Effect of river inputs on environmental status and potentially harmful phytoplankton in a coastal area of eastern Mediterranean (Maliakos Gulf, Greece)

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Research Article

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Abstract

This paper examines the response of phytoplankton and potentially harmful species to river inflows in an eastern Mediterranean coastal area, within the context of environmental status assessment suggested by the European Commission's Water Framework Directive (WFD, 2000/60/EC) and the Marine Strategy Framework Directive (MSFD, 2008/56/EC). The spatio-temporal distribution of phytoplankton communities and biomass (as chlorophyll a), potentially harmful species, nutrient levels, dissolved oxygen, salinity and temperature were studied. A marginal good to moderate physicochemical status was assigned to downstream of River Spercheios that flows into Maliakos Gulf. Silicates and nitrates were indicated as proxies of freshwater influence in Maliakos Gulf, whereas ammonium, nitrites and phosphates as proxies of pollutants from non-point sources. Phytoplankton biomass and abundances reached high levels throughout Maliakos Gulf inter-seasonally. High silicates favoured the dominance of Diatoms. The potentially harmful species bloomed frequently, with higher levels in the estuary, and they were associated with low salinity, thus demonstrating the riverine influence on them. *Pseudo-nitzschia* was the most frequent potentially harmful genus with an interesting strong linkage with low silicates and nitrates. Maliakos Gulf demonstrated an overall mesotrophic condition and failed to achieve good ecological status.

Keywords: Marine Strategy Framework Directive (MSFD); Water Framework Directive (WFD); environmental status assessment; phytoplankton parameters; Harmful Algal Blooms (HABs); nutrients; river discharges; Eutrophication Index (EI); Maliakos Gulf; Spercheios river, Mediterranean Sea.

Introduction

The presence of potentially harmful phytoplankton and algal blooms has been increasing in frequency, expansion and duration in oceanic and coastal waters over the last decades (Anderson *et al.* 2002; Howarth, 2008). Eutrophication is considered one of the main reasons triggering Harmful Algal Blooms (HABs) worldwide since the 1980s (Anderson *et al.*, 2002; Granéli, 2004; Glibert *et al.*, 2005). Coastal eutrophication is globally attributed to human-induced nitrogen pollution and less to phosphorus inputs. Surface and underground freshwater runoffs are the main way of N and P transfer from agricultural fertilizers, animal breeding sewage and domestic waste to estuaries and coasts. N and P human enrichment in coastal waters is well-known to alter the Redfield ratio (Si:N:P = 16:16:1, Redfield *et al.*, 1962), with subsequent changes in the composition, abundance and succession of phytoplankton communities, e.g. the dominance of dinoflagellates over diatoms (Smayda, 1990; Moncheva *et*

al., 2001; Pagou, 2005), or the dominance of small-sized flagellates (Smayda and Reynolds, 2001), or the proliferation of harmful microalgal species v.s. non-harmful ones (Anderson *et al.*, 2002).

The Marine Strategy Framework Directive requires that European Union Member States achieve Good Environmental Status (GES) in marine waters by 2020 (MSFD, 2008/56/EC). Following an ecosystem-based approach, MSFD refers to the adverse effects of eutrophication in marine waters, involving human drivers, pressures and their resulting alterations. Eutrophication assessment requires combining information on phytoplankton composition and biomass, HABs and nutrient levels, as ecologically relevant primary and secondary effects. Phytoplankton is also one of the Biological Quality Elements (BQE) of the Water Framework Directive for the coastal waters quality assessment (WFD, 2000/60/EC). Statistical evaluation, based on chlorophyll a concentrations, has determined the reference and critical values for the five classes of ecological quality (High,

Good, Moderate, Poor, Bad) for European coastal waters according to WFD, and has been widely used as an indicator ever since (Caroppo *et al.*, 2013). Furthermore, the composition of phytoplankton communities, the dominance of taxonomic groups (e.g. diatoms v.s. dinoflagellates or microflagellates) and the frequency of HABs are also considered for overall assessment of coastal eutrophication status (EC 2005).

Despite the well-studied oligotrophic character of the eastern Mediterranean (Gotsis-Skretas *et al.*, 1999; Ignatiades *et al.*, 2002; 2009; Siokou-Frangou *et al.*, 2002), there are coastal areas in Greece characterized by high pollution levels, restricted water mass circulation and riverine inflows, in which HABs frequently occur. Pavlidou *et al.* (2015) suggested that agriculture and mariculture activities are most related to eutrophication in many Greek coastal areas. In Thermaikos Gulf, Northern Greece, toxic microalgal species of the "*Dinophysis acuminata* complex" have been mostly responsible for intoxications of *Mytilus galloprovincialis* and long harvest closures of mussel farms over the last fifteen years (Koukaras & Nikolaidis, 2004; Pagou, 2005; Papaefthimiou *et al.,* 2010; Varkitzi *et al.,* 2013). Maliakos Gulf is another coastal area in central Greece that is affected by riverine discharges, high pollution levels and eutrophication (Kormas *et al.*, 2001; 2002; Akoumianaki & Nikolaidou, 2007; Akoumianaki *et al.*, 2013). Human pressures close to the coast involve intensive agriculture, partially treated domestic and industrial sewage, fish and mussel farming (*Mytilus galloprovincialis*) and fishing (Dimitriou *et al.*,

2015). In 2009, a bloom of the toxic phytoplankton species *Chattonella* sp. caused massive fish-killings in the area with serious economic losses (HCMR, 2009; Pagou *et al.,* 2010; Yiagnisis *et al.,* 2010).

This study focuses on the impact of river discharges (as pressure) on the distribution of coastal phytoplankton communities, with an emphasis on potentially harmful species (as impact). These assessment metrics were tested in a complex coastal environment with fluctuations due to riverine inflows, but still within the oligotrophic environment of E. Mediterranean. To our knowledge, studies on the direct riverine impact on potentially harmful phytoplankton species in the oligotrophic E. Mediterranean are rather scarce within the framework of the WFD and MSFD quality assessment process. The spatio-temporal distribution of phytoplankton parameters (biomass, community composition and potentially harmful species) were studied in relation to river flow, nutrient loads and other environmental parameters in a network of stations in Spercheios river and the adjacent Maliakos Gulf, central Greece.

Materials and Methods

Study area

A network of stations was sampled downstream and in the estuary of River Spercheios and in the adjacent coastal area of Maliakos Gulf from April 2014 to September 2015, monthly or bimonthly (Fig. 1). Coordinates
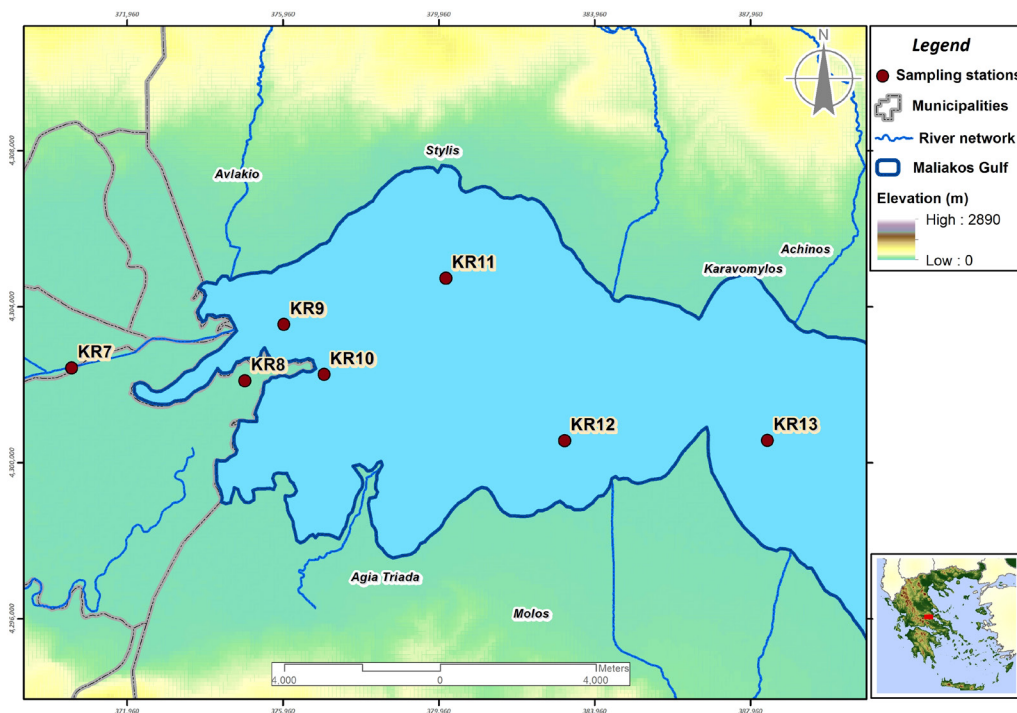

Fig. 1: Network of sampling stations in the study area: KR7 and KR8 were located downstream of Spercheios River, KR9 and KR10 close to the river estuary, KR11 and KR12 in the inner part and KR13 in the outer part of the Gulf.

327

Medit. Mar. Sci., 19/2, 2018, 326-343

and depths of the river and coastal sampling stations are presented in Table 1. Downstream station KR7 was located in an anti-flood and irrigation canal, which also receives treated urban sewage. The second downstream station (KR8) was located in the natural river mouth. Two sampling stations (KR9 and KR10) were located close to the estuary, two in the inner part of the Gulf (KR11 and KR12) and one in the outer Gulf (KR13). Nutrient loads from the river were expected to enrich mostly the inner part of the Gulf and for this reason most coastal sampling stations were located there. Stations KR8 (in the river estuary) and KR9a (a mussel-farm close to KR9; 4303785.7143W, 377539.9390N) were sampled for phytoplankton analysis only once after a request we received to check the increasing colouring of the seawaters there in April 2014.

Some characteristic features of River Spercheios and Maliakos Gulf are presented in Table 2. Spercheios River has the 6th in size Delta in Greece and its area of river-mouth-area is included in the Natura 2000 network. Maliakos is a shallow semi enclosed Gulf that covers an area of ca 200 km². It is separated into an inner-west part (maximum depth 25 m) and an outer-east part (maximum depth 50 m) by two headlands. The estuary of River Spercheios is located in western Maliakos Gulf. Through the north-eastern part, an anti-clockwise current brings Aegean Sea waters into the gulf and constantly mixes the water column (Christou et al., 1995). For example, during winter, mean temperature and salinity values were 11.5°C and 35 psu at the surface, and 12°C and 37.5 psu at 20m depth (Kontoyannis et al., 2005). There are two different river outflows in Maliakos Gulf, the natural river mouth and an anti-flood canal (see Table 1).

Table 1. Coordinates and depths of river (KR7-KR8) and coastal (KR9-KR13) sampling stations.

Sampling station	Latitude	Longitude	Depth	Location
KR 7	4302437.947W	370537.502N	0 m	Spillway used as anti-flood and irrigation canal, also receives partially treated domestic and industrial sewage
KR 8	4302105.852W	374977.1551N	0 m	Spercheios river mouth
KR 9	4303547.9633W	375979.5485N	2 m	Estuary (close to spillway)
KR 10	4302266.4525W	377011.3436N	2 m	Estuary (close to natural river mouth)
KR 11	4304733.8372W	380139.9624N	8 m	WFD sampling network
KR 12	4300568.0239W	383187.8807N	18 m	WFD sampling network
KR 13	4300577.3450W	388391.9204N	18 m	Close to fish farm

Table 2. Main characteristics of River Spercheios and the adjacent Gulf of Maliakos, central Greece, eastern Mediterranean (Akoumianaki et al., 2013; Efthimiou et al., 2014 and references therein).

Spercheios River	Maliakos Gulf
Location: Central Greece	Location Central: Greece (38450 N; 22310 E)
Length: 85 km, tenth in Greece for its annual flow and seventh for annual sediment discharge	Area: ca 200 km²
Catchment area: 1,907.2 km²	East-west direction, separated by two headlands into inner-west and outer-east part
Average slope of catchment area:33%	Maximum depth in the inner part: 25 m Maximum depth in the outer part: 50 m
Mean annual water discharge: 62 m³ /s	Receives Spercheios river discharges
Min-Max water discharge: 22 m³ /s (in August) – 110 m³ /s (in January)	Average seawater salinity in inner Maliakos: close to 38, as in the open sea
Deltaic area: 196 km² (6th in size in Greece), Natura 2000 network	Wind direction (from the west and the northwest), anti-clockwise water mass circulation, tidal movements (tidal range: 1 m)
Drainage basin and delta uses/pressures: intensive agriculture, irrigation, untreated industrial sewage and waste disposal, fishing	Uses/pressures in the coastal front: intensive agriculture, partially treated industrial and domestic sewage discharges, mussel and fish farming, fishing

Medit. Mar. Sci., 19/2, 2018, 326-343

328

Water sampling and in situ measurements

At the river stations, an Aquameter™-Aquaread portable instrument was used to measure dissolved oxygen concentrations in situ. At the coastal stations, temperature, salinity and dissolved oxygen were measured in situ with an SBE 19plus V2 SeaCAT profiler CTD. Seawater samples were collected from the surface and near-bottom layers with 20 L Niskin bottles. All the analyses were performed at HCMR laboratories.

Nutrients and chlorophyll-a analyses in water samples

River water samples were collected in polyethylene bottles, which had previously been cleaned with HCl 1%. $HgCl_2$ solution was added to each bottle as a preservative (1 ml $HgCl_2$ per Litre of sample). Samples were kept cool and transferred to the laboratory. Concentrations of inorganic nutrients (NO_3^-, NO_2^-, NH_4^+, PO_4^{3-} and SiO_4^{4-}) were determined in the soluble fraction using a Metrohm ion analyzer, a Radiometer automatic analyser and a Merck Nova 400 photometer.

Nutrient analyses for seawater samples were performed at the ISO 17025 (366-2) certified biogeochemical laboratories of HCMR, using standard methods. Ammonium and phosphates were measured with a UV–VIS Perkin Elmer 20 Lambda spectrophotometer (Koroleff, 1970). Nitrate, nitrite and silicate concentrations were measured with a SEAL nutrient autoanalyzer III (Mullin and Riley, 1955; Murphy and Riley, 1962; Strickland and Parsons, 1977). Sea water samples for chlorophyll a (chl-a) analysis were collected with NIO samplers. Usually 1 L of seawater was filtered through Whatman Glass Fibre Filters (GF/F) immediately after collection and the filters were stored in a dry and dark environment at −25°C untill analysis. Chl-a concentrations were used as a proxy for phytoplankton biomass and they were determined with a TURNER 00-AU- 10U fluorometer, according to the method of Holm-Hansen *et al.* (1965), modified by Welschmeyer (1994).

Phytoplankton parameters and potentially harmful species in the coastal environment

Samples for the study of phytoplankton populations were collected simultaneously with water samples for chl-a determination and from the same depths. Seawater samples (120-150 mL) were fixed with Lugol's iodine solution and stored in the dark at 4°C. The qualitative and quantitative analysis of phytoplankton community structure was performed with the sedimentation method of Utermöhl (1958), using an OLYMPUS IX70 inverted fluorescence microscope. According to the quantitative results of the phytoplankton composition analysis, we determined the species number and abundance (cells L^{-1}). We also determined the species number and the abundance of the phytoplankton taxonomic groups.

Statistical analyses

In order to test the response of phytoplankton and potentially harmful species to anthropogenic pressures we performed multiparametric statistical routines (Legendre & Legendre, 1998). Data on chl-a concentration, phytoplankton abundance and potentially harmful species occurrence were analyzed in relation to environmental variables, such as temperature, salinity and nutrients, and an anthropogenic pressure index (Eutrophication Index EI, developed by Primpas *et al.*, 2010). In order to simplify the interpretation of results, depth integrated mean values were used. Since biotic data (chl-a concentration, total phytoplankton abundance, Eutrophication index and potentially harmful species abundance) didn't follow a normal distribution, the Box-Cox transformation coefficient was applied (Box & Cox, 1964). Shapiro-Wilk, Anderson-Darling, Lilliefors and Jarque-Bera tests were used for testing data normality. Moreover, abiotic data (Temperature, Salinity, NO_3^-, NO_2^-, NH_4^+, PO_4^{3-} and SiO_4^{4-}) were standardized: [(data value-mean)/standard deviation] in order to be dimensionally homogeneous and allow comparison of regression coefficients, which may have different scales otherwise.

Pearson correlations were implemented among biotic and abiotic variables. One-way ANOVA and t-test were used to determine differences among stations (coastal versus estuary) in relation to the biotic variables, along with various abiotic variables. The least significant difference (LSD) Post hoc test was implemented in order to highlight the origin of observed differences. Redundancy Analysis (RDA) was performed in order to visualize the relationship between the potentially harmful species, biotic variables and abiotic variables (Ter Braak, 1986). The abundances of the most frequent species (*Pseudo-nitzschia galaxiae, P. multiseries, P. pseudodelicatissima* and *P. delicatissima*) were pooled to genus level in order to reduce the number of zeros, and were square root transformed.

Water quality classification schemes

The assessment of the environmental status of the river and marine waters followed the five-step classification scheme according to the WFD guidelines. Physicochemical assessment of the river was based on the five-step scale of Skoulikidis *et al.* (2006) for nitrates, nitrites, ammonium and phosphates, and Cadorso *et al.* (2001) for dissolved oxygen in the freshwater samples. The assessment of the trophic status and the ecological quality of coastal waters was based on the five-step quality scale, extrapolated from the computation of the Eutrophication Index (EI) of Primpas *et al.* (2010), which involves nitrates, nitrites, ammonia, phosphates and chl-a concentrations.

329

Medit. Mar. Sci., 19/2, 2018, 326-343

Results

Responses of phytoplankton parameters to environmental pressures, with an emphasis on potentially harmful species

The distribution of inorganic nutrients and dissolved oxygen at downstream and coastal stations is presented in Figure 2, in order to illustrate the environmental pressures in the coastal environment from the riverine inflows. The anti-flood canal (KR7), which receives the effluents from the wastewater treatment plant of Lamia city (population approximately 76.000) also, had higher nitrate and ammonium concentrations compared to the natural river branch (KR8), and this pattern continued in the near estuary sites. The distribution of silicates, nitrates and phosphates with decreasing values from the coastal sites near the estuary (KR9, KR10) to the middle inner and the outer gulf was most indicative of the freshwater inflows. Dissolved oxygen was higher at KR7, due to the higher freshwater influence, but it was relatively constant at the coastal stations (KR9-KR13).

The range of values (minimum-maximum values) for a set of phytoplankton parameters in the coastal waters of Maliakos Gulf are presented in Table 3. In most cases, maximal values were observed near the estuary (KR9) in spring. Diatoms and Dinoflagellates were the most abundant groups. Silicoflagellates, Coccolithophores and Raphidophytes ranged at low levels, with the exception of a blooming event with *Dictyocha fibula* (see comments below). The maximum value of potentially harmful phy-

toplankton species abundance was recorded in the surface waters of KR12 (WFD sampling network) in April 2014.

The spatial distribution of several phytoplankton parameters in Maliakos Gulf during 2014-2015 is presented in Figure 3. The interannual average per sampling station demonstrated relatively high chl-a concentrations close to the river outflows and moderate values in the outer Gulf. Interannual average phytoplankton abundance exceeded 130 10^3 cells L^{-1} close to the anti-flood canal (KR9) in 2014, while it was approximately half that in the rest of the study area (Fig. 3B). The number of phytoplankton species increased constantly as we moved away from the estuary (Fig. 3C). The potentially harmful phytoplankton species spread throughout the area with higher values (5000-7000 cells L^{-1}) in the outer Gulf (Fig. 3D).

Figure 4 presents the actual abundances of potentially harmful phytoplankton species per depth, station and sampling cruise (values >5 10^3 cells L^{-1}). It shows that they were blooming in the whole sampling area during 2014-2015. Stations KR8 (in the river estuary) and KR9a (a mussel-farm near the estuary and KR9) were sampled for phytoplankton analysis only after a notice we received about increasing colouring of the seawaters there (see Materials and Methods). Most frequent potentially toxic Diatoms above the alert levels were species of the genus *Pseudo-nitzschia* (alert levels at 10^3-10^5 cells L^{-1}, Todd 2003). *Alexandrium tamarense*, *A. minutum* and *Dinophysis caudata* were the most frequent potentially toxic Dinoflagellates. Alert levels for toxic *Alexandrium* species range from detection to 10^3 cells L^{-1} and *Dinophysis* at 10^2-10^3 cells L^{-1} (Todd, 2003). For the potentially
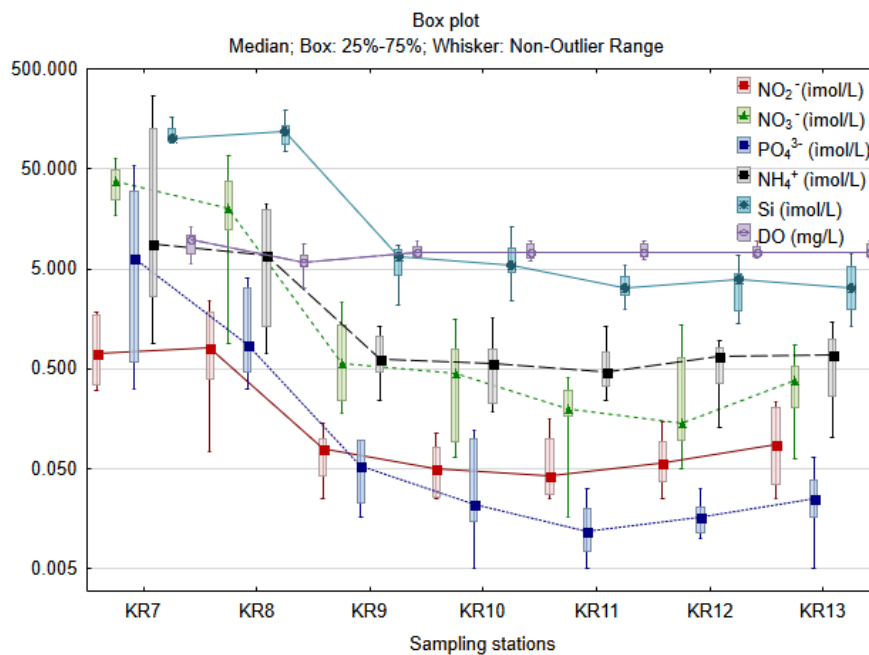

Fig. 2: Box plot of nutrient and dissolved oxygen distribution in downstream Spercheios River (stations KR7, KR8) and Maliakos Gulf (stations KR9-KR13) during 2014-2015. The large boxes represent 25%-75% percentiles, the small boxes mark the median value and the Whiskers show the non-outlier range of each variable.

Medit. Mar. Sci., 19/2, 2018, 326-343

330

Table 3. Range of values and their 95th percentile for the phytoplankton parameters in Maliakos Gulf during the period 2014-2015.

|	minimum		maximum		95%
		2014-2015			
Chlorophyll a concentration (μg L^{-1})	0.056	KR13, surface waters, 5/2014	4.983	KR9, surface waters, 9/2014	1.923
Phytoplankton abundance (cells L^{-1})	4.48 10^3	KR11, surface waters, 1/2015	1.07 10^6	KR9, surface waters, 4/2014	2.31 10^5
Number of phytoplankton species	5	KR9, bottom waters, 8/2014	49	KR12, mid-column waters, 3/2014	40
Diatoms abundance (cells L^{-1})	2.96 10^3	KR10, bottom waters, 7/2015	1.04 10^6	KR9, surface waters, 4/2014	2.30 10^5
Dinoflagellates abundance (cells L^{-1})	0	KR11, bottom waters, 9/2015	3.79 10^4	KR9a, surface waters, 4/2014	1.48 10^4
Silicoflagellates abundance (cells L^{-1})	0	In all stations	18.4 10^3	KR9, surface waters, 7/2015	1472
Coccolithophores (cells L^{-1})	0	In all stations	690	KR13, surface waters, 5/2015	80
Abundance of potentially harmful phytoplankton (cells L^{-1})	1.6 10^2	KR12, bottom waters, 7/2015	3.58 10^4	KR12, surface waters, 4/2104	1.60 10^4
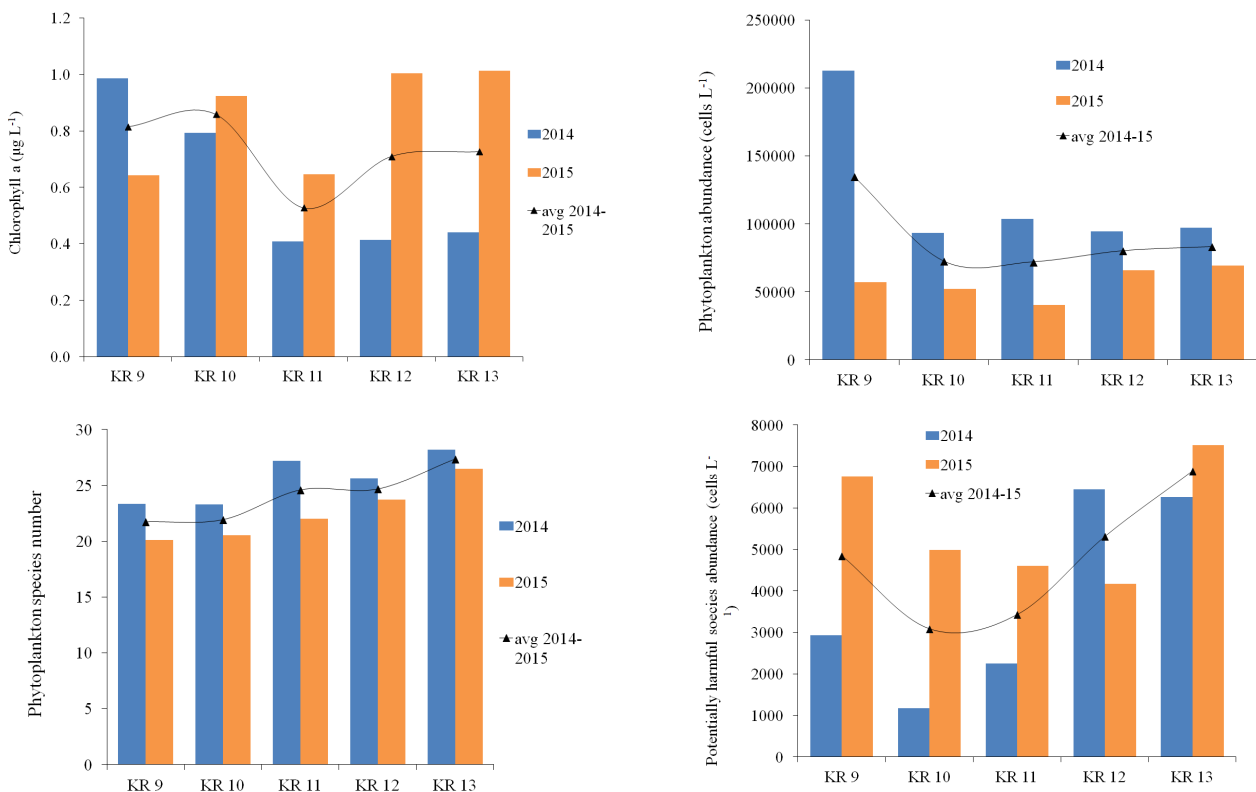
Fig. 3: Spatial distribution of chlorophyll a concentrations (0.4 μg Chl-a L^{-1} is the Good/Moderate ecological status threshold by Simboura *et al*. 2005) (a); phytoplankton abundance (b); phytoplankton species numbers (c); and potentially harmful microalgae abundance (d) in Maliakos Gulf during 2014-2015. The annual average values for 2014 and 2015 respectively per sampling station are presented in bars, while the inter-annual average values for the whole sampling period 2014-2015 per station are presented in a solid line.
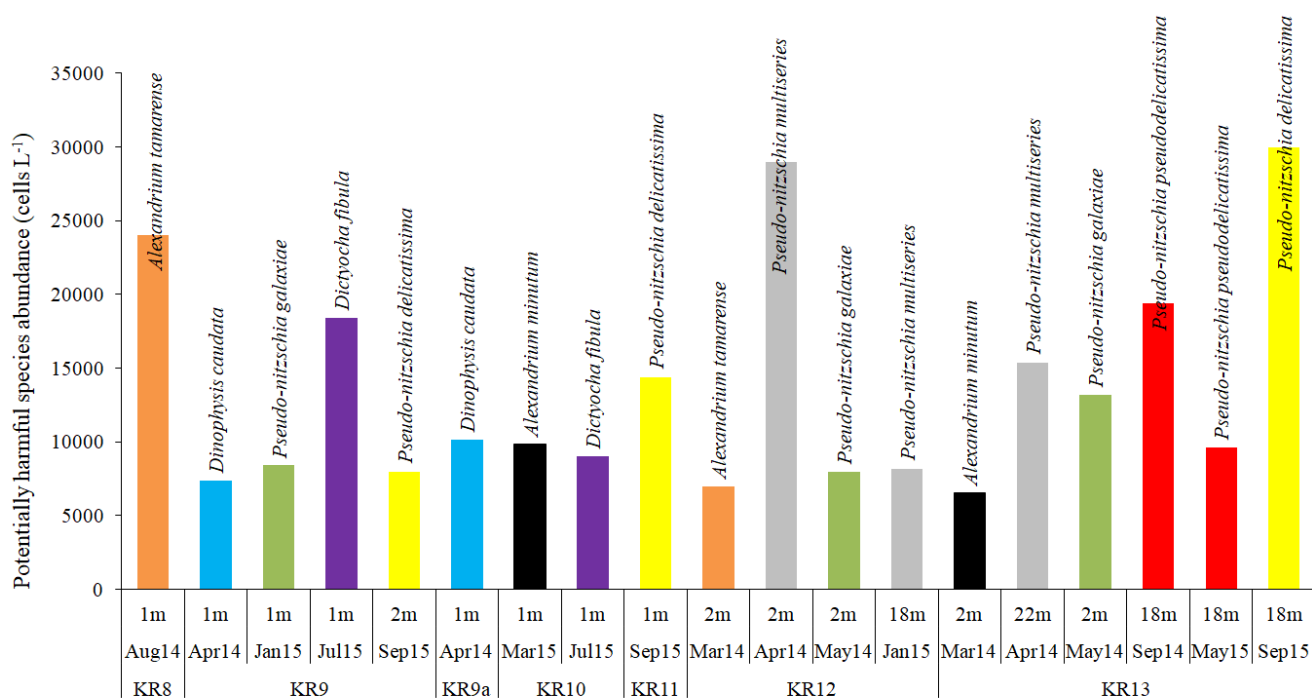
331

Medit. Mar. Sci., 19/2, 2018, 326-343

Fig. 4: Blooming potentially harmful/toxic phytoplankton species in Maliakos Gulf during 2014-2015. Cell numbers greater than 5 10³ cells L⁻¹ are presented. The x'x axis presents the sampling stations, depths and months.

harmful Silicoflagellate *Dictyocha fibula* that was found, there are no alert levels in the literature so far, but for *Dictyocha speculum* they reach 10^4-10^6 cells L⁻¹ (Prego *et al.,* 1998).

Among the potentially harmful species presenting high abundances quite frequently but below the alert levels (Table 4), we found *Chaetoceros* spp. (*C. socialis* with bloom levels 10^6-10^7 cells L⁻¹, according to Booth 2002), *Skeletonema costatum* (with bloom levels 10^6-10^7 cells L⁻¹, Shikata, 2008), *Thalassiosira rotula* (with bloom levels 10^5-10^7 cells L⁻¹), and *Prorocentrum micans* (with bloom levels 10^6-10^7 cells L⁻¹, Heil *et al.,* 2005). We also found the potentially toxic Dinoflagellates *Dinophysis rotundata, D. acuminata* and *Prorocentrum lima* at low levels (≤600 cells L⁻¹). The potentially toxic Dinoflagellate *Vulcanodinium rugosum* was also found throughout the sampling area from May to Sep 2015. However, it was not included in the statistical analyses of the potentially harmful species because it has not been linked with any intoxication incident so far. The potentially ichthyotoxic raphidophyte *Chattonella* sp. was found at low levels (≤400 cells L⁻¹).

The contribution of Diatom species was higher than 90% of the total abundance of phytoplankton in the whole study area. Dinoflagellates were the second most abundant group followed by Silicoflagellates, Coccolithophores and Raphidophytes (Fig. 5A). Potentially toxic Diatoms comprised the most abundant group among the potentially harmful species overall and their % contribution increased towards the outer Gulf (Fig. 5A, B). However, the potentially toxic Dinoflagellates reached 47.1%

of the total abundance of potentially harmful species in 2014 near the estuary (KR9 and KR10, Fig. 6A). Similarly, the potentially harmful Silicoflagellate *Dictyocha fibula* peaked at 40% in 2015 in the estuary (Fir. 6B). As a general spatial pattern, potentially harmful Dinoflagellates and Silicoflagellates seemed to prefer the coastal waters affected directly by river discharges, while potentially toxic Diatoms were distributed mostly towards the outer Gulf (Fig. 4 and 6).

The temporal variation of phytoplankton parameters is presented in Figures 7 and 8. Chl-a concentrations and phytoplankton abundances presented two maxima in spring and autumn (Fig. 7A). Diatoms thrived over the rest of the phytoplankton functional groups throughout 2014-2015, ranging from 9.8 10³ in August to 380.8 10³ cells L⁻¹ in April (Fig. 7B, C), reaching 99.4% contribution to the whole phytoplankton community and following closely the total phytoplankton temporal distribution. Small-sized Diatoms dominated in spring (*Chaetoceros socialis* and *Pseudo-nitzschia* spp.), while large-sized Diatoms dominated in autumn (*C. affinis, C. costatus* and *C. Compressus*). Dinoflagellates were constantly the second most abundant group and they peaked in April (Fig. 7B, C), mostly due to the contribution of the potentially toxic species *Dinophysis caudata, Prorocentrum micans* and *Alexandrium minutum*. The rest of the phytoplankton groups were poorly represented, except for the Silicoflagellates in July 2015 (25.8%).

The potentially harmful phytoplankton followed the temporal distribution of total phytoplankton closely (Fig. 8A) with max values in April and September.

Medit. Mar. Sci., 19/2, 2018, 326-343

332

Table 4. Potentially toxic microalgae species and high biomass producers with their maximum abundances in Maliakos Gulf.

Potentially harmful microalgae species in Maliakos Gulf	Maximum abundances (cells L^{-1})	Month/ Depth/Station
Potentially toxic species		
Diatoms		
Pseudo-nitzschia multiseries (Hasle) Hasle	29 10^3	Apr/2m/KR12
P. delicatissima (Cleve) Heiden	30 10^3	Sep/18m/KR13
P. galaxiae Lundholm & Moestrup	13.2 10^3	May/2m/KR13
P. pseudodelicatissima (Hasle) Hasle	19.4 10^3	Sep/18m/KR13
Dinoflagellates		
Alexandrium minutum Halim	9.8 10^3	Mar/1m/KR10
Alexandrium tamarense (Lebour) Balech	24 10^3	Aug/1m/KR8
Protoperidinium steinii (Jørgensen) Balech	1.4 10^3	Apr/2m/KR12
Dinophysis caudata Saville-Kent	10.2 10^3	Apr/1m/KR9a
Dinophysis rotundata Claparède & Lachmann	320	Mar/10m/KR10
D. acuminata Claparède & Lachmann	160	Mar/10m/KR10
Prorocentrum lima (Ehrenberg) F. Stein	600	May/8m/KR11
Vulcanodinium rugosum Nézan & Chomérat	1520	May/1m/KR9
Silicoflagellates		
Dictyocha fibula Ehrenberg	18.4 10^3	Jul/1m/KR9
Raphidophytes		
Chattonella sp. Biecheler	400	Mar/1m/KR9
High biomass producers		
Diatoms		
Chaetoceros socialis Lauder	7.86 10^5	Apr/1m/KR9
Chaetoceros affinis Lauder	40.4 10^3	Apr/2m/KR11
Chaetoceros costatus Pavillard	63 10^3	Nov/2m/KR11
Chaetoceros compressus Lauder	45 10^3	Nov/11m/KR11
Skeletonema costatum (Greville) Cleve	36 10^3	Apr/18m/KR12
Thalassiosira rotula Meunier	36 10^3	Mar/10m/KR13
Dinoflagellates		
Prorocentrum micans Ehrenberg	20.9 10^3	Apr/2m/KR13

The contribution (%) of potentially harmful microalgae to the whole phytoplankton community increased in May and July (24% and 28%), and was due primarily to *P. galaxiae* and *D. Fibula*, respectively, in combination with the low levels of total phytoplankton abundance then (Fig. 8A, B). Potentially toxic Diatoms were the dominant HAB group that followed closely the overall HAB species distribution during 2014-2015 (67% on average) (Fig. 8C, D). Potentially toxic Dinoflagellates followed with high contributions in March (50-56%) due mostly to *Alexandrium minutum*, while potentially harmful Silicofalgellates were low in general but peaked significantly in July-August with *D. fibula* (maximum 91%).

Multiparametric analyses of pressures and responses in the coastal environment

A Pearson correlation matrix (Table 5) demonstrated a positive correlation of Chl-a with phosphates, silicates and EI (p-value<0.05). Total phytoplankton was positively correlated with potentially harmful species and negatively correlated with Si and salinity. Potentially harmful species were also negatively correlated with salinity. Nitrates were found to be positively correlated with ammonium, silicates and dissolved oxygen, whereas they were negatively correlated with temperature and salinity. Phosphates and ammonium were both positively correlated with silicates. Temperature and salinity were found to
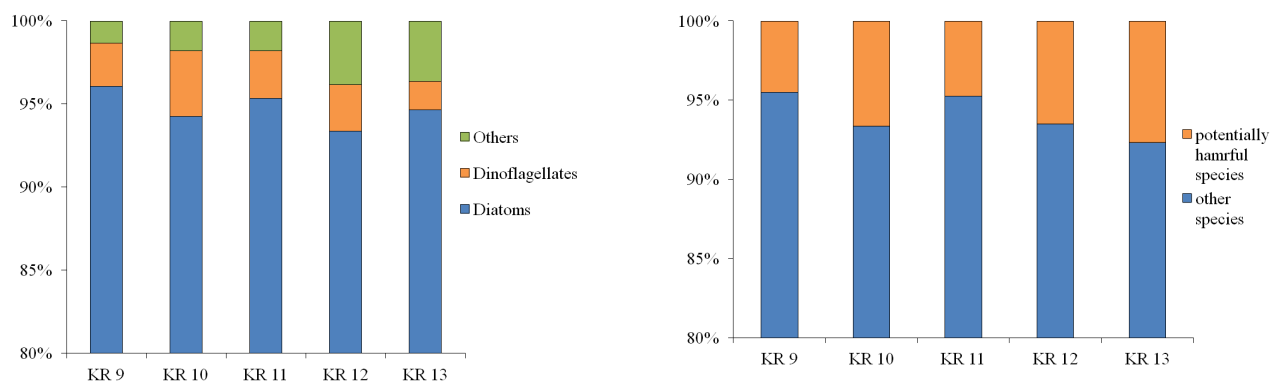
333

Medit. Mar. Sci., 19/2, 2018, 326-343

Fig. 5: Contribution (%) of taxonomic groups to total phytoplankton abundance (a) and potentially harmful species (b) in Maliakos Gulf. Average values per station for the whole sampling period 2014-2015 are presented.
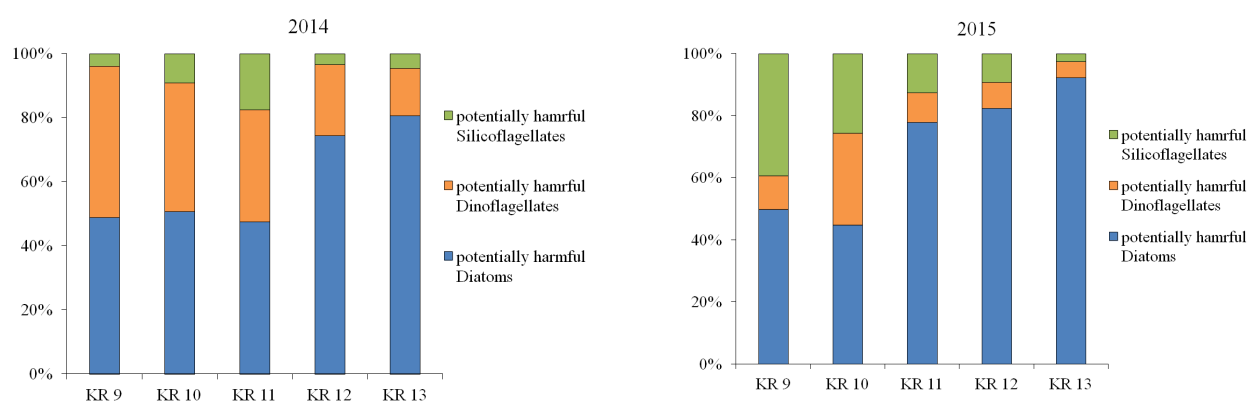

Fig. 6: Contribution (%) of potentially harmful Diatoms, Dinoflagellates and Silicoflagellates to total abundance of potentially harmful species in Maliakos Gulf in 2014 (a) and 2015 (b). Annual average values per station are presented.

Table 5. Pearson's Correlation matrix. Statistically significant correlations are presented in bold (p-value<0.05). Values in bold are different from 0 with a significance level alpha=0,05.

|	Chla	EI	TOTAL Phytoplankton abundance	Potentially HAB species	NO2	NO3	PO4	NH4	Si	Temperature	Salinity	DO
Chla	**1**	**0.627**	0.066	0.027	-0.056	0.151	**0.338**	0.058	**0.262**	-0.054	0.163	-0.149
EI	**0.627**	**1**	0.087	0.082	0.175	**0.699**	**0.361**	**0.641**	**0.493**	-0.234	-0.059	0.089
TOTAL Phytoplankton abundance	0.066	0.087	**1**	**0.450**	-0.114	0.162	-0.241	-0.075	**-0.267**	-0.184	**-0.521**	0.235
Potentially HAB species	0.027	0.082	**0.450**	**1**	-0.150	0.122	-0.234	0.019	-0.149	-0.085	**-0.321**	0.165
NO2	-0.056	0.175	-0.114	-0.150	**1**	0.126	0.037	-0.018	0.173	-0.193	0.071	0.172
NO3	0.151	**0.699**	0.162	0.122	0.126	**1**	0.056	**0.499**	**0.314**	**-0.429**	**-0.284**	**0.388**
PO4	**0.338**	**0.361**	-0.241	-0.234	0.037	0.056	**1**	0.090	**0.693**	0.197	0.055	-0.207
NH4	0.058	**0.641**	-0.075	0.019	-0.018	**0.499**	0.090	**1**	**0.356**	-0.039	0.034	-0.069
Si	**0.262**	**0.493**	**-0.267**	-0.149	0.173	**0.314**	**0.693**	**0.356**	**1**	0.169	0.020	-0.147
Temperature	-0.054	-0.234	-0.184	-0.085	-0.193	**-0.429**	0.197	-0.039	0.169	**1**	**0.319**	**-0.904**
Salinity	0.163	-0.059	**-0.521**	**-0.321**	0.071	**-0.284**	0.055	0.034	0.020	**0.319**	**1**	**-0.525**
DO	-0.149	0.089	0.235	0.165	0.172	**0.388**	-0.207	-0.069	-0.147	**-0.904**	**-0.525**	**1**
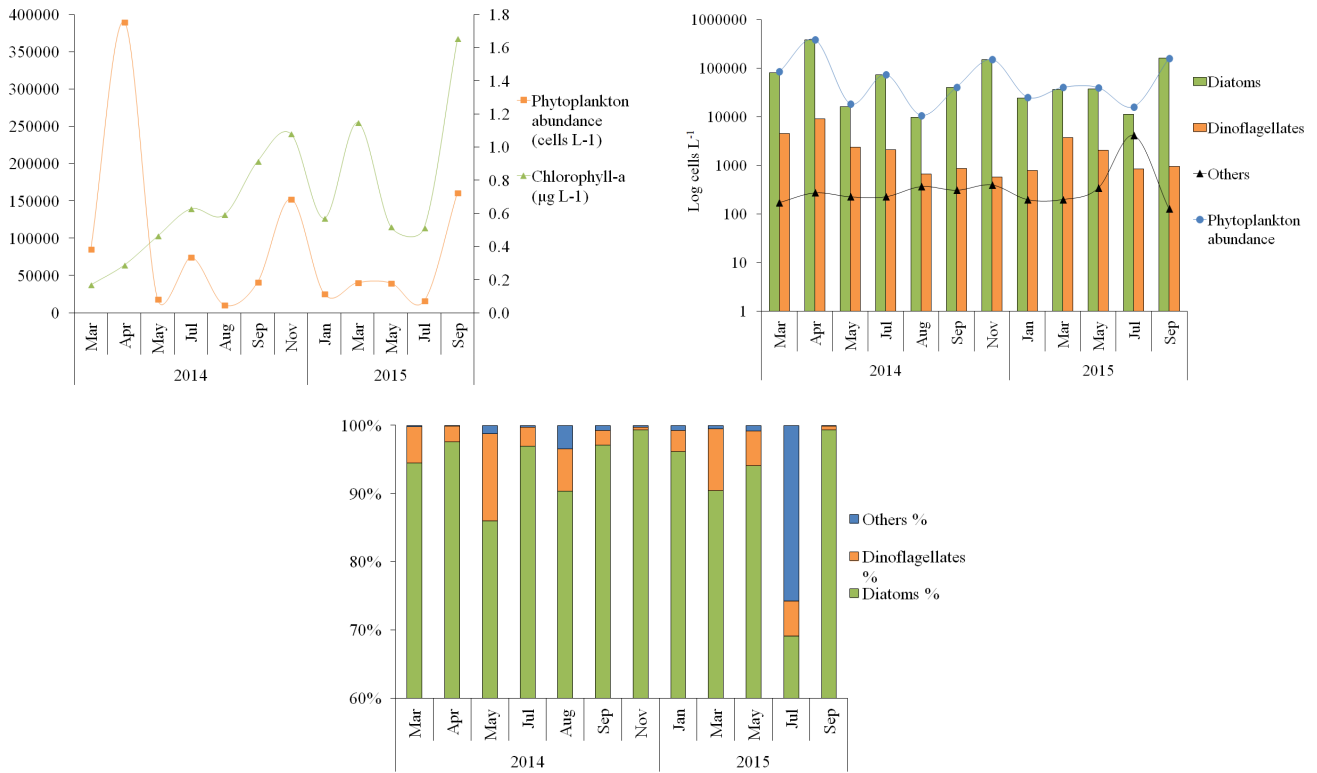
Fig. 7: Temporal variation of phytoplankton abundance on the primary y'y axis and chlorophyll a concentration on the secondary y'y axis (a), taxonomic groups abundance (b) and their % contribution (c) in Maliakos Gulf during 2014-2015. Average values for the whole study area per sampling effort are presented here.
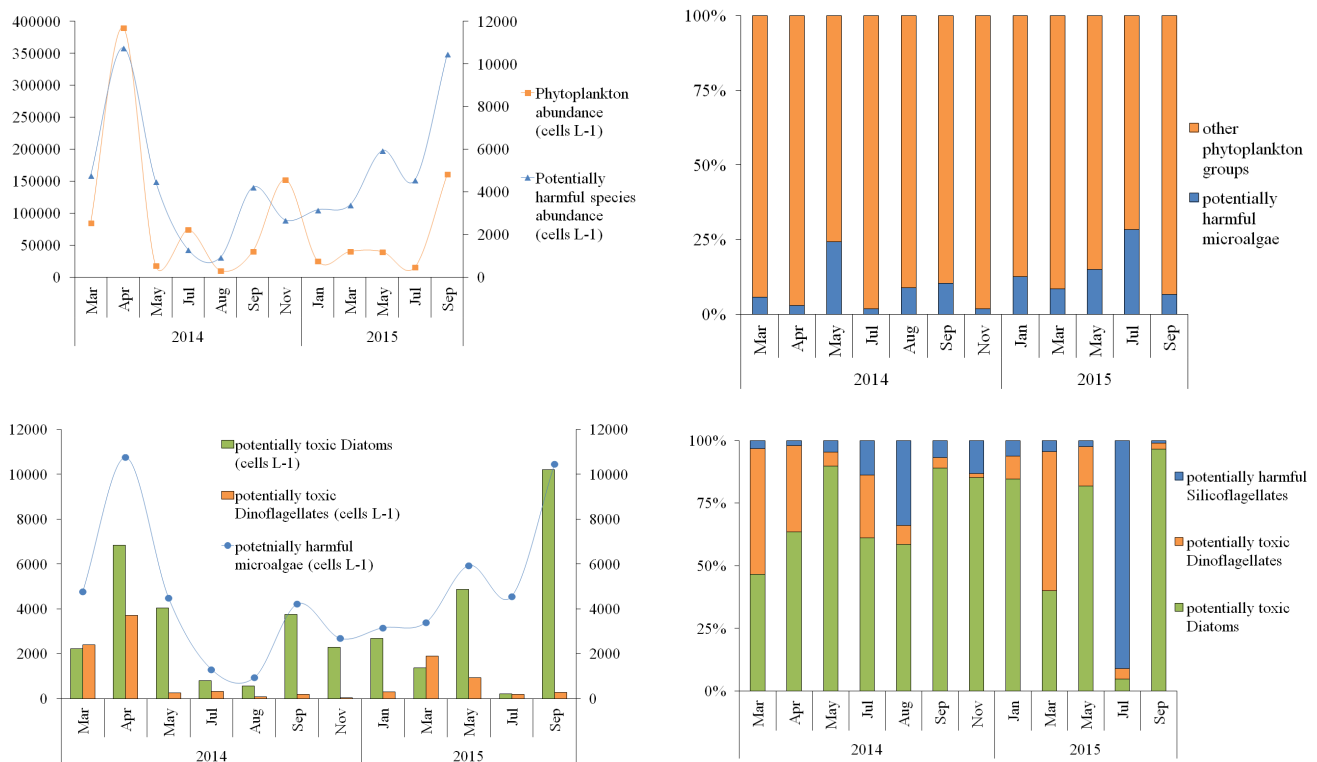

Fig. 8: Temporal variation of potentially harmful microalgae abundance on the secondary y'y axis (a) and % contribution to total phytoplankton communities (b), functional HAB groups abundance (c) and % contribution to HAB species in total (d) in Maliakos Gulf during 2014-2015. Average values for the whole study area per sampling effort are presented here.

335

Medit. Mar. Sci., 19/2, 2018, 326-343

be positively correlated with each other and negatively correlated with DO. Finally, EI was positively correlated with chl-a, nitrates, phosphates, ammonium and silicates.

According to the t-test results of the comparison between the two groups of coastal and estuary stations, higher values of chl-a, EI, nitrates, phosphates and silicates (p-value<0.05) were observed at the estuary stations than at the coastal stations (Table 6). For total phytoplankton abundance, potentially harmful species abundance, nitrites, ammonium, DO, salinity and temperature, no statistically significant differences were found between coastal and estuary stations. According to the t-test results of the comparison between the two groups of high and low river flow period, all N nutrients, DO and EI were significantly higher during the high flow period, whereas salinity and temperature were significantly higher during the low flow period.

Since *Pseudo-nitzschia* was the most frequent and abundant genus among the potentially harmful genera, a deeper insight was attempted by applying the Redundancy Analysis (RDA) method. RDA was applied in order to determine which environmental factors (as explanatory variables) were the most significant to explain the variation (*Pseudo-nitzschia* genus distribution, chl-a, total phytoplankton abundance and EI (as response variables). Figure 9 illustrates that both the first and second axes (F1 and F2) represented 99.98% of the total variation of the multivariate system. The first axis is the component that accounts for nearly the totality of the system variation (99.96%). According to Table 7 (explanatory variables), the most contributing variables to the first axis were nitrates and silicates, whereas the least contributing ones were temperature and salinity. For the second axis, most contributing variables were nitrates and ammonium, while nitrites were the least contributing. The response variables in Table 7 were mainly explained by the *Pseudo-nitzschia* genus regarding the first axis, and slightly by EI regarding the second axis.

Integrated assessment of the status in downstream Spercheios river and Maliakos Gulf

Taking into consideration the average values of DO and nutrient concentrations, the overall physicochemical status of downstream stations KR7 and KR8 in Spercheios river, for the entire sampling period (2014-2015), was assessed according to Skoulikidis *et al.* (2006) and Cardoso *et al.* (2001) (Fig. 10A). The bad quality at KR7 for both ammonium and phosphates is a strong indication of the impacts of the Lamia city waste water treatment plant that outflows near this site. Moreover, the moderate quality at KR8 for ammonium and nitrites indicates potential impacts mainly from agricultural runoff and untreated domestic sewage disposal outside Lamia city, which is also confirmed by the relatively low DO levels detected. The overall physicochemical status of KR8 station is marginally characterized as good due to low phosphate concentrations.

The assessment of the trophic conditions and the ecological quality status in Maliakos Gulf was based on the Eutrophication Index (EI) of Primpas *et al.* (2010). EI decreased as we moved away from the estuary towards the outer Gulf (Fig. 10B). The ecological quality was mainly moderate; it turned poor in spring and therefore failed to achieve Good quality status (Fig. 10C). The trophic status ranged at mesotrophic levels in most of the coastal area, except for KR11 that presented an oligotrophic status (Fig. 10D). On average, for the whole study area and the entire sampling period, EI was 0.522, which indicates an overall mesotrophic status and moderate ecological quality for the whole Gulf in 2014-2015.

Discussion

Spercheios River is characterized by significant pollution pressures, including fertilizers from agricultural areas, small factories and domestic wastewater. The mark of these pollutants was detected at the river's lower part, especially at KR7 and KR8 stations, causing high nitrite, ammonium and phosphate concentrations. KR7 is located in an anti-flood canal (spillway) of Spercheios River, which also receives treated domestic and industrial sewage from the wastewater treatment plant of the city of Lamia. This anti-flood canal also transfers irrigation water for the croplands in the deltaic area during the dry period of the year. Furthermore, KR8 is located on the natural river branch a few hundred meters before the river mouth and receives pollutants originating from the river catchment area. The increase in phosphates, ammonium and nitrites in the outer Gulf might be an indication of non-point pollution sources, such as streams and nearby aquafarms. The statistical analysis further indicated silicates and nitrates as proxies of freshwater influence, whereas ammonium, nitrites and phosphates as proxies of pollutants from non-point sources.

Chlorophyll a (chl-a) concentrations and phytoplankton abundances reached high levels throughout Maliakos Gulf. Chl-a can increase significantly in the area, and in during this study we found similar values (0.06-4.98 µg L^{-1}) to those reported more than a decade ago (0.1-5.9 µg L^{-1} in Akoumianaki & Nikolaidou, 2007, 0.05-4.9 µg L^{-1} in Kormas *et al.,* 2003). These chl-a levels exceed the oligotrophic range of the eastern Mediterranean (Pagou *et al.,* 2002; Siokou-Frangou *et al.*, 2010) but they are in the range of other delta front areas in Greece (Varkitzi *et al.,* 2013) and the Mediterranean (Bernardi Aubry *et al.,* 2004). The same authors report maximal chl-a levels in Maliakos Gulf from winter till late spring, whereas we found that chl-a and phytoplankton abundances peaked during spring, as expected, but they also peaked in autumn (Sep 2015), when river inflows were low. This finding highlights the importance of nutrient inputs from domestic/industrial sewage through the spillway/anti-flood canal, streams and other non-point sources dispersed along the coastline of Maliakos Gulf.

Medit. Mar. Sci., 19/2, 2018, 326-343

336

Table 6. Differences between river discharge periods (high *vs.* low) and between station (coastal *vs.* estuary), in relation to biotic and abiotic variables (95% confidence interval). Values in bold shading correspond to statistically significant values (p-value < 0.05).

| Phytoplankton and environmental parameters		High vs. low river discharge period — Levene's Test for Equality of Variances		t-test for Equality of Means		Coastal vs. estuarine stations — Levene's Test for Equality of Variances		t-test for Equality of Means	
		F	Sig.	t	Sig. (2-tailed)	F	Sig.	t	Sig. (2-tailed)
Chl-a	Equal variances assumed	.563	.456	-1.059	.294	.020	.888	-3.590	**.001**
	Equal variances not assumed			-1.049	.299			-3.622	.001
Eutrophication Index	Equal variances assumed	.865	.356	2.454	**.017**	.019	.892	-3.842	**.000**
	Equal variances not assumed			2.529	.014			-3.831	.000
Total phytoplankton abundance	Equal variances assumed	.543	.464	1.680	.099	.769	.384	.317	.752
	Equal variances not assumed			1.665	.102			.305	.762
Potential HAB species	Equal variances assumed	2.326	.133	1.384	.172	.089	.766	.690	.493
	Equal variances not assumed			1.339	.187			.703	.485
NO2	Equal variances assumed	16.079	.000	2.949	.005	.076	.784	.180	.858
	Equal variances not assumed			3.424	**.002**			.180	.858
NO3	Equal variances assumed	27.468	.000	4.587	.000	11.431	.001	-2.868	.006
	Equal variances not assumed			5.300	**.000**			-2.513	**.018**
PO4	Equal variances assumed	6.288	.015	-1.312	.195	13.991	.000	-3.184	.002
	Equal variances not assumed			-1.144	.263			-2.548	**.018**
NH4	Equal variances assumed	6.959	.011	2.097	.040	.975	.328	-.911	.366
	Equal variances not assumed			2.262	**.028**			-.862	.394
Si	Equal variances assumed	.076	.784	.668	.507	7.543	.008	-4.747	.000
	Equal variances not assumed			.660	.512			-4.129	**.000**
Temperature	Equal variances assumed	15.091	.000	-9.214	.000	.921	.341	.391	.697
	Equal variances not assumed			-10.051	**.000**			.396	.694
Salinity	Equal variances assumed	7.303	.009	-2.511	.015	1.536	.220	.087	.931
	Equal variances not assumed			-2.880	**.006**			.098	.922
DO	Equal variances assumed	120.638	.000	9.728	.000	.043	.836	.236	.814
	Equal variances not assumed			11.173	**.000**			.236	.815

RDA Map
(axes F1 and F2: 99,98 %)
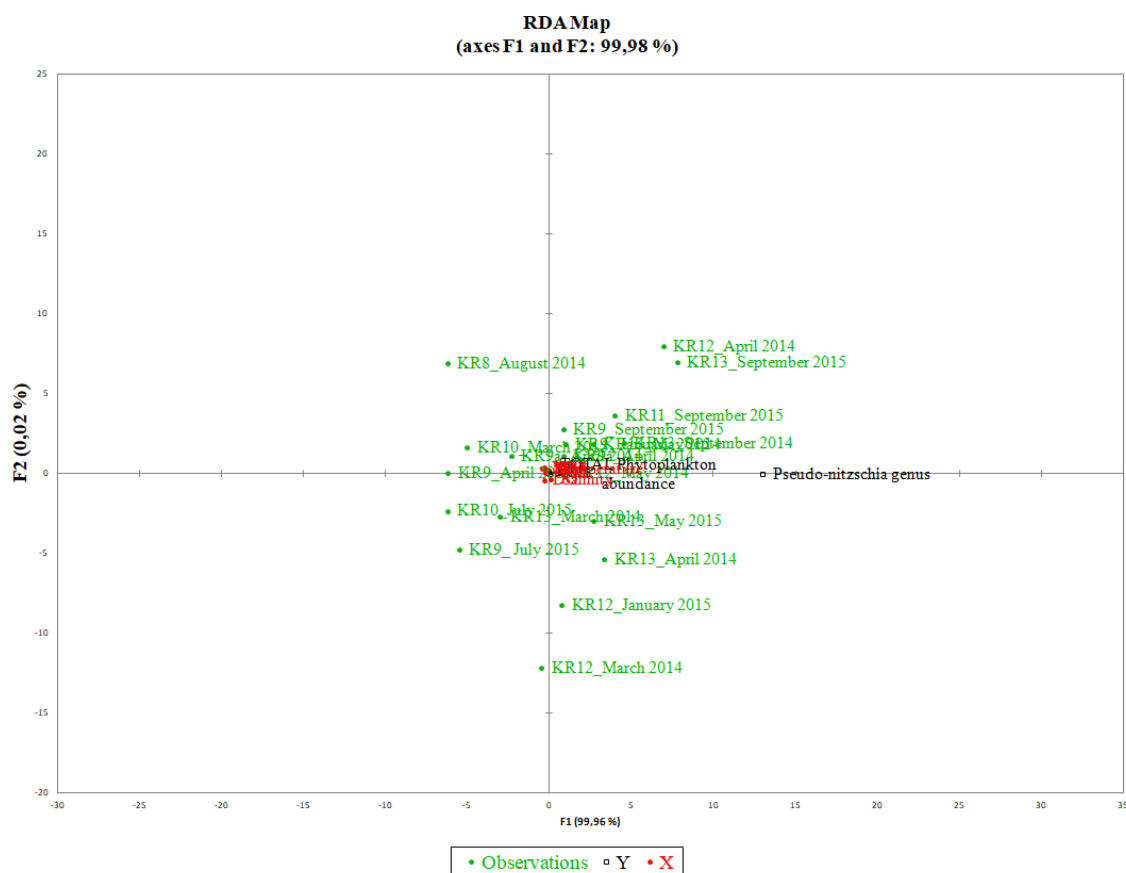
Fig. 9: Redundancy Analysis (RDA) biplot.

Table 7. Explanatory and response variables - scores for the first two axes of the Redundancy Analysis (RDA).

Explanatory variables	F1	F2
NO2	-0.296	0.097
NO3	**-0.467**	**0.378**
PO4	-0.344	0.322
NH4	-0.306	**0.417**
Si	**-0.374**	0.321
Temperature	-0.135	0.310
Salinity	0.062	-0.337
DO	-0.350	-0.363

Response variable	F1	F2
Pseudo-nitzschia genus	**12.966**	0.003
Chla	-0.119	0.097
EI	-0.157	**0.179**
TOTAL Phytoplankton abundance	0.004	0.009

Chl-a was regulated mostly by the availability of phosphates and silicates, as confirmed by the statistical analysis. The river enriches the area with Si while phosphates are suggested to be a limiting factor in Maliakos (Christou *et al.,* 1995). During our study, phytoplankton abundance was associated with low silicates and salinity. These findings show that high cell numbers were fuelled by silicates and nitrates from riverine inputs, while sampling was performed in the aftermath of the conditions that triggered cell growth. Bargu *et al.* (2016) reported the same pattern with high *Pseudo-nitzschia* abundances associated with low nutrient conditions in the Mississippi delta. Higher levels of chl-a and phytoplankton abundance were distributed mainly in the estuary and decreased seawards in our study. Chla was significantly higher in the estuary than at the coastal stations. In contrast, the number of phytoplankton species increased as we moved away from the estuary towards the outer Gulf. The same trend has been reported for the number of zoobenthic species in Maliakos Gulf (Akoumianaki *et al.,* 2013).

Diatoms dominated over the rest of the phytoplankton taxonomic groups in the whole study area inter-seasonally. This can explain why phytoplankton distribution was mainly determined by Si availability, as mentioned above. Dinoflagellates were the second most abundant

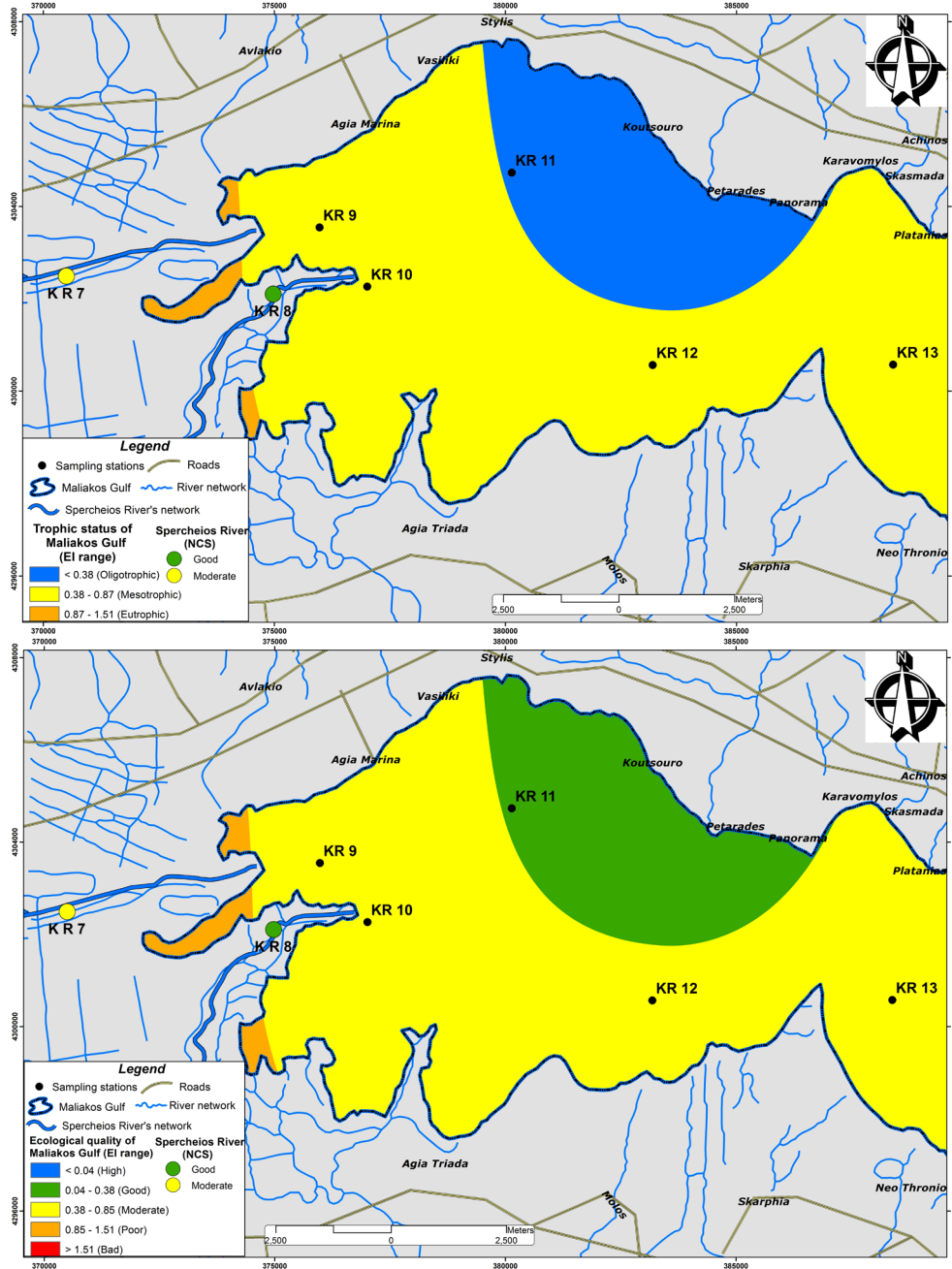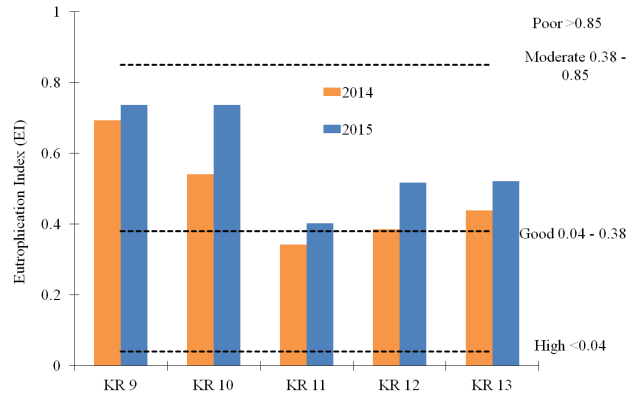*Medit. Mar. Sci.*, 19/2, 2018, 326-343

338

Fig.10: Physicochemical assessment in downstream Spercheios River (a), Eutrophication Index (by Primpas *et al*., 2010) in Maliakos Gulf with quality classes presented on the graph (b), assessment of trophic conditions (c) and ecological status (d) in the study area during 2014-2015.

group and they peaked in spring. Siliconflagellates, Coccolithophores and Raphidophytes followed. Diatoms are known to prefer nutrient rich environments (especially Si) with high turbulence in order to keep their cells in suspension (Bates & Trainer, 2006). Diatoms take advantage of available Si, grow rapidly and dominate the phytoplankton assemblage, forming a "bloom". This ecological success may lie in their use of Si to form siliceous cell walls, which requires less energy to synthesize compared to organic cell walls (Raven, 1983). Maliakos Gulf is a high nutrient environment due to inputs from River Spercheios, e.g. high silicates and fast mixing/homogenization of water masses.

Previous findings report that the mean annual concentrations of nitrates and phosphates range at moderate levels in Maliakos (Christou *et al.,* 1995; Kormas *et al.,* 2003; Ignatiades *et al.,* 1992). In our study, nitrates were linked to high DO and low temperature and salinity, thus indicating their main origin, i.e. freshwater inputs. The ΣN:P ratio diverges from 16:1, indicating P as the limiting factor in winter and N in summer in Maliakos, according to Christou *et al.* (1995). Unbalanced N:P ratios are well-known to affect phytoplankton composition, the proliferation of HABs and toxin production (Anderson *et al.,* 2002; Varkitzi *et al.,* 2010). This is the first time that phytoplankton composition and potentially harmful species are studied in this area, where fishing, aquaculture and tourism are important economic activities. The potentially harmful species bloomed frequently in the whole sampling area during the 2014-2015 period. They were associated with low salinity, thus demonstrating the high influence of river inflows. They comprised a small fraction of the phytoplankton communities (less than 7.7%) due to high total phytoplankton abundances, but they could still cause bloom incidents because they exceeded the defined alert levels by far on some occasions. Furthermore, they were an important component of the phytoplankton communities because they were strongly associated with total phytoplankton abundance, as further confirmed by our statistical findings.

As a general spatial pattern, potentially harmful Dinoflagellates and Siliconflagellates seem to prefer estuarine waters that are directly affected by river inflows, while potentially toxic Diatoms are distributed mostly towards the outer Gulf. High silicate and nitrogen levels were dispersed from the estuary to the whole Gulf due to water mass circulation and shallow depths. Therefore, potentially toxic Diatoms might prefer the outer Gulf due to the stronger mixing of the water column there, which keeps their cells in suspension. *Pseudo-nitzschia* species were strongly linked with low nitrates and silicates and poorly linked with temperature and salinity in our study. We can suggest that *Pseudo-nitzschia* species consumed nitrates and silicates in order to increase their cell numbers and form blooms. Therefore, *Pseudo-nitzschia* blooms were fuelled and determined mostly by the availability of nitrates and silicates and less by the temperature or salinity of Maliakos Gulf.

The potentially toxic Diatom *Pseudo-nitzschia multi-series* reached $2.9 \cdot 10^4$ cells L^{-1} in April in the outer Gulf (KR12), exceeding the alert levels ($10^3 - 10^5$ cells L^{-1} by Todd, 2003). This species can produce the toxin domoic acid and its analogues, which cause Amnesic Shellfish Poisoning (ASP) to humans with serious and sometimes fatal symptoms (Landsberg, 2002). All strains of *P. multiseries* found so far are toxic (Bates & Trainer, 2006). The distribution of *P. multiseries* followed the general pattern pertaining to the distribution of Diatoms in Maliakos Gulf. This emphasises the hypothesis that HABs are generated close to the estuary and then further dispersed seawards. In addition, the nutrient-rich Spercheios run-offs circulate throughout the Gulf, because it is a shallow coastal environment with fast mixing, and this favours HAB proliferation.

The potentially toxic Dinoflagellate *Alexandrium tamarense,* which reached $2.4 \cdot 10^4$ cells L^{-1} in the estuary in late summer (August), is a characteristic case with the alert levels of this potentially toxic species ranging between $1 - 10^3$ cells L^{-1}. These species can produce toxins, e.g. saxitoxins, gonyautoxins etc, which cause Paralytic Shellfish Poisoning (PSP) to humans, with serious symptoms, even death (Landsberg, 2002). It is noteworthy that both potentially toxic *A. tamarense* and *A. minutum* were distributed throughout the Gulf, with cell numbers exceeding the alert levels. This pattern shows that even if Dinoflagellate blooms start in the estuary, they can disperse to the rest of the Gulf due to the circulation regime, possibly with future consequences for public health and the economy. The high loads of particulate matter in delta front areas have been found to be associated with and favour toxic Dinoflagellates, as in the case of toxic *Dinophysis* spp. in Thermaikos Gulf, northern Greece (Varkitzi *et al.,* 2013; Zervoudaki *et al.,* 2014).

Another potentially toxic Dinoflagellate was *Dinophysis caudata,* reaching $1.1 \cdot 10^4$ cells L^{-1} (alert levels 10^2 to 10^3 cells L^{-1}) in April, again in the estuary close to a mussel farm. This species can produce okadaic acid and pectenotoxins, which cause Diarrhetic Shellfish Poisoning (DSP) to humans, with serious gastrointestinal and other symptoms (Landsberg, 2002). The potentially harmful Dinoflagellate *Vulcanodinium rugosum* was also found practically throughout the Maliakos Gulf from May to July (maximum 1520 cells L^{-1}). This is a newly found species, which was first described from Igril lagoon in southern France in 2009 by Nezan & Chomerat (2011). It produces pinnatoxins, which can accumulate in the tissues of mussels, oysters etc, but no poisoning of humans has been reported so far.

In the estuary, we also found the potentially icthyotoxic Siliconflagellates *Dictyocha fibula* in high numbers in July (up to 18400 cells L^{-1}) and less often *D. speculum*. The most frequent *Dictyocha* species in Europe is *D. speculum,* which causes massive fish-kills in high numbers ($10^4 - 10^6$ cells L^{-1}), either due to the characteristic spines of their cell walls, that block the fish gills and cause asphyxia, or due to hypoxia or anoxia in the environment be-

Medit. Mar. Sci., 19/2, 2018, 326-343

340

cause of high *Dictyocha* biomass decomposition (Prego *et al.* 1998). For example, high numbers of *D. speculum* (660 10^3 cells L^{-1}) have been reported from the N. Adriatic Sea in August (Fanuko, 1989) and have been linked to anoxia and mass mortalities in the benthic communities (Stachowitsch, 1984). Lower *D. fibula* abundances are reported from Europe and therefore icthyotoxic events are rare (Vila & Maso, 2005). Despite the high *D. fibula* numbers, no icthyotoxic event was reported from Maliakos Gulf during that period.

Since the 1990s, a lot of work has been done towards an ecological evaluation system based on the pressure-impact relationship in Eastern Mediterranean waters (Ignatiades *et al.,* 1992; Karydis, 1999; Pagou *et al.,* 2002). For chl-a, the original scale was adapted to the WFD five-step scale for ecological status assessment of Greek coastal waters (Simboura *et al.* 2005; 2015). The Eutrophication Index (EI) by Primpas *et al.* (2010) was another step forward and the extrapolated five-step ecological scale, which has been developed for the oligotrophic waters of the eastern Mediterranean and has been widely used ever since, mainly because it combines both pressures (nutrients) and responses (chl-a) in its formula. For river waters in Greece, a combined five-step quality scale by Skoulikidis *et al.* (2006) and Cardoso *et al.* (2001) is used extensively and is based on physicochemical characteristics. Both scales aim to assess and classify the physicochemical status of river waters in one of the five quality levels suggested by the WFD. The efficiency of these assessment scales has been discussed by the scientific community (Gritzalis *et al.,* 2015; Tzoraki *et al.,* 2013; Markogianni *et al.,* 2017; Dimitriou *et al.,* 2015a; b; Pavlidou *et al.,* 2015; Simboura *et al.,* 2015; 2016).

In our study, the physicochemical status of the deltaic sites (KR7, KR8) was marginally good to moderate and this indicates degradation along the whole river, from upstream (high status) to the river delta according to Markogianni *et al.* (2017). This is the result of pollution pressures accumulating along the river and the significant point pollution sources encountered in the lowland areas (croplands, wastewater treatment plant, paper and olive mill industries, etc). As regards the Maliakos Gulf eutrophication and ecological quality assessment, EI demonstrated an overall mesotrophic status and a moderate ecological quality. EI was significantly higher in the estuary that at the coastal stations and this is attributed to the significantly higher levels of chl-a, nitrates, phosphates and silicates there as well. Both the trophic status and the ecological quality deteriorated during the period of high river inflows (Mar, May 2015). According to Markogianni *et al.* (2017), our classification of ecological quality, based on the WFD five-step chl-a scale (Simboura *et al.* 2005; 2015), demonstrated an overall poor status in the estuary and moderate status in the outer Maliakos Gulf. According to both chl-a and EI classification scales, Maliakos Gulf failed to achieve good ecological status, which EU Member States are supposed to achieve in marine waters by 2020, according to the MSFD (2008/56/EC).

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341

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Medit. Mar. Sci., 19/2, 2018, 326-343

342

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343

Medit. Mar. Sci., 19/2, 2018, 326-343