypothesis, which predicts large species number in areas subjected to intermediate levels of disturbance (Huston, 1979; Sommer et al., 1993). Accordingly, in situations where disturbance is minimal, species diversity is reduced because of competitive exclusion between species, whereas with slight increase of disturbance competition is relaxed, thus resulting in increased diversity. With further disturbance increase, diversity drops again because of species reduction due to stress. The study of Spatharis et al. (2007) in the Aegean Sea supports the view that moderate nutrient inputs stimulate taxonomic diversity while high nutrient concentrations have a negative effect on diversity through the dominance of a single species. Minimal diversities during both, monospecific blooms which are characterized with highest density and during the lowest densities, were reported from Orbetello lagoon (Tuscany) (Nuccio et al., 2003). Low diversity during monospecific blooms is also well documented (Nuccio et al., 2003; Coelho et al., 2007). Besides nutrients, phytoplankton community composition is strongly influenced by turbulence (Wyatt, 2013). Diversity of phytoplankton community depends on phylogenetic phytoplankton group *inoculums* at investigated area. Dinoflagellates exhibit high diversity in habitat preference, but low bloom-species diversity within these habitats, while diatoms show low habitat diversity but high bloom-species diversity (Smayda and Reynolds, 2003). Since our results are in accordance with the above-mentioned theory, and knowing that environmental parameters other then nutrients also determine the phytoplankton community, we believe that diversity index is not a sufficiently clear indicator of eutrophication process to be used for water quality assessment.

CCA analysis revealed the phytoplankton taxa whose abundances could be used in water quality assessment in relation to eutrophication process. Although the problem of indicator species, which could be present at one place and absent at other due to their spatial, geographic and natural variability is under discussion (Mouillot et al., 2006; Degerlund and Eilertsen, 2010), there are less impediments if these species are to be used in areas where they occur, i.e., locally. According to our results, increased abundances of *Skeletonema marinoii*, *Scrippsiella trochoidea*, *Guinardia flaccida*, *Leptocylindrus* spp., *Prorocentrum* spp., *Proboscia alata*, *Eutreptiella* spp., and *Pseudonitzschia* spp. are indicative of nutrient loads and increasing eutrophication process in type II waters in the eastern Adriatic. Fast growth of *Pseudonitzschia* species during nutrient-rich conditions is reported in the Eastern Mediterranean (Spatharis et al., 2007). The present study confirmed diatoms as the opportunistic group when nutrient availability is concerned, as was recognized in previous studies (Fogg, 1991; Sebastiá et al., 2012). Selected species are common in the Mediterranean region and show the intensive growth in nutrient rich embayments (Moncheva et al., 2001) suggesting that they are good indicators of eutrophication process. The present study differentiated *Skeletonema marinoii* as the indicator of freshwater influence and dinoflagellate *Scrippsiella trochoidea* as the indicator of urban influence, probably due to diatoms' demand for silica.

Water quality assessment in Croatian waters through phytoplankton indices uses concentration of chl *a*. The method is described in Section 3.5. Concentration of chl *a* is used because the factor analyses associated chl a and phytoplankton abundance with anthropogenic pressure indicator LUSI and transparency, thus showing that these parameters have similar variability. In addition, concentrations of chl *a* differed significantly among stations under different anthropogenic pressure degrees. Phytoplankton abundances did not differentiate between stations under anthropogenic pressure quantified by LUSI index values of 3 and 5. Problems with abundances arise from different sizes of counted cells, as smaller cells could be overlooked when microplankton dominates in the community. If phytoplankton abundances are to be used in the water quality assessment, it is necessary to define the size of phytoplankton cells that will be counted (Revilla et al., 2009).

Boundary values according to WFD requests for concentration of chlorophyll *a* obtained in this study are much higher than those obtained in the eastern Mediterranean Sea (Simboura et al., 2005; Pagou et al., 2002) and lower than those published for UK marine waters (Devlin et al., 2007). Differences in the productivity of the cited areas, vicinity of the investigated stations to land and freshwater source, as well as different methods applied on data set for boundary settings could be some of the reasons for this divergence. For example, the Eastern Mediterranean Sea is often characterized as one of the least productive seas of the world, on the basis of prevailing low nutrient levels, impoverished phytoplankton populations, and low productivity (Kimor and Wood, 1975; Berman et al., 1984; Dowidar, 1984; Azov, 1991; Krom et al., 1991; Ignatiades et al., 1995; Ignatiades, 1998). Concentrations of chlorophyll *a* obtained in the present study are very similar to those obtained in the Bay of Biscay (northern Spain) (Revilla et al., 2009). The values were determined in the same period of investigation, but different methods for boundary settings were applied on the data sets.

The results of the environmental research obtained in this study revealed that the concentration of chlorophyll *a* and abundance of indicator species distinctly respond to the anthropogenic pressure. However, because of the ambiguous relationship with nutrients loads, phytoplankton diversity is not a reliable indicator of anthropogenic pressure. Further analyses to determine the boundary values for selected indicator species should be performed. Water quality assessment in accordance with EU water-related directives has shown the importance of distance from the coast when selecting a referent station and stations for quality assessment.

Acknowledgments

This study was supported by the Croatian Ministry of Science, Education and Sports in the frame of projects 001-0010501-0848 and 001-0013077-0845. The authors wish to thank the Croatian water management company “Hrvatske vode” for funding and supporting participation in MED GIG working group. We are extremely grateful for the guidance and useful discussion provided by the members of phytoplankton MED GIG working group. Especially thank to my colleague Olja Vidjak for providing language help.

References

Ahel, M., Barlow, R.G., Mantoura, R.F.C., 1996. Effect of salinity gradients on the distribution of phytoplankton pigments in a stratified estuary. Mar. Ecol. Prog. Ser. 143, 289–295.

Azov, Y., 1991. Eastern Mediterranean – a marine desert? Mar. Pollut. Bull. 23, 225–232.

Berman, T., Townsend, J.D.W., El Sayed, S.Z., Trees, C.C., Azov, Y., 1984. Optical transparency, chlorophyll and primary production in the Eastern Mediterranean near the Israeli coast. Oceanol. Acta 7, 367–372.

Carlson, R.E., 1977. A trophic state index for lakes. Limnol. Oceanogr. 22, 361–369.

Carstensen, J., Camacho, M.S., Duarte, C.M., Jensen, D.K., Marba, N., 2011. Connecting the dots: response of coastal ecosystems to changing nutrient concentrations. Environ. Sci. Technol. 45, 9122–9132.

Clarke, K.R., Warwick, R.M., 1994. Change in marine communities: an approach to statistical analysis and interpretation. Natural Environmental Research Council, UK, pp. 144.

Coelho, S., Gamito, S., Ruzafa, A.P., 2007. Trophic state of Foz de Almargem coastal lagoon (Algarve, South Portugal) based on the water quality and the phytoplankton community. Estuar. Coast. Shelf Sci. 71, 218–231.

Degerlund, M., Eilertsen, H.C., 2010. Main species characteristics of phytoplankton spring blooms in NE Atlantic and Arctic waters (68–80°N). Estuar. Coasts 33, 242–249, 10.1007/s12237-009-9167-7.

Devlin, M., Best, M., Coates, D., Bresnan, E., O'Boyle, S., Park, R., Silke, J., Cusack, C., Skeats, J., 2007. Establishing boundary classes for classification of UK marine waters using phytoplankton communities. Mar. Pollut. Bull. 55, 91–103.

Dowidar, N.M., 1984. Phytoplankton biomass and primary production of the south-eastern Mediterranean. Deep-Sea Res. 31, 983–1000.

Flo, E., Camp, J., Garcés, E., 2011a. Assessment pressure methodology: land uses simplified indeks (LUSI). In: BQE Phytoplankton. Work dokument (MedGiG meeting, Rome), Spain, Catalonia.

Flo, E., Garcés, E., Manzanera, M., Camp, J., 2011b. Coastal inshore waters in the NW Mediterranean: physicochemical and biological characterization and management implications. Estuar. Coast. Shelf Sci. 93, 279–289.

Fogg, G.E., 1991. The phytoplankton way of life. N. Phytol. 118, 191–232.

Garmendia, M., Borja, A., Franco, J., Revilla, M., 2012. Phytoplankton composition indicators for the assessment of eutrophication in marine waters: present state and challenges within the European directives. Mar. Pollut. Bull., http://dx.doi.org/10.1016/j.marpolbul.2012.10.005.

Grasshoff, K., 1976. Methods of Seawater Analysis. Verlag Chem, Weinheim, pp. 307.

Huston, M., 1979. A general hypothesis of species diversity. Am. Nat. 113, 81–101.

Ignatiades, L., 1998. The productive and optical status of the oligotrophic waters of the Southern Aegean Sea (Cretan Sea), Eastern Mediterranean. J. Plankton Res. 20, 985–995.

Ignatiades, L., Georgopoulos, D., Karydis, M., 1995. Description of phytoplanktonic community of the oligotrophic waters of SE Aegean Sea (Mediterranean). P.S.Z.I. Mar. Ecol. 16, 13–26.

Kimor, B., Wood, E.J.F., 1975. A plankton study in the Eastern Mediterranean. Mar. Biol. 29, 321–333.

Krom, D.M., Kress, N., Brenner, S., 1991. Phosphorus limitation of primary production in eastern Mediterranean Sea. Limnol. Oceanogr. 36, 424–432.

Krstulović, N., Šolić, M., Marasović, I., 1997. Relationship between bacteria, phytoplankton and heterotrophic nanoflagellates along the trophic gradient. Helgol. Meersunters. 51, 433–443.

Levandowsky, M., 1972. An ordination of phytoplankton populations in ponds of varying salinity and temperature. Ecology 53, 398–407.

Marasović, I., Gačić, M., Krstulović, N., Kušpilić, G., Pucher-Petković, T., Odžak, N., Šolić, M., 1991. Development of the red tide in the Kaštela Bay (Adriatic Sea). Mar. Chem. 32, 375–385.

Marasović, I., Ninčević-Gladan, Ž., Kušpilić, G., Marinović, S., Marinov, S., 2005. Long-term changes of basic biological and chemical parameters at two stations in the middle Adriatic. J. Sea Res. 54, 3–14.

Marshall, H.G., Lacouture, R.V., Buchanan, C., Johnson, J.M., 2006. Phytoplankton assemblages associated with water quality and salinity regions in Chesapeake Bay, USA. Estuar. Coast. Shelf Sci. 69, 10–18.

Moncheva, S., Gotsis-Skretas, O., Pagou, K., Krastev, A., 2001. Phytoplankton blooms in Black Sea and Mediterranean coastal ecosystems subjected to anthropogenic eutrophication: similarities and differences. Estuar. Coast. Shelf Sci. 53, 281–295.

Mouillot, D., Spatharis, S., Reizopoulou, S., Laugier, T., Sabetta, L., Basset, A., Do Chi, T., 2006. Alternatives to taxonomic-based approaches to assess changes in transitional water communities. Aquat. Conserv. 16, 469–482.

Mozetič, P., Malačič, V., Turk, V., 2008. A case study of sewage discharge in the shallow coastal area of the Northern Adriatic Sea (Gulf of Trieste). Mar. Ecol. Evol. Persp. 29, 483–494.

Muylaert, K., Sabbe, K., Vyverman, W., 2000. Spatial and temporal dynamics of phytoplankton communities in a freshwater tidal estuary (Schelde, Belgium). Estuar. Coast. Shelf Sci. 50, 673–687.

Nuccio, C., Melillo, C., Messi, L., Innamorati, M., 2003. Phytoplankton abundance, community structure and diversity in the eutrophicated Orbetello lagoon (Tuscany) from 1995 to 2001. Oceanol. Acta 26, 15–25.

Pagou, K., Siokou-Frangou, I., Papathanassiou, E., 2002. Nutrients and their ratios in relation to eutrophication and HAB occurrence. The case of Eastern Mediterranean coastal waters. In: Second Workshop on “Thresholds of Environmental Sustainability: The case of nutrients”, 18–19 June 2002, Brussels, Belgium.

Penna, N., Capellacci, S., Ricci, F., 2004. The influence of the Po River discharge on phytoplankton bloom dynamics along the coastline of Pesaro (Italy) in the Adriatic Sea. Mar. Pollut. Bull. 48, 321–326.

Revilla, M., Franco, J., Bald, J., Borja, A., Laza, A., Seoane, S., Valencia, V., 2009. Assessment of the phytoplankton ecological status in the Basque coast (northern Spain) according to the European Water Framework Directive. J. Sea Res. 61, 60–67.

Sebastiá, M.T., Rodilla, M., Sanchis, J.A., Altur, V., Gadea, I., Falco, S., 2012. Influence of nutrient inputs from a wetland dominated by agriculture on the phytoplankton community in a shallow harbour at the Spanish Mediterranean. Agr. Ecosyst. Environ. 152, 10–20.

Sebilo, M., Mayer, B., Nicolardot, B., Pinay, G., Mariotti, A., 2013. Long-term fate of nitrate fertilizer in agricultural soils. Proc. Natl. Acad. Sci. U. S. A. 110, 18185–18189, http://dx.doi.org/10.1073/pnas.1305372110.

Simboura, N., Panayotidis, P., Papathanassiou, E., 2005. A synthesis of the biological quality elements for the implementation of the European Water Framework Directive in the Mediterranean ecoregion: the case of Saronikos. Gulf. Ecol. Indic. 5, 253–266.

Smayda, T.J., Reynolds, C.S., 2003. Strategies of marine dinoflagellate survival and some rules os assembly. J. Sea Res. 49, 95–106.

Smith, V.H., Samantha, B.J., Howarth, W.R., 2006. Eutrophication of freshwater and marine ecosystems. Limnol. Oceanogr. 51, 351–800.

Sommer, U., Padisak, J., Reynolds, C.S., Juhász-Nagy, P., 1993. Hutchinson's heritage: diversity-disturbance relationship in phytoplankton. Hydrobiologia 249, 1–7.

Spatharis, S., Tsirtsis, G., 2010. Ecological quality scales based on phytoplankton for the implementation of Water Framework Directive in the Eastern Mediterranean. Ecol. Indic. 10, 840–847.

Spatharis, S., Tsirtsis, G., Danielidis, D.B., Chi, T.D., Mouillot, D., 2007. Effects of pulsed nutrient inputs on phytoplankton assemblage structure and blooms in an enclosed coastal area. Estuar. Coast. Shelf Sci. 73, 807–815.

Strickland, J.D.H., Parsons, T.R., 1972. A practical handbook of seawater analysis. J. Fish. Res. Board Can. 167, 1–310.

Tudor, M., Beg Paklar, G., 2014. Numerical modelling. In: Marasovic, I. (Ed.), The Adriatic Sea monitoring program, 2nd phase: consulting services for the definition of the monitoring and observation system for ongoing assessment of the Adriatic Sea. Institute of Oceanography and Fisheries, Split, pp. 255–308.

UNEP/MAP, 2011. Review of the Methods, Criteria and Limit Values for the Assessment of Eutrophication (Biological Quality Element “Phytoplankton”) as Developed in the Framework of the Intercalibration Exercise of the MED GIG (Mediterranean Eco-region) Water Framework Directive 2000/60 EC., pp. 41.

Üthermöhl, H., 1958. Zur Vervollkommnung der quantitativen Phytoplankton Methodik. Mitt. Int. Verein. Theor. Angew. Limnol. 9, 1–38.

Viličić, D., 1989. Phytoplankton population density and volume as indicators of eutrophication in the eastern part of the Adriatic Sea. Hydrobiologia 174, 117–132.

Vukadin, I., 1981. Hydrographic properties of Mali Ston Bay and the adjacent Sea. In: Roglić, J., Meštrov, M. (Eds.), Proceedings of the Symposium on Mali Ston Bay. Dubrovnik, Croatia, pp. 52–65.

Water Framework Directive Intercalibration Technical Report, 2009. Part 3: coastal and transitional waters. In: Carletti, A., Heiskanen, A.S. (Eds.), OPOCE. European Commission Joint Research Centre Institute for Environment and Sustainability, Fermi, Italy.

Weinbauer, M.G., Fuks, D., Peduzzi, P., 1993. Distribution of viruses and dissolved DNA along a Coastal Trophic gradient in the Northern Adriatic Sea. Appl. Environ. Microbiol., 4074–4082.

Wyatt, T., 2013. Margalef's mandala and phytoplankton bloom strategies. Deep-Sea Res. II 101, 32–49.

Zavatarelli, M., Baretta, J.W., Baretta-Bekker, J.G., Pinardi, N., 2000. The dynamics of the Adriatic Sea ecosystem. An idealized model study. Deep-Sea Res. 47, 937–970.