Mediterranean Marine Science

Vol 15, No 3 (2014)

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doi: 10.12681/mms.866

To cite this article:

SKEJIĆ, S., BOJANIĆ, N., MATIJEVIĆ, S., VIDJAK, O., GRBEC, B., NINČEVIĆ, Ž G., ŠESTANOVIĆ, S., & ŠANTIĆ, D. (2014). Analysis of phytoplankton community in the vicinity of domestic sewage outflow during stratified conditions. *Mediterranean Marine Science*, *15*(3), 574–586. https://doi.org/10.12681/mms.866

Research Article

Mediterranean Marine Science
Indexed in WoS (Web of Science, ISI Thomson) and SCOPUS
The journal is available on line at http://www.medit-mar-sc.net
DOI: http://dx.doi.org/10.12681/mms.866

Analysis of the phytoplankton community in the vicinity of domestic sewage outflow during stratified conditions

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Handling Editor: Lydia Ignatiades

Received: 9 April 2014; Accepted: 30 April 2014; Published on line: 18 September 2014

Abstract

The response of phytoplankton community structure to anthropogenically-derived nutrients from the submarine sewage outflow was analysed in a shallow coastal area of the middle Adriatic Sea. The study was conducted during an eight-year period (2002-2010) at six stations in the Brač Channel, aiming to assess the potential change in the state of biological and environmental parameters before and after commissioning of the sewage plant. Research was focused on phytoplankton producers in particular, during the summer, which is considered to be critical due to water stratification and low water dynamics. The results show an increase in phytoplankton biomass in the deep layer after discharge activation that was significant (M-W test; U=584.5, $p<0.01$), but not related to any intense phytoplankton bloom or oxygen deficiency. Temporal variability of total phytoplankton abundances throughout the research period indicated a significant increase in 2005, the year following sewage outflow activation. The Pairwise Dunn's multiple comparison test emphasised the difference in the years before sewage activation (2002, 2003) compared to 2005 (2002 vs. 2005, R=-60.92 $p<0.001$; 2003 vs. 2005, R=-63.92 $p<0.001$). This situation was soon followed by the rapid equalization in phytoplankton abundances. Considering the entire research period, there were no changes in the contribution of major phytoplankton groups, but the increase in overall species diversity was recorded, thus supporting the view that moderate inputs may stimulate plankton taxonomic diversity in coastal ecosystems. Increased ciliate and copepod nauplii abundance was also observed but was considered to be caused by natural variability.

Keywords: Phytoplankton, biomass, community composition, sewage outflow, Adriatic Sea.

Introduction

Coastal marine ecosystems are increasingly affected by nutrient loadings from a variety of anthropogenic sources, including industrial and domestic wastewater and agricultural runoff (Nixon, 1995; Justic *et al.*, 1995; Smith *et al.*, 1999; Spatharis *et al.*, 2007). Untreated domestic and industrial wastewater is considered as one of the main causes of the deterioration of the state of the Mediterranean Sea (UNEP, 2008). Among biological indicators, plankton biomass and population structure provide an early warning of eutrophication-induced change, while other frequently used eutrophication indicators, such as benthic community, often provide a delayed response, hindering the application of preventive measures and effective protection (Mee, 2005; McQuatters-Gollop *et al.*, 2009).

It is argued that a continuous supply of nutrients results in a steady state assemblage, where some species successfully compete for limited nutrients and develop high abundances, while weaker competitors are scarce or completely excluded (Hardin, 1960; Sommer, 1985; Capblancq, 1990). According to the intermediate disturbance hypothesis (Conell, 1978; Shea *et al.*, 2004), when disturbance becomes very significant, most species are exterminated and diversity decreases (Polishchuk, 1999).

For several decades, the middle Adriatic Kaštela Bay has been among the most eutrophicated coastal environments in the eastern Mediterranean, due to considerable inflow of industrial wastewater and the combined effects of domestic sewage from the cities of Split, Trogir and Solin situated along its coast (Marasović, 1989; Marasović & Pucher-Petković, 1991; Marasović *et al.,* 1991, 1995; Krstulović *et al*., 2011). With the aim of improving the environmental status in the Bay, a new sewage treatment plant was constructed and the outflow moved to the wider area of the Brač Channel at the end of 2004. The possible effects of the submarine sewage outflow at this new location were assessed through a comprehensive monitoring programme encompassing chemical and biological parameters and conducted both prior to sewage outflow commissioning and after its activation.

Previous results from the Brač Channel, considering the analysis of the water column, indicated a low trophic status for the area, which was especially evident during the summer period when nutrient levels and phytoplankton biomass and population structure were similar to the open sea oligotrophic conditions. Consequently, we were particularly interested in phytoplankton occurrences during the summer season, which is generally considered as critical due to low water dynamics and strong water column stratification, and in which the possible negative consequences of eutrophication could be clearly detected. Therefore, this study aims to assess the phytoplankton response before and after activation of the new sewage outflow, in terms of detectable changes in abundance and population structure.

Material and Methods

Study area

The Brač Channel is a marine area between the northern coast of Brač Island and the mainland coast. This area, including the microlocation of the coastal town of Stobreč, is under the influence of the prevailing western winds and pronounced cyclonic activity. Summer and winter climate conditions in this area differ considerably; winters are characterized by frequent changes of cyclones and anticyclones, resulting in frequent changes in the Bora and Sirocco winds, while during the summer the area is influenced by a subtropical field of high air pressure characterised by the 24-hour land-sea circulation activity.

The thermohaline characteristics of the Brač Channel are subject to strong atmospheric influences, especially as regards the wind direction, as well as under the influence of freshwater inflows from the land. Both parameters show pronounced seasonal variability. Isothermal conditions in the water column are observed from mid-November to early spring, after which increased atmospheric warming initiates the heating of the water surface and causes the formation of a thermocline. Earlier observations of sea temperature in this area show that a thermocline in July is usually observed at approximately 10 m depth (Grbec *et al.*, 2009).

Surface salinity varies considerably at the spatial scale as a result of freshwater inflow from the mainland. The thermohaline characteristics of the narrow coastal area near the town of Stobreč are also quite complex, characterized by certain estuarine features and by permanent halocline conditions.

The water circulation regime in the Brač Channel is highly dependent on wind direction and the existence of two underwater reefs located southwest of Stobreč town. The reefs represent a physical barrier preventing the free movement of water masses and affecting the overall circulation pattern (Grbec *et al.*, 2006).

Sampling and measurement methods

The study was conducted during the 2002-2010 period at six stations in the Brač Channel (middle Adriatic Sea) in the vicinity of the submarine sewage outflow. Physical, chemical and biological parameters were determined in July at discrete depths of 0, 5, 10, 20, 30 and 40 meters. The period from 2002 to 2005 is designated as "before" and the period from 2005 to 2010 as "after" (Fig. 1). Considering the differences in depth of the investigated stations as well as the hydrographic characteristics in the area, two layers in the water column were considered: the surface layer (0 and 5 m depths) and the deep layer (below 10 m depth).

The phytoplankton population structure was analysed in all samples collected from discrete depths. Samples were immediately preserved in 0.4% glutaraldehyde-seawater solution and the subsamples (25 ml) were settled overnight in sedimentation chambers. Cell counting was performed using the inverted microscope (Olympus IX 51) following the Utermöhl sedimentation method (Utermöhl, 1958), at magnifications of 200x and 400x. In order to determine the taxo-

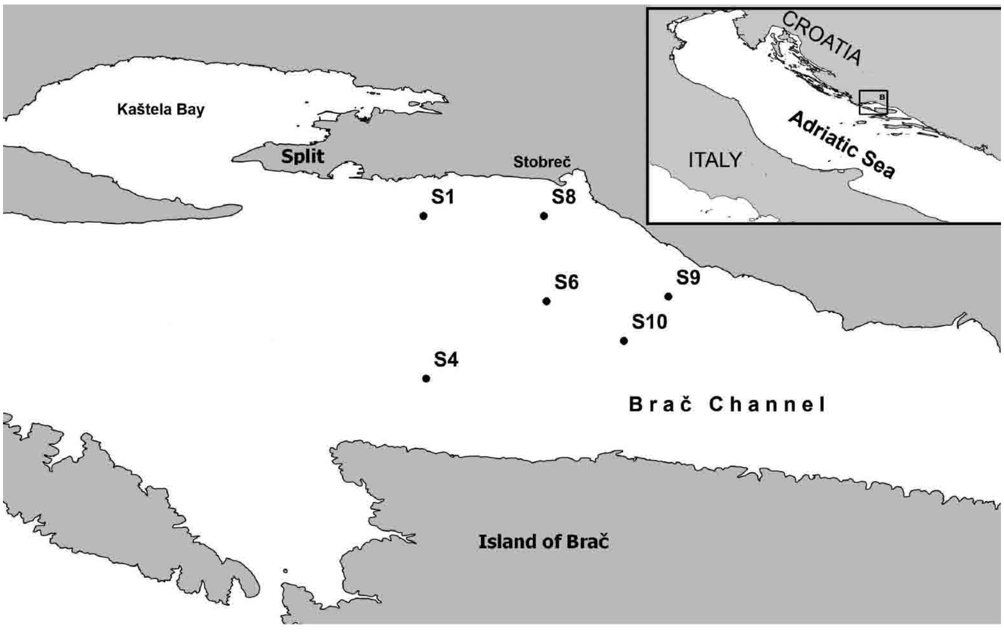

Fig. 1: Position of sampling stations in the Brač Channel.

nomic composition of phytoplankton species, the following references were used: Hustedt, 1930, 1931; Schiller, 1933, 1937; Tomas, 1993, 1996; Horner, 2002; Viličić *et al.*, 2002. Chlorophyll *a* (Chl *a*) concentrations were determined in all samples according to Strickland and Parsons (1972).

Samples for bacterial counts were collected in parallel with phytoplankton sampling. Samples were poured into sterile acid-washed glass bottles and fixed with a formaldehyde solution (final concentration 2.0%). Bacterial counts were performed under an epifluorescence microscope (Olympus BX50) at 1000x magnification, using the standard DAPI staining technique (Porter & Feig, 1980).

Samples for microzooplankton counts were collected in parallel with phytoplankton sampling, with the exception of 2006, due to technical difficulties. Microzooplankton samples were collected at 5 m depth intervals from the water surface to the sea bottom using 5 L Niskin bottles. The samples were preserved in a 2.5% formaldehyde-seawater solution, previously buffered with $CaCO_3$, since Lugol's solution stains the detritus that can be abundant in this area and would thus reduce visibility (Fonda-Umani & Beran, 2003). Preparation of the samples for microscopic analysis was performed as described in detail in Bojanić *et al.* (2005). Counting and species identification were carried out using an inverted microscope (Olympus IMT-2) at 100x and 400x magnification. The entire bottom of the sedimentation chamber was analysed and the abundances were expressed as the number of individuals per litre (ind. L^{-1}). Heterotrophic dinoflagellates were not included in this group.

Vertical temperature and conductivity profiles were measured to an accuracy of >±0.01°C and ±0.02 psu, respectively, using CTD multiparameter probes (Idronaut and Sea-Bird). The dissolved oxygen concentration was determined using the Winkler titration method (Grasshoff *et al.*, 1983). Nutrient concentration (orthophosphates HPO_4^{2-}, nitrates NO_3^-, nitrites NO_2^-, ammonia NH_4^+), was determined colorimetrically with an Auto-Analyzer III and Seal Analytical system, using automated methods (Grasshoff *et al.*, 1983).

Statistical analysis

The Kruskal-Wallis ANOVA test (K-W test) was performed to analyse differences in phytoplankton abundances among sampling years (2002-2010) for each station and layer. The Mann-Whitney test statistic (U) was used to test differences among values of chlorophyll *a*, as well as mikrozooplankton abundances, before and after activation of the sewage system.

The Shannon-Wiener diversity index (Shannon & Wiener, 1949) $H' = \Sigma\, p i \log (p i)$ and Margalef's evenness index $d = (S-1)/\log(N)$ were used to analyse phytoplankton diversity. In order to reveal individual taxa contributions to dissimilarities among the sampling stations, layers and years, Similarity Percentage Analysis (SIMPER) was performed on the log (x+1) transformed phytoplankton abundance data matrix (Clark & Gorley, 2001).

Redundancy analysis (RDA) was used to evaluate the linear and cause-effect relations between the abundance of the most abundant phytoplankton taxa (as response variables) and the environmental parameters (as explanatory variables). Response variables were: *Chaetoceros curvisetus*, *Chaetoceros spp*., *Hemiaulus hauckii, Leptocylindrus danicus,* Pennatae indeterm, *Proboscia alata*, *Pseudonitzchia spp.* and *Gymnodinium spp.*, while explanatory variables were: heterotrophic bacteria abundance, chlorophyll a, total phytoplankton abundance, temperature, salinity, O_2, NO_3^-, NO_2^-, NH_4^+, TIN, HPO_4^{2-}, SiO_4). Since the length of the gradient present in our data set (interannual changes of variables) was <4 SD units, a linear response model was appropriate (Van den Brink *et al.*, 2003). RDA explicitly models response variables as a function of explanatory variables (Zuur *et al.,* 2007). The analysis was based on correlation data matrices of surface and deep layer values for representative station S8 in every year, in which each the variable was standardised to a zero mean and unit variance in order to eliminate numerical differences between study parameters, leaving only relative year-to-year changes in parameters. RDA was performed using XLS-Biplot 1. software 1 (1999-2002) created by E.P. Smith and I.A. Lipkovich of the Statistics Department at Virginia Tech.

Results

Environmental parameters

Environmental parameters (nutrients concentration and oxygen saturation) are presented in Table 1 as comparisons of two investigated periods "before" (2002-2005) and "after" sewage outfall commissioning (2005-2010) for the entire investigated area (Fig. 1). The results show an increase in total inorganic nitrogen ($TIN = NO_3^- + NO_2^- + NH_4^+$) concentrations in both surface and deep layers, with the range values rising from 0.10 -1.47 µmol L^{-1} ("before") to 0.5-4.29 µmol L^{-1} ("after"). A similar situation was noticed for the average TIN values, which spanned from 0.96±0.32 µmol L^{-1} ("before") to 1.20±0.70 µmol L^{-1} ("after"). Moreover, an increase in all inorganic nitrogen species was observed: nitrates, nitrites and ammonia after sewage outfall activation, both in the surface and deeper layer. Conversely, orthophosphate concentrations (HPO_4^{2-}) showed a decrease in concentrations during the period "after" (2005-2010). Oxygen saturation values (%) indicate good oxygenation of both layers of the water column, as well as an increase in the period after sewage outfall activation (Table 1).

Biological parameters

Phytoplankton biomass

Chlorophyll *a* (Chl *a*) values were relatively low during the entire investigated period. "Before" and "after" comparisons showed an expanded range of Chl *a* values in the period after activation of the sewage outfall. The Kruskal-Wallis test showed significant differences in the Chl *a* con-

Table 1. Nutrient concentrations (μmol L-1) and oxygen saturation (%) presented as ranges and average values with standard deviations in the surface layer (0-5 m depth) and deep layer of the water column (10m to the bottom) in the period before (2002-2005) and after (2005-2010) sewage outflow activation.

Parameter [μmol L^{-1}]		**Before**	**After**
Nitrate (NO_3^-)	surface	0.007-0.902 0.192±0.170	0.002-0.893 0.197±0.180
	deep	0.0-0.780 0.170±0.128	0.003-2.834 0.336±0.575
Nitrite (NO_2^-)	surface	0.006-0.051 0.024±0.011	0.009-0.139 0.065±0.037
	deep	0.002-0.108 0.025±0.018	0.0-0.43 0.089±0.076
Ammonia (NH_4^+)	surface	0.09-1.02 0.78±0.49	0.06-5.66 1.25±1.09
	deep	0.04-1.91 0.77±0.57	0.09-5.36 1.32±0.99
Orthophosphate (HPO_4^{2-})	surface	0.031-0.767 0.097±0.134	0.0-0.128 0.052±0.028
	deep	0.006-0.310 0.078±0.049	0.0-0.143 0.048±0.025
Oxygen saturation (%)	surface	83.2-106.8 102.4±4.6	98.6-119.7 106.7±5.3
	deep	89.3-113.9 100.4±4.8	71.1-117.5 101.4±8.9

centrations among the investigated years (K-W test=37.88; p<0.0001). Separate analyses of the surface (0 and 5) and deep layers (below 10 m) showed an increase in phytoplankton biomass in the deep layer at all stations after sewage outfall activation (Fig. 2), corroborated by the significant result of the Mann-Whitney test (M-W test; U=584.5, p<0.01).

Phytoplankton abundance

Temporal variability of total phytoplankton abundances throughout the 2002-2010 period indicated a visible increase in the year following sewage outflow activation (i.e. 2005). The K-W test showed a significant difference in average phytoplankton abundance among the investigated years (K-W test=59.86; p<0.001). Dunn's Pairwise multiple comparison test emphasised the difference in the years before sewage outfall activation (2002, 2003) compared to 2005. These results (2002 vs. 2005, R=-60.92 p<0.001; 2003 vs. 2005, R=-63.92 p<0.001) were mainly affected by the variability in two major microphytoplankton groups, diatoms and dinoflagellates (Fig. 3). The overall abundance increase in 2005, in diatoms as well as dinoflagellates, was particularly pronounced at 0 and 5 m depths (Fig. 3).

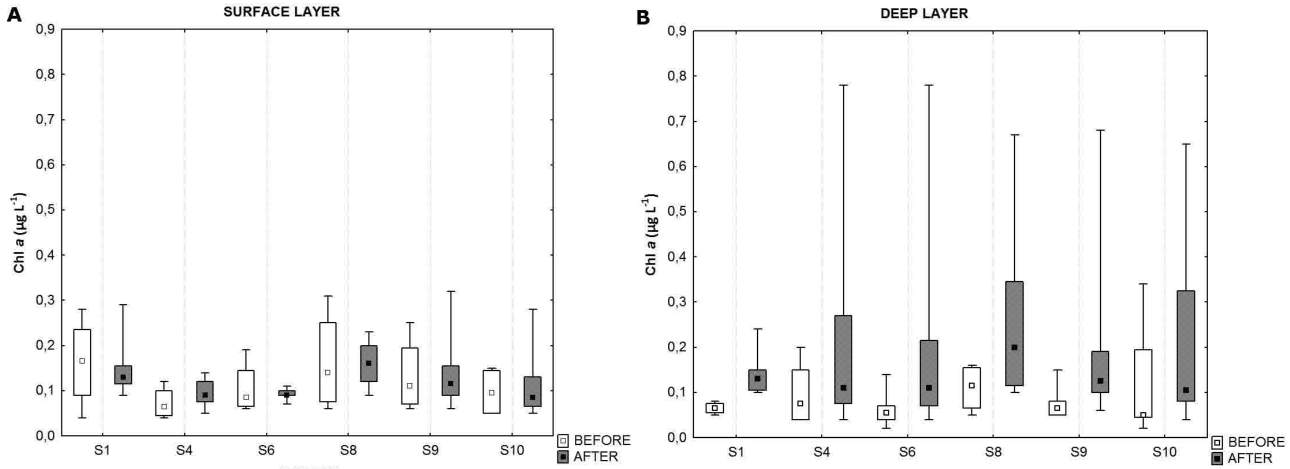

Fig. 2: Spatial distribution of chlorophyll *a* concentrations in (A) the surface layer and (B) the deep layer during the period 2002-2005 (B - before sewage outfall activation) and 2005-2010 (A - after sewage outfall activation) at six stations in the Brač Channel illustrated by box and whisker diagrams (values indicate median; box: 25%-75%. whisker: minimum-maximum).

Since all investigated stations showed fairly similar total phytoplankton abundance (K-W test=4.712; p<0.452), we chose to present the temporal distribution of two microphytoplankton groups for one representative station only, namely S8 (Fig. 3). After the peak in 2005, the abundances became more balanced. Another phytoplankton abundance peak was observed in 2009, but it was less pronounced and spatially restricted to stations S6 and S10.

Considering the entire research period, diatoms prevailed in the microphytoplankton in terms of abundance, followed by dinoflagellates. Coccolithophorids and silicoflagellates were poorly represented, contributing 1% to total phytoplankton abundance, on average.

Phytoplankton population structure

Qualitative analysis of the phytoplankton community revealed a similar number of diatom (54) and dinoflagellate taxa (58) in the investigated area. A detailed list of recorded phytoplankton taxa including the individual abundances and frequencies of occurrence is presented in Table 2.

The majority of recorded diatom taxa belonged to the genus *Chaetoceros* (15 taxa). The most frequently recorded taxa were *Leptocylindrus danicus* and *Pseudonitzschia* spp. (*delicatissima* group), and various small pennate diatoms. Regarding the taxonomic variety of dinoflagellates, the dominant and the most frequently occurring genus (occurring in 47% of samples) was *Gymnodinium*.

In 2005, the dominant phytoplankton taxa were *Chaetoceros curvisetus* (1.2x10^5 cell L^{-1}; 53% in total phytoplankton abundance), *Ch. compressus* (1.8x10^4 cell L^{-1}; 8.2%) and *Pseudonitzschia spp.* (1.9x10^4 cell L^{-1}; 8%). Compared to the period before sewage outfall activation, the change was observed in increased abundances, but not in the frequency of occurrence of the species.

The phytoplankton taxa occurring with a frequency >10%, but in low abundances (less than 8.0x10^3 cell L^{-1}) were *Cylindrotheca closterium, Guinardia striata, G. flaccida, Hemiaulus hauckii, Navicula spp.* and *Coccolithhophoridae* spp. Conversely, the species *Skeletonema marinoi* occurred with low frequency but in high abundances (Table 3).

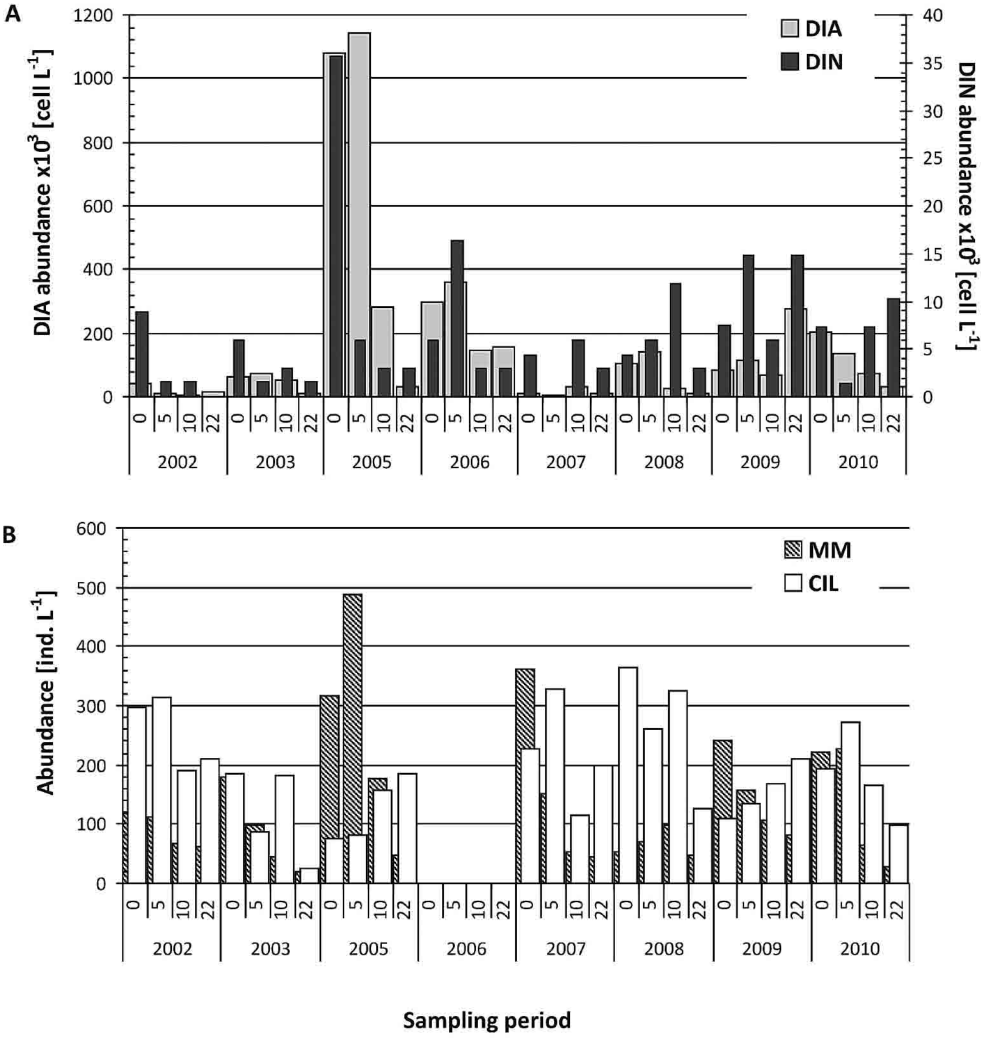

Fig. 3: Temporal and vertical distribution of total abundances of (A) diatoms and dinoflagellates and (B) ciliates and micrometazoans at station S8 from 2002 until 2010 (DIA. diatoms; DIN. dinoflagellates; CIL. ciliates; MM. micrometazoans).

Table 2. List and numerical data for the phytoplankton taxa identified in the Brač Channel (see Figure 1) from 2002 until 2010.

Phytoplankton taxa	**Average value** [cell L^{-1}]	**Maximum** [cell L^{-1}]	**Frequency** [%]
Diatoms			
Amphiprora sulcata O'Meara	1480	1480	0.4
Amphora sp. Ehrenberg ex Kützing	2960	2960	0.4
Asterionellopsis glacialis (Castracane) Round	9860	16280	1.3
Bacteriastrum delicatulum Cleve	4933	7400	1.3
Biddulphia sp. Grey	1480	1480	0.9
Cerataulina pelagica (Cleve) Hendey	5440	42920	16.5
Chaetoceros affinis Lauder	33866	273800	12.2
C. atlanticus Cleve	6370	8820	1.3
C. compressus Lauder	65801	207200	10.4
C. costatus Pavillard	9960	14800	1.7
C. curvisetus Cleve	155605	919000	19.6
C. decipiens Cleve	9748	14800	2.2
C. diversus Cleve	5145	7350	0.9
C. gracilis Pantocsek	2467	4440	1.3
C. holsaticus Schütt	5820	5920	0.4
C. peruvianus Brightwell	1480	1480	0.9
C. pseudocurvisetus Mangin	39238	91140	5.7
C. socialis Lauder	5900	5900	0.9
C. vixvisibilis Schiller	8533	11760	1.3
C. wighamii Brightwell	13245	30870	2.6
Chaetoceros spp. Ehrenberg	65305	505000	22.2
Cocconeis sp. Ehrenberg	1470	1470	0.9
Cyclotella sp. Kützing	6905	27930	5.7
Cylindrotheca closterium (Ehr) Reim. et Lewin	1966	5880	15.7
Dactyliosolen fragilissimus (Bergon) Hastle	4072	27930	14.8
Diploneis sp. Ehrenberg ex Cleve	1470	1470	0.4
Eucampia cornuta (Cleve) Grunow	2943	4410	1.3
Guinardia delicatula (Cleve) Hasle	4144	8880	2.2
Guinardia flaccida (Castracane) Peragallo	2415	5920	13.0
G. striata (Stolterfoth) Hasle	3344	8880	17.8
Hemiaulus hauckii Grunow	2928	7400	24.3
H. sinensis Greville	2956	2960	2.2
Leptocylindrus adriaticus Schroder	10360	10360	0.4
Leptocylindrus danicus Cleve	27993	379260	48.3
L. mediterraneus (Peragallo) Hastle	11790	22050	1.3
L. minimus Gran	9087	26640	5.2
Licmophora sp Agardh	1477	1480	1.3
Melosira distans (Ehrenberg) Kützing	5880	5880	0.4
Navicula spp.	2181	5880	11.7
Nitzschia longissima (Brelb.) Ralfs	5280	26640	3.0
Nitzschia sigma (Kützing) Smith	1480	1480	0.4
Pennatae indeterm	3950	11840	44.8
Pleurosigma angulatum (Quekett.) Smith	2940	5880	1.3
Pleurosigma sp.	1475	1480	1.7
Proboscia alata (Brightwell) Sundstrom	3714	11800	13.5
Proboscia indica (Peragallo) Hernández-Becerril	2104	4400	3.0
Pseudosolenia calcar avis (Schultze) Sundström	1470	1470	0.4
Pseudo-nitzschia spp. Peragallo	14553	122840	72.2
R. imbricata Brightwell	1893	2960	3.0
Skeletonema costatum (Greville) Cleve	19472	124350	7.8
Thalassionema frauenfeldii (Grunow) Hallegraeff	1570	2940	1.7
Thalassionema nitzschioides Schrader	4006	7400	3.0
Thalassiosira rotula Meunier	5920	5920	0.4
Thalassiosira sp.	2383	4440	5.7
Dinoflagellates			
Alexandrium tamarense (Lebour) Balech	1480	1480	3.0
Alexandrium sp.	1480	1480	0.9
Amphidinium acutissimum Schiller.	1628	2940	8.3
A. acutum Lohmann	2960	2960	0.4

(continued)

Table 2. *(continued)*

Phytoplankton taxa	**Average value** [cell L^{-1}]	**Maximum** [cell L^{-1}]	**Frequency** [%]
A. schröderi Schiller	1850	2960	1.7
Amphidinium sp.	1480	1480	0.9
Ceratium candelabrum (Ehrenberg) Stein	1480	1480	0.4
Ceratium furca (Efrenb.) Claparéde et Lachmann	1125	1480	1.7
C. fusus (Ehrenberg) Dujardin	1190	1480	2.2
C. macroceros (Ehrenberg) Cleve	1123	1470	1.7
C. trichoceros (Ehrenberg) Kofoid	1480	1480	0.4
C. tripos (Müller) Nitzsch	1480	1480	1.3
Ceratocorys armata (Schütt) Kofoid	1480	1480	0.4
Cochlodinium sp.	1480	1480	0.9
Dinophysis caudata Saville-Kent	80	80	0.4
Dinophysis rotundata Claparède et Lachmann	775	1470	0.9
Dinophysis sacculus Stein	1480	1480	1.3
Gonyaulax polygramma Stein	1198	1480	2.2
Gonyaulax spinifera (Claparède et Lachmann) Diesing	80	80	0.4
Gymnodinium conicum Kofoid & Swezy	1470	1470	0.4
G. simplex Lohmann	1470	1470	0.4
G. uberimum (Allman) Kofoid et Swezy	1470	1470	2.6
Gymnodinium sp.	4178	20600	47.0
Gyrodinium fulvum Kofoid & Swezy	2220	2960	0.9
G. fusiforme Kofoid et Swezy	1500	2960	8.3
G. opimum (Schütt) Lebour	1970	2960	2.6
G. pinque (Schütt) Kofoid et Swezy	1768	2960	2.2
Gyrodinium sp.	1639	2960	7.8
Hermesinum adriaticum Zacharias	1470	1470	0.4
Heterocapsa sp.	2220	4440	1.7
Karenia sp.	1475	1480	0.9
Lingulodinium polyedrum (Stein) Dodge	1470	1470	0.4
Noctiluca scintilans (Macartney) Kofoid et Swezy	1470	1470	0.4
Oxytoxum caudatum Schiller	1480	1480	0.4
Oxytoxum globosum Schiller	1470	1470	0.4
O. scolopax Stein	1480	1480	0.4
Oxytoxum sp.	1477	1480	1.3
Podolampas palmipes Stein	1470	1470	0.4
Pronoctiluca spinifera (Lohmann) Schiller	1470	1470	0.9
Prorocentrum compressum (Bailey) Abe et Dodge	1480	1480	0.9
P. lima (Ehrenberg) Stein	80	80	0.4
P. gracile Schütt	1480	1480	0.4
P. micans Ehrenberg	1498	2940	2.6
P. minimum (Pavilard) Schiller	2012	2960	2.6
P. rostratum Stein	1480	1480	0.4
P. triestinum Schiller	1772	2960	2.2
P. bispinum (Schiller) Balech	1470	1470	0.4
P. diabolus (Cleve) Balech	1480	1480	0.4
P. pellucidum Bergh	1480	1480	0.4
P. pyriforme (Paulsen) Balech	160	160	0.4
P. steinii (Jörgensen) Balech	1475	1480	0.9
P. tuba (Schiller) Balech	1476	1480	3.0
Protoperidinium sp.	1125	1480	1.7
Pseliodinium vaubanii Sournia	1480	1480	0.4
Scrippsiella spinifera Honsell et Cabrini	1475	1480	0.9
Scrippseilla sp.Balech ex.Loeblich	1475	1480	0.9
S. trochoidea (Stein) Loeblich	1611	2960	5.2
Dinofl <20µm	6297	52400	41.3
Coccolithophorids			
Coccolithophoridae spp.	3011	8880	10.4
Syrachosphaera pulchra Lohmann	1896	4410	3.0
Rhabdosphaera tignifer Schiller	3430	7340	1.3
Silicoflagellates			
Dictyocha fibula Ehrenberg	1475	1480	0.9

A detailed list of the most frequent and the most abundant species during the entire investigated period is presented in Table 3.

Similarity percentage analysis (SIMPER) identified *Pseudonitzschia spp.* and *Leptocylindrus danicus* as key taxa that mostly contributed to before/after differences in the surface layer. In the deep layer, *Pseudonitzschia spp.* and small dinoflagellates (< 20 μm) were extracted as key elements contributing to these differences (Table 4).

Diversity indices

The observed variability of the Shannon-Wiener's diversity index (H') indicated a change in the microphytoplankton population structure. In the surface layer, in the "before" period H' ranged between 0.32 and 1.79, with an average value of 1.30±0.42. In the "after" period, the number of species per sample increased, and H' ranged from 0.54 to 2.36, with an average value of 1.43±0.41. Quite a similar trend was recorded in the deep layer. Before sewage outfall activation, the average H' value of 0.90±0.45 was recorded with a maximum of 1.78, while in the "after" period both values increased to 1.21±0.42 and 2.2, respectively.

Margalef's index (d) standardises the number of species encountered against the total number of specimens encountered. In the surface layer, this index revealed an average biodiversity of 0.49±0.19 in the period "before" and an average of 0.77±0.22 in the period "after". Min-max values ranged between 0.19 and 0.91 ("before") in comparison with the values of 0.12-1.37 ("after"). Additionally, in the deep layer increases in averages and min-max

Table 3. Average values, maximum abundances and frequencies of occurrence (>10% samples) of the dominant phytoplankton taxa under stratified conditions in the period from 2002 until 2010.

Phytoplankton taxa	**Average value** [cell L^{-1}]	**Maximum** [cell L^{-1}]	**Frequency** [%]
Cerataulina pelagica	5440	42920	16.5
Chaetoceros affinis	33866	273800	12.2
Ch. compressus	65801	207200	10.4
Ch. curvisetus	155605	919000	19.6
Chaetoceros spp.	65305	505000	22.2
Dactyliosolen fragfilissimus	4072	27930	14.8
Leptocylindrus danicus	27993	379260	48.3
Pennatae indeterm	3950	11840	44.8
Proboscia alata	3145	11800	53.5
Pseudonitzschia spp.	14553	122840	72.2
Gymnodinium sp.	4178	20600	47.0
Dinoflagellates <20μm	6297	52400	41.3

Table 4. Similarity percentage analysis (SIMPER). Groups are combined by factors: layer (surface-S/deep-D) and treatment (before-B/after-A).

Groups BS & AS						
Ave dissimilarity = 71,35	**Group BS Av.Abund**	**Group AS Av.Abund**	**Av.Diss**	**Diss/SD**	**Contrib%**	**Cum.%**
Pseudonitzschia spp.	3.65	8.46	4.64	1.24	6.50	6.50
Leptocylindrus danicus	4.11	6.76	4.17	1.10	5.84	12.34
Gymodinial dinofl<20μm	1.88	4.37	3.85	0.99	5.39	17.74
Chaetoceros compressus	0.83	4.62	3.54	0.86	4.97	22.70
Gymnodinium sp.	5.71	4.40	3.50	1.03	4.91	27.61
Chaetoceros curvisetus	0.85	4.29	3.40	0.78	4.77	32.38
Groups BD & AD						
Ave dissimilarity = 87.24	**Group BD Av.Abund**	**Group AD Av.Abund**	**Av.Diss**	**Diss/SD**	**Contrib%**	**Cum.%**
Pseudonitzschia spp	1.48	7.15	8.72	1.35	9.99	9.99
Gymodinial dinofl<20μm	1.91	4.51	7.48	0.94	8.58	18.57
Navicula sp.	0.87	5.04	7.18	1.02	8.23	26.80
Hemiaulus hauckii	3.06	1.10	5.25	0.74	6.02	32.81
Leptocylindrus danicus	1.58	3.98	5.06	0.84	5.80	38.61
Proboscia alata	3.24	2.36	5.00	0.85	5.73	44.34

ranges for both periods were recorded (averages 0.26±0.19 ("before") and 0.46±0.25 ("after"); min-max 0 - 0.61 ("before") and 0-1.28 ("after")).

Microzooplankton and heterotrophic bacteria

Increased abundances of ciliates (CIL) and copepod nauplii (NAUP) populations as the main components of the microzooplankton community were recorded in the surface as well as in the deep layer in the "after" period (Table 5). However, a statistically significant change of CIL abundance was recorded only in the deep layer (U=96, p=0.0186). After sewage outflow activation the significant increase in NAUP category was recorded in the surface layer (U=78, p=0.0037) and the deep layer (U=99, p=0.0235). A similar increase was not recorded in the abundances of heterotrophic bacteria (Table 5).

Analysis of environmental influences on the phytoplankton community

The results of RDA, used to identify an environmental basis for phytoplankton community ordination at the representative station, are presented on the triplot (Fig. 4). The RDA triplot shows that the first two ordination axes explained 58.58% of the data variability of phytoplankton taxa in the ordination of environmental parameters. Among environmental parameters, the abundance of bacteria was negatively associated with axis 1, while copepod nauplii were strongly positively correlated with axis 1. Additionally, the temperature and concentration of total inorganic nitrogen showed a strong negative association with the second axis, while chlorophyll *a* and dissolved oxygen were strongly associated with the same axis. Taking into account the determining effect of temperature on dissolved oxygen, we assume that temperature is the main abiotic parameter responsible for the environmental gradient along axis 2. The abundances of *Chaetoceros curvisetus*, *Pseudonitzschia spp.* and *Proboscia alata* were significantly positively associated with axis 1 in the surface layer in 2005 and 2008. Axis 2 positively affected the abundance of Pennatae diatoms especially in the deep layer in 2006, while it negatively affected the abundance of *Hemiaulus hauckii* in the surface layer in 2005 and 2010. Axis 3 (not displayed) explained 17.05% of the data variability and, in addition to temperature, it was positively related to orthophosphates and ciliates. Phytoplankton taxa related to this axis included *Chaetoceros* species and dinoflagellates in the surface layer in 2006 and 2002, respectively. Additional axes explained significantly less of the overall variability in the phytoplankton community compared to the first three axes.

Discussion

Understanding the relationship between pollution caused by domestic wastewater and ecosystem responses is important for the evaluation of the coastal marine trophic state in regional seas (Tsirtsis *et al.,* 2008). The relationship between nutrient enrichment and phytoplankton biomass has often been shown to be proportional (Fanuko, 1984; Pan & Subba Rao, 1997). Nevertheless, many studies dealing with the effects of additional nutrient supply on marine ecosystems (such as aquaculture) have not determined a positive correlation between the increased nutrient concentrations and phytoplankton growth (Beveridge, 1996; Pitta *et al.,* 1999; La Rosa *et al.,* 2002; Mozetič *et al.,* 2008; Skejić *et al.,* 2011). The effect of nutrient supply on phytoplankton population structure is even more unpredictable. While some authors note the negative consequences of increased nutrient input in terms of reduced species diversity and increased occurrences of harmful algal bloom events (Fanuko, 1984; Pan & Subba Rao, 1997; Wang *et al.,* 2006), others emphasize that moderate nutrient inputs can promote biodiversity by stimulating the growth of different taxonomic groups (Spatharis *et al.,* 2007). These theories become even more complex when we consider that zooplankton can obscure changes in the phytoplankton population structure through selective grazing. In general, pulsed nutrient inflows can modify plankton fluctuations by stimulating an increase of zooplankton biomass, which consequently prevents excessive accumulation of phytoplankton biomass and controls phytoplankton diversity (Arhonditsis *et al.,* 2000; Buyukates & Roelke, 2005).

Table 5. Average values, with standard deviations (SD), of microzooplankton (CIL=ciliates; Naup= copepod nauplii) and heterotrophic bacteria in the surface and deep layer from 2002 until 2010.

		Before	After
Microzooplankton (ind. L^{-1})			
CIL	surface	174.33 ±58.82	210.77±100.70
	deep	105.83 ±47.86	143.13±41.90
NAUP	surface	71.29±31.14	158±105.78
	deep	25.38±10.93	38.89±21.72
Heterotr. Bacteria (cells mL^{-1})			
	surface	0.40-2.10 x 10^6	0.25-1.05 x 10^6
	deep	0.62-1.20 x 10^6	0.13-0.70 x 10^6

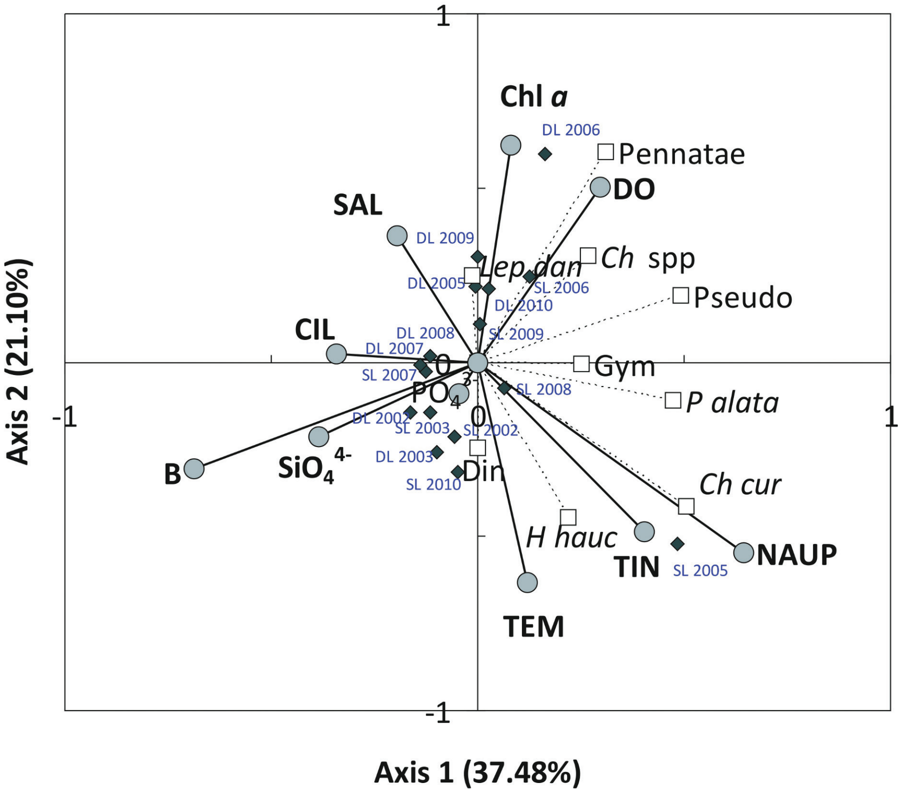

Fig. 4: Redundancy analysis (RDA) correlation triplot for phytoplankton taxa abundances (Ch spp-*Chaetoceros* spp.; Ch cur-*Chaeroceros curvisetus*; Din- Dinoflagellates<20µm; Gym-Gymnodinium sp. ; H hauc- *Hemiaulus hauchii*; Lep dan- *Leptocylindrus danicus*; Pennatae -Pennatae indeterm; Pseudo- *Pseudonitzschia* spp.;) and environmental parameters at station S8 in the Brač Channel (SL, surface layer; DL, deep layer; TEM, temperature; SAL, salinity; DO, dissolved oxygen; TIN, total inorganic nitrogen; PO_4^{3-}, orthophosphate; SiO_4^{4-}, silicate; Chl *a*, concentration of chlorophyll *a*; B, bacteria; CIL, ciliates; NAUP, copepod nauplii). Results are presented as comparisons of two layers; surface layer (SL) and deep layer (DL) for two investigated periods, i.e. "before" (2002-2005) and "after" (2005-2010) for each investigated year.

The increase in total inorganic nitrogen concentrations determined through this study is considered to be due to anthropogenic causes mainly in comparison to certain sites investigated in the nearby middle Adriatic area during the same period (Kušpilić *et al.*, 2010). An unexpected decrease in orthophosphate concentrations after sewage outflow could be due to fast HPO_4^{2} uptake by the phytoplankton during the photosynthesis process considering the limiting role of orthophosphate for primary production in this part of the middle Adriatic (Kušpilić *et al.*, 2010). This assumption is also in accordance with the determined increase in biomass in the investigated area during the "after" period, and also the increase in oxygen saturation.

The importance of Chl *a* concentration as a phytoplankton biomass proxy is emphasized by the fact that, at present, it is the only plankton parameter for the the Mediterranean Region that is taken into consideration for the implementation of the Water Framework Directive (Simboura *et al.,* 2005; Spatharis & Tsirtis, 2010). In the present study, the observed range of phytoplankton biomass values in the Brač Channel (Fig. 2) was relatively narrow throughout the investigated period, particularly when compared to other Adriatic coastal waters that are subject to anthropogenic pressure (Kušpilić *et al*., 2011; Mozetič *et al.,* 2008; Ninčević Gladan *et al.*, 2010; Skejić *et al.,* 2011; Bužančić *et al.*, 2012; Vidjak *et al.,* 2012). This shows that the increased nutrient availability in the period after sewage outfall commissioning, although coupled with a mild increase in phytoplankton biomass in the deep layer, does not necessarily point towards an alteration of the area's trophic status.

In the surface layer, rapid dispersion of phytoplankton biomass into adjacent areas is probably enhanced by favourable current dynamics and water circulation, additionally intensified by local winds (Dadić*,* 1998). The observed increase in phytoplankton biomass in the deep layer after sewage outfall activation was correlated with the increase in nutrients but could not be clearly connected to a specific phytoplankton bloom or oxygen deficiency. One of the plausible explanations for the observed increase in Chl *a* concentration could be the contribution of additional Chl *a* sources, such as chloroplast-retaining oligotrich ciliates (Stoecker, 1991). In wild populations, retention of functional chloroplasts seems to be a consistent characteristic, particularly in a subset of choreotrich (oligotrich and tintinnid) taxa, characteristic of estuarine and marine plankton species of genera *Laboea*, *Strombidium* and *Tontonia*, all of the oligotrich family Strombidiidae (Stoecker, 1991). Those plastidic ciliates are a major component of the ciliate community in a variety of marine water habitats. They represent 17-54% of ciliate abundance and 13-62% of ciliate biomass in the oligotrophic eastern Mediterranean Sea (Pitta & Giannakourou, 2000). At present, it is not clear which factors regulate the relative contribution of mixotrophic forms to the ciliate community (Dolan, 1992).

Previous ecological studies of the adjacent Kaštela Bay (Pucher-Petković & Marasović, 1980; Marasović & Pucher-Petković, 1991) demonstrated that a summer increase in the Chl *a* concentration due to eutrophication was accompanied by changes in community structure. Considerable changes were recorded in the proportions of major phytoplankton groups, leading to a marked reduction in diatom abundances. However, in this study the continuous prevalence of diatoms was observed, and the ratio of major phytoplankton group abundances was constant. Phytoplankton displayed the characteristics of an open-sea community in terms of community composition and the domination of short chain-forming diatoms such as *Chaetoceros* spp. (Ninčević Gladan *et al.,* 2010).

Phytoplankton abundance is naturally affected by microzooplankton grazing, primarily of ciliates and copepod nauplii (Calbet & Landry, 2004; Irigoien *et al*., 2005). Considering the size fraction of the phytoplankton community (micro), larger nonloricate ciliates and tintinnids, as well as small copepods could play a role in the top-down control of phytoplankton populations. However, the statistical coupling between phytoplankton and ciliates was weak (Spearman R=-0.205), probably since we investigated nonloricates as a single ecological category. The effect of micrometazoans on phytoplankton could also be indirect, *via* trophic cascade. Earlier investigation in the adjacent Kaštela Bay suggested that predator activities of small copepods are primarily directed towards nonloricate ciliates (Bojanić *et al*., 2005), which in turn may increase phytoplankton biomass through the suppression of direct grazers. This was confirmed by the positive and significant correlation between overall phytoplankton abundance and small metazoans (mainly copepod nauplii: Spearman R=0.502 $P<0.01$). Additionally, the importance of top-down control is confirmed by the fact that a higher percentage of primary production is consumed in more oligotrophic environments compared to the more productive estuarine or bay systems (Calbet & Landry, 2004).

The RDA results suggest that among the abiotic parameters, temperature and inorganic nitrogen availability had the greatest influence on phytoplankton variability during our investigation. An even higher impact was recorded for biotic variables such as bacteria, copepod nauplii, phytoplankton biomass and ciliates, which determined a great part of the overall variability in the summer phytoplankton community. These indicated that the nature of relationships within the plankton community was equally affected by the supply of nutrients and predator-prey interactions and competitors.

Besides phytoplankton biomass and abundance, diversity is an additional quantitative method that provides valuable information about the condition of the community (Tsirtsis & Karidys, 1998), and in conjunction with other parameters it is important for assessing the eutrophication level. According to the present study, sewage effluents in the Brač Channel did not cause negative effects on phytoplankton diversity. On the contrary, a mild increase in phytoplankton diversity was observed throughout the investigated period, confirming the hypothesis that moderate nutrient enrichment stimulates diversity (Spatharis *et al.,* 2007). This is in accordance with the intermediate disturbance theory proposed by Conell and Sousa (Conell, 1978; Conell & Sousa, 1983) that localized pulse disturbance is not necessarily considered as a threat to ecosystem health.

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