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PRIMARY RESEARCH PAPER

Impact of nutrient availability on the trophic strategies of the planktonic protist communities in a disturbed Mediterranean coastal lagoon

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Abstract The impact of changes in nitrogen (N) and phosphorus (P) availability on the trophic strategies of planktonic protists was evaluated in a disturbed Mediterranean lagoon (Biguglia lagoon, France) using short-term bioassays. Natural communities were collected in three periods, i.e., autumn, spring and summer, to address the influence of the different environmental conditions. The responses of autotrophic plankton communities to experimentally induced N and/or P limitations were assessed as changes in chlorophyll *a* (Chl *a*) concentrations and in the abundances of potentially mixotrophic protists taxa. We observed blooms ($> 10^5$ cells l^{-1}) of nanoflagellates in autumn, and of phycocyanin-rich picocyanobacteria in summer. Communities showed a co-limitation by N and P at the three sampling periods, despite high N:P ratios in autumn and spring. The high abundances of potentially mixotrophic dinoflagellates during these periods suggest the involvement of alternative trophic pathways for their maintenance in the lagoon. After bioassay incubations using different nutrient enrichment treatments, we often observed reduced abundances of mixotrophic protists containing Chl *a* with a concomitant increased abundance of protists without Chl *a*. This indicates a loss of chloroplasts and photoautotrophic abilities in protists cells, possibly reflecting a shift towards heterotrophy that could be sustained by phagotrophy.

Keywords Growth rate · Dinoflagellates · Nutrient limitation · Mixotrophy

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Introduction

In the last few decades, eutrophication upon nutrient over-enrichment has dramatically altered the functioning of many coastal waters (Cloern, 2001; Boesch, 2002). Coastal lagoons are particularly vulnerable to

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this threat due to their confinement from the marine water that leads to reduced water turnover and nutrient accumulation. De-eutrophication or re-oligotrophication of these semi-enclosed ecosystems by reducing nutrient over-enrichment is a challenging issue for managers and scientific communities because eutrophication alters the services provided by these ecosystems. Indeed, nutrient over-enrichment may result in significant environmental disturbances (e.g. hypoxia and harmful algal blooms) that cause mass mortality in the whole food web.

To fully understand the impact of coastal lagoon eutrophication and de-eutrophication, the effect of nutrient availability must be evaluated at the different scales of the trophic web, particularly autotrophic plankton that quickly responds to nutrient availability changes and constitutes the basis of food webs (Schramm, 1999; Boesch, 2002). For instance, reduction of inorganic nitrogen (N) and/or phosphorus (P) load could lead to drastic changes in the community composition, in favor of organisms that can functionally adapt and thrive despite the increasing nutrient limitation. In this respect, strictly autotrophic plankton and potentially mixotrophic protists need to be considered together. Mixotrophic protists, also known as mixoplankton, contain chloroplasts with chlorophyll *a* (Chl *a*) and can be autotrophic when performing photosynthesis and heterotrophic by phagotrophy (Mitra et al., 2016). Several mixotrophic protists possess their own chloroplasts, while others are heterotrophic protists that have the capacity to acquire chloroplasts from phototrophic preys (Mitra et al., 2016). Some mixotrophic protists can thus lose chloroplasts and Chl *a* and become operational heterotrophic organisms. We use the term "potential mixotroph protists" to include all (i) Chl *a*-containing protists belonging to species with known heterotrophic capacities, and (ii) heterotrophic protists of species known to be capable of hosting a chloroplast and of autotrophy (Mitra et al., 2016; Flynn et al., 2018). Mixotrophic organisms have been reported to be more successful than strict heterotrophic or strict autotrophic species in coastal water ecosystems under nutrient-limiting conditions, and where increased runoff of nutrients and organic matter promotes high N:P ratios (Leles et al., 2018). Blooms of potentially harmful algal species with mixotrophic abilities have been increasingly observed in several coastal lagoons following the reduction of nutrients inputs (Yamamoto, 2003; Collos et al., 2009; Leruste et al., 2016). This community composition modification can lead to changes in the interactions between nutrient stocks and the different organisms in the community, and between organisms, particularly concerning competition and predation (Flynn & Mitra, 2009). The overall structure and dynamics of food webs are greatly affected by these changes that can alter the ecosystem functioning, for example through the occurrence of mixotrophic harmful algal blooms (Yamamoto, 2003; Burkholder et al., 2008; Leles et al., 2018).

For decades, Biguglia lagoon, the largest coastal lagoon in Corsica (France), has been experiencing an important eutrophication process, mainly linked to the development of agricultural activities on its watershed. This degradation intensified since the seventies, with the densification of human populations due to the increasing urbanization of the whole Biguglia catchment and also summer tourism. Significant changes in the composition of the primary producer communities have been documented, particularly a net reduction of the aquatic angiosperm cover (Pasqualini et al., 2017). Since 2009, hydrological management interventions have been implemented to increase water fluxes and reduce the confinement of Biguglia lagoon. Nevertheless, nutrient input must be reduced to strengthen these management efforts and to support the lagoon restoration (Pasqualini et al., 2017). To plan future actions for improving the lagoon ecological state, it is important to understand how the protist communities might physiologically and behaviorally respond to nutrient limitation. Indeed, in Biguglia lagoon, blooms of potentially mixotrophic dinoflagellates have been increasingly observed after the modification of the lagoon hydrology in 2009 (Cecchi et al., 2016; Garrido et al., 2016; Leruste et al., 2019b). The environmental causes of these blooms need to be identified to avoid management actions that might lead to potentially harmful algal blooms produced by mixotrophic dinoflagellates species, such as *Prorocentrum cordatum* (Ostenfeld) J.D. Dodge, 1975, formerly known as *Prorocentrum minimum* (Pavillard, 1916) J. Schiller, 1933.

Therefore, the aim of this study in the Biguglia coastal lagoon (Corsica) was to investigate the potential impact of changes in N and P availability on the trophic responses of planktonic protist communities, focusing on strictly autotrophic and potentially

mixotrophic species. We focused on two particular objectives: (i) determining whether the local communities preferentially used N or P resources (internal, external and regenerated pools) to thrive in conditions of nutrient limitation; (ii) testing whether experimentally induced N and/or P limitation promotes the use of mixotrophic strategies by protists, particularly dinoflagellates. We hypothesized that co-limitation by N and P induced development of potentially mixotrophic dinoflagellates suggesting the involvement of alternative trophic pathways for their maintenance in the lagoon. The key questions to be addressed are: (i) do planktonic protist communities vary among seasons? (ii) Are there variations of trophic strategies for N or P resources among seasons? (iii) Do these mixotrophic strategies play a significant role in the development of potentially harmful blooms in the lagoons?

Materials and methods

Study site

Biguglia lagoon (42°36′ N; 9°28′ E) is a shallow brackish coastal lagoon (14.5 km^2, average depth 1.2 m), separated from the Tyrrhenian Sea by a sandy beach barrier (Fig. 1). This choked lagoon (sensu Kjerfve, 1994) is connected to the sea by a long, narrow and shallow natural inlet at the north end (1.5 km). The inlet morphology and its natural inclination to silt up limit the marine water input and lead to a long water residence time ranging from several days near the sea inlet to several weeks or months in the southern basin (Mouillot et al., 2000; Pasqualini et al., 2017). Freshwater inputs (mainly from sewage plants, several rivers, and pumping stations draining the agricultural plain) dominate the water budget of Biguglia lagoon (Fig. 1). These inputs are directly controlled by the inter-annual and inter-seasonal climatic variability and they drastically shape the lagoon salinity. Salinity steeply decreases from the North to the South of the lagoon, because of the artificial freshwater inputs from the Golo River that is connected to the lagoon through the Fossone canal in the South (Garrido et al., 2016). Consequently, Biguglia lagoon displays a highly variable hydrological functioning which affects salinity and nutrient inputs, directly influencing the phytoplankton community structure and composition (Lafabrie et al., 2013; Garrido et al., 2016). This seasonal variability generally determines three hydrological periods characterized by differences in nutrient origin and availability, and in phytoplankton biomass, size class structure and photosynthetic performance (Cecchi et al., 2016; Garrido et al., 2016).

Since the 1980s, increasing nutrient inputs from the watershed has gradually eutrophicated Biguglia lagoon. This anthropogenic pressure is especially high during the touristic summer period (Lafabrie et al., 2013). The lagoon sometimes presents higher nutrient concentrations in the water column (NH_4, NO_2, NO_3, DIN, Si, TN) compared with other Mediterranean lagoons (Orsoni et al., 2001; Souchu et al., 2010). This phenomenon is enhanced by the reduced exchanges with the sea. Compared with other Mediterranean coastal lagoons, the sediment compartment displays a silting with high nutrient concentrations (total nitrogen and total phosphorus) and organic matter content that reflect the lagoon eutrophication (Souchu et al., 2010). Moreover, the southern basin of the lagoon is more eutrophicated than the northern basin (Garrido et al., 2016).

Sampling procedures

Water samples were collected in two stations representatives of the northern (NB) and the southern basins (SB) (Fig. 1, 42°38′12″ N, 9°27′15″ E and 42°35′00″ N, 9°29′18″ E, respectively). Experiments for two stations were carried out in autumn 2013 (26/27 November and 4/5 December), spring 2014 (2/3 and 7/8 April), and summer 2014 (9/10 and 11/12 September). At each sampling station, sub-surface salinity, temperature, turbidity and percentage of dissolved oxygen (DO) were measured with a multiparameter Water Quality Probe (YSI® 6600 V2-2). At each station, 70 l of water was sampled at sub-surface (20 cm depth) and kept in the dark. All samples were pre-filtered through a 1000-µm mesh to remove larger debris but not the zooplankton and larger phytoplankton cells (Collos et al., 2005). At the laboratory, water samples were immediately stored at − 20 °C after homogenization. Sampling time for filtration and storage was within one hour. Measures of NH_4^+, PO_4^{3-}, NO_3^-, NO_2^-, TN, and TP concentrations (µM) were performed on duplicates of 80 ml previously filtered (0.7 µm) with Whatman GF/F glass fiber

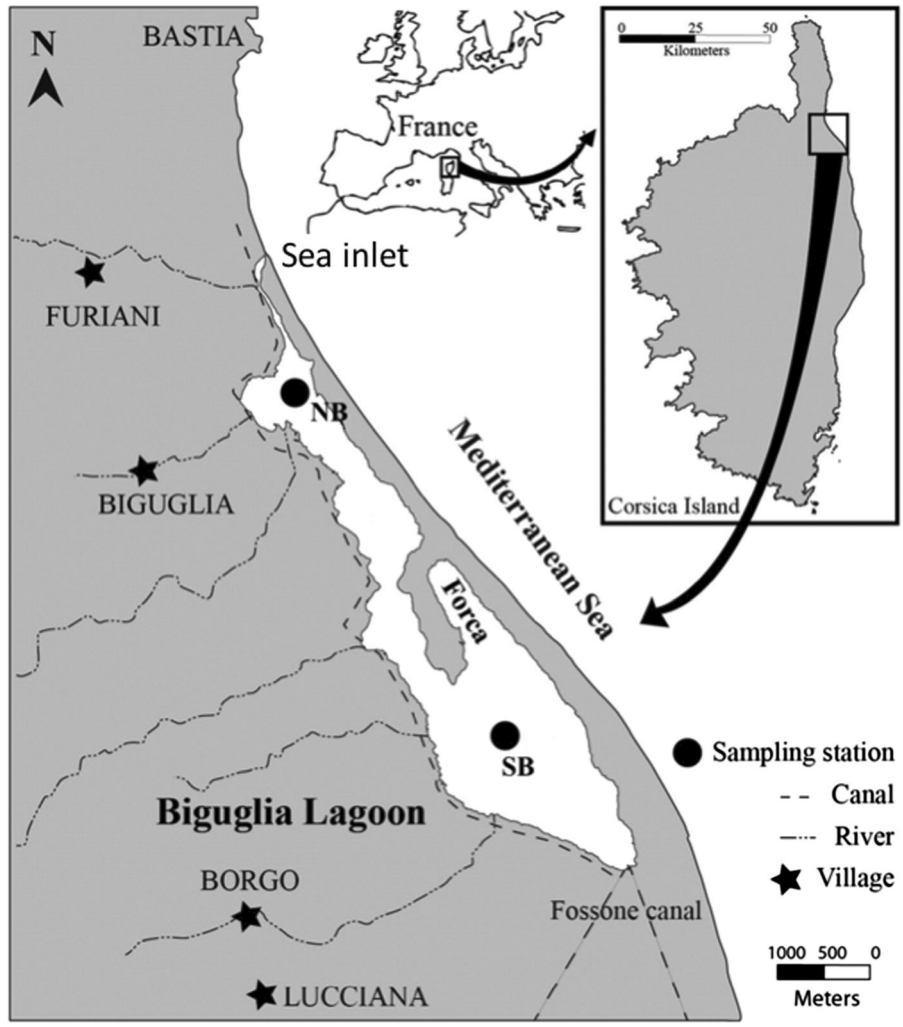

Fig. 1 Location of the two stations representatives of the Northern Basin (NB) and the Southern Basin (SB) of Biguglia lagoon

filters (Aminot & Chaussepied, 1983). For calculating the Redfield ratio (DIN:DIP), DIN values corresponded to the sum of the concentrations of the different dissolved inorganic nitrogen forms (NH_4^+, NO_3^- and NO_2^-), and DIP values corresponded to the concentration of dissolved reactive inorganic phosphorus (PO_4^{3-}).

Autotrophic plankton biomass and community composition

Chlorophyll *a* (Chl *a*) concentration ($\mu g\ l^{-1}$) was used as a proxy for the photoautotrophic plankton biomass (Neveux & Lantoine, 1993). Size fractioning of water samples with nylon filter meshes allowed estimating the biomasses of micro- (> 20 µm), nano- (between 5 and 20 µm in size), and ultraphytoplankton (< 5 µm in size) according to the protocol described in Leruste et al. (2019b).

The taxonomic composition of the phytoplankton communities was analyzed by optical microscopy for cells > 5 µm, and by flow cytometry for cells < 5 µm, as described by Leruste et al. (2018). Identification of the phytoplankton communities was done using a Zeiss Axiolab microscope, at × 400 or × 600 depending on phytoplankton cell size, after sedimentation (Utermöhl, 1958). At least 200 cells per sample were counted to obtain a relevant assessment of the assemblage. Taxonomic resolution was realized at species level whenever possible (Bourrelly, 1990;

Tomas, 1997a, b; Bérard-Therriault et al., 1999; Loir, 2004; Bellinger and Sigee, 2015), and taxonomy was verified using several databases such as the World Register of Marine Species (http://www.marinespecies.org/, databases available online). Abundances of picocyanobacteria, autotrophic picoeukaryotes, and ultraphytoplankton individuals were estimated using a FACSCalibur flow cytometer (Becton–Dickinson), fitted with a 15 mW argon laser (488 nm excitation). For sample processing, the sheath fluid was prepared from filtered (pore size 0.2 µm) artificial seawater (NaCl) whose salinity was adjusted to that of the samples (± 2 units) to avoid alterations of refractive indices of the cells and changes in the measured Forward Side Scatter. Two protocols have been used depending on cell size. Sample acquisition was done at a flow rate of 25 to 30 µl min^{-1}. Samples were diluted when events reached 1,000 s^{-1}. The two eukaryotic groups were distinguished on the basis of optical properties including FSC, related to cell size, and red fluorescence emissions (FL3), a proxy for Chl *a*-content. Among picocyanobacteria, phycoerythrin-rich and phycocyanin-rich populations were identified and distinguished by their orange and/or red fluorescence emissions using beads for size calibration, but they were not identified at a more precise taxonomic level.

Experimental procedure to induce and evaluate phytoplankton nutrient limitation

At the three sampling periods, dilution experiments using the «all minus one» technique and dilution experiments with a full enrichment (FE) were performed to assess the physiological N and P limitation of autotrophic plankton communities in Biguglia lagoon (Andersen et al., 1991; Landry et al., 1998). For a detailed description of the protocol, see Leruste et al. (2019b). For each sample, three series of five dilutions (9, 17, 43, 74 and 100%) were prepared by dilution with water sampled at the site and filtered on 0.2 µm. The different dilutions were then incubated in different enrichment conditions, according to Andersen et al. (1991). The FE condition consisted of adding DIN and PO_4^{3-} at final concentrations of 20 µM and 0.8 µM, respectively. For each of the five dilutions, the first series was incubated with the FE, the second with FE minus N (-N), and the third with FE minus P (-P). Duplicates were performed for the different treatments, i.e. FE, -N and -P, and in triplicate for the water sampled at the beginning of the experiment (see section "Trophic mode of potentially mixotrophic taxa" hereafter). N was supplied as nitrate and/or ammonium, depending on the season. For the April 2014 samples, N was supplied as nitrate (20 µM final concentration), on the basis of the assumption that nitrate inputs from watershed leaching represented the main N source in that period. For the September 2014 samples, N was supplied as ammonium (20 µM final concentration), assuming that this should have been the predominant form provided by the sediments as an internal source related to the remineralization of organic matter (Collos et al., 2003; Cecchi et al., 2016). For the November to December 2013 samples, N was supplied in both forms (10 µM each) because, in this period, temperatures can be sufficiently high to allow ammonium regeneration from the sediments. Moreover, flash floods could bring nitrates from the watershed (Cecchi et al., 2016). All samples, including two bottles without enrichment (WE) for each sample, were incubated simultaneously in Biguglia lagoon (in situ temperature and light conditions) at 30 cm depth for 24 h. In the end, 32 bottles of 1 l are used, i.e. 30 bottles for the dilutions with enrichment and 2 bottles for the control (100% water sample without enrichment).

After 24 h incubation, the changes of total and size-fractionated Chl *a* in each bottle were used to calculate the apparent growth rate $k(x)$ of autotrophic plankton at each dilution x. The relationship between the apparent growth rate and the dilution factor x allowed calculating the maximal growth rate μ_{max} and the mortality rate g. All rates were expressed on a per day basis (day^{-1}). In -N and -P treatments, the mean growth rates μ_{-N} and μ_{-P} were estimated as follows: $\mu_{-N} = g + k_{-N}$ and $\mu_{-P} = g + k_{-P}$. (Andersen et al., 1991). The g:μ_{max} ratio gave indications about the potential biomass transfer to higher trophic levels (g:$\mu > 1$) and about biomass accumulation (g:$\mu < 1$) (Calbet & Landry, 2004).

Trophic mode of potentially mixotrophic taxa

The trophic mode of taxa that could be mixotrophic according to the literature was investigated at the two stations and for the three periods before and after incubation in the different treatments (WE, FE, -N and -P) (undiluted samples). The analysis focused on

dinoflagellates, because most autotrophic dinoflagellates display phagocytic activity, and on autotrophic Euglenophyceae that are commonly observed in eutrophicated brackish waters and benefit from the high amounts of organic matter through their phagocytic activities (Willey et al., 1988; Stoecker, 1999). Triplicates of 250 ml of water samples fixed in glutaraldehyde (0.4% final concentration) and one sample per bottle at T24 (two bottles per treatment) were stored at 4 °C in the dark before analysis. Epifluorescence microscopy was used to identify cells showing Chl *a* red fluorescence and to distinguish strictly heterotrophic organisms from those capable of autotrophy at sampling time (Seoane et al., 2011; Leruste et al., 2018). More precisely for nanoflagellates, samples were stained with DAPI (diaminido phenyl indole) for 15 min in the dark and counted under an epifluorescence microscope. For dinoflagellates, samples were counted directly under an optical microscope with or without light filter.

Type of limitation

The "all minus one" experiment targeted the type of resource limitation of the total phytoplankton and of the three size classes at the two stations and for the three periods. These limitations were described using interaction plots representing the phytoplankton communities' response (biomass increase) to factorial addition of N and P resources, with one line representing N addition (without enrichment − enrichment minus P), the other representing P addition (enrichment minus N − full enrichment) (Harpole et al., 2011). The *Y*-axis represents the biomass responses to the factorial addition of N and/or P relative to the bottles without enrichment. The trends of these plots allow hypothesizing about the co-limitation type (simultaneous, independent, serial and synergistic limitation), the negative response, and the absence of response to nutrient addition (Harpole et al., 2011).

Contributing nutrient resources under experimentally induced N and P limitations

Three potential nutrient sources were considered for phytoplankton growth during experimentally induced nutrient limitations: (1) external source, including the nutrients dissolved in the water at the beginning of incubation; (2) internal nutrient pools present in cells at the start of incubation; (3) nutrients supplied by recycling through grazing, such as excretion, egestion and 'sloppy feeding' (i.e., release of organic matter during physical phytoplankton cell breakage) (Andersen et al., 1991). In this study, their relative contributions to the biomass production during incubation were estimated using Eq. (1):

$$\exp(k(x)t) - 1 = K_{\mathrm{I}} + K_{\mathrm{R}}x + K_{\mathrm{E}}x^{-1}, \quad (1)$$

where $k(x)$ is the apparent phytoplankton growth rate at dilution x, and K_{E}, K_{I}, and K_{R} are the potential production coefficients of the three different nutrient pools. These coefficients represent the relative yields of external, internal and remineralized nutrients, respectively. The values of K_{E}, K_{I}, and K_{R} were then obtained by multiple linear regression, with x and x^{-1} as independent variables and exp $(k(x)\ t) - 1$ as the dependent variable. Equations were fitted with the "lmer" function of the "lme4" library (Bates et al., 2015), and model selection was based on parsimony using the small-sample corrected Akaike's information criterion (Burnham & Anderson, 2004) and the 'dredge' function of the MuMIn package (Bartón, 2013). We used equation models proposed in the cited literature and modelized them on R. We used the MuMin package for model selection by $\mathrm{AIC_c}$ (see Leruste et al., 2019a).

Statistical analysis and interpretation

Statistical analyses were performed using R (R Core Team, 2013). The effects of the four treatments on Chl *a* concentrations and the abundances of potentially mixotrophic taxa were assessed using parametric or non-parametric analyses of variance according to the data. Parametric multifactorial variance analyses using 'anova.lm' function (Chambers, 1992) were assessed when data fulfilled the conditions of application (normal distribution, homoscedasticity and independence of residuals). When significant effects were observed, Tukey post hoc tests using 'TukeyHSD' function were used to determine significant differences in pairwise comparisons. If conditions of application were not fulfilled, logarithm transformation was tested, and then in the last option, non-parametric variance analyses were assessed using

'kruskal test' and posthoc 'kruskalmc' test of the 'pgirmess' package (Giraudoux, 2013).

As detailed below, 'lmer' function from the 'lme4' library (version 1.1-10, Bates et al., 2015), 'dredge' function of the 'MuMIn' package (Bartón, 2013) were used to explore the growth rate and limiting nutrient characteristics of the studied communities.

N and P consumptions were estimated by the percentage of variation of their concentration between the beginning and the end of the 24 h incubation (WE, FE, -N and -P).

Results

Environmental variables and autotrophic plankton community composition

The environmental conditions and nutrient concentrations measured before the dilution experiments (T0) were very different according to the sampling period (autumn, spring and summer). Salinity was more variable at the northern station (NB) (from 2 in spring to 10.9 in summer) than at the southern station (SB) (from 6 in spring and summer, to 6.1 in autumn). The dissolved oxygen (DO) percentage was always more elevated at NB (from 97.0% in summer to 113% in spring) than at SB (from 86.2% in summer to 101.5% in autumn). Conversely, turbidity was lower at NB (from 0.8 in spring to 2.9 in autumn) than at SB (from 4.6 in spring to 16.8 in autumn). Temperature was lowest in autumn (8 °C) and highest in summer (average 25 °C).

Nutrient concentrations and phytoplankton biomasses in the water column at T0 showed the highest PO_4^{3-} values in autumn at both stations (Table 1). The ratios of dissolved inorganic nitrogen to dissolved inorganic phosphorus (DIN:DIP ratios) were much higher than the Redfield ratio (i.e., 16:1) in samples collected in autumn and spring (the two wet seasons), while they were lower in summer (Table 1). These elevated DIN:DIP ratios were caused by high NO_3^- concentrations. Chlorophyll *a* (Chl *a*) concentrations ranged from 3.6 and 5.8 µg l^{-1} at both stations for the three sampling periods, except at SB in autumn when it peaked at 20.6 µg l^{-1} (Table 1).

The percentages of micro-, nano- and ultraphytoplankton biomasses are presented in Table 1, and the taxonomic composition of the phytoplankton communities in the two stations at the three sampling periods is summarized in Fig. 2. Ultraphytoplankton represented the highest biomass fraction at both stations and at all sampling times (until 88.0% at SB in summer). The proportions of micro- and nanophytoplankton were highest at NB in autumn and at SB in spring (Table 1).

In autumn, nanophytoplankton represented the highest proportion of the total biomass (39.8%) at NB. This was caused by a bloom of Dictyochophyceae *Apedinella radians* (Lohmann) P.H. Campbell, 1973 (1.3×10^6 cells l^{-1}, representing 100% of the Dictyochophyceae abundance) and Dinoflagellate *P. cordatum* (2.9×10^5 cells l^{-1}, representing 87.6% of the Dinoflagellates abundance) (Fig. 2). At SB, the community was dominated by a bloom of Dinoflagellate *Heterocapsa minima* Pomroy, 1989 (5.1×10^6 cells l^{-1}, representing 98.2% of the Dinoflagellates abundance), but also showed high abundance of Dictyochophyceae *A. radians* (4.8×10^6 cells l^{-1}, representing 99.9% of the Dictyochophyceae abundance) and of the ciliate *Mesodinium rubrum* Lohmann, 1908 (2.3×10^5 cells l^{-1}). Picoeukaryotes and Cryptophyceae were also abundant (Fig. 2). In spring, the community at NB was dominated by dinoflagellates with a bloom of *H. minima* (3.7×10^6 cells l^{-1}, representing 97.7% of the Dinoflagellates abundance), and by diatoms (3.1×10^6 cells l^{-1}) (Fig. 2). Community in SB was dominated by a bloom of phycocyanin-rich picocyanobacteria (PC-picocyanobacteria) and picoeukaryotes (6.4×10^7 cells $l^{-1}$and 5.6×10^7 cells l^{-1}, respectively) (Fig. 2). *Mesodinium rubrum* was also abundant at this station (1.6×10^5 cells l^{-1}). In summer, ultraphytoplankton was dominant (more than 80% of the total biomass at both stations) due to a bloom of PC-picocyanobacteria (9.4×10^8 cells l^{-1} in NB, 3.0×10^8 cells l^{-1} at SB) and picoeukaryotes (6.5×10^7 cells l^{-1} in NB, 1.7×10^7 cells l^{-1} at SB) (Fig. 2).

At T0, potentially mixotrophic protists containing Chl *a* (+Chl *a* protists) were significantly more abundant than those without Chl *a* (−Chl *a* protists) in the communities of both stations at all sampling dates (Fig. 3). The abundances of the two size classes varied significantly between stations and sampling seasons (two-way ANOVA, P value < 0.05).

At all sampling dates and at both stations, +Chl *a* protists between 10 and 20 µm in size were mainly

Table 1 Nutrient concentrations, mean Chlorophyll *a* (Chl *a*) concentrations, and percentages of the total Chl *a* concentrations represented by microphytoplankton > 20 µm in size (Micro), nanophytoplankton between 5 and 20 µm in size (Nano) and ultraphytoplankton < 5 µm in size (Ultra) in the two stations (NB and SB) of Biguglia lagoon for the three samplings

Date of sampling	Station	NH_4^+ (µM)	NO_3^-	NO_2^-	PO_4^{3-}	DIN:DIP	Chl *a* ± SD (µg l^{-1})	Micro (%)	Nano	Ultra
26/11/2013	NB	7.52	70.01	0.49	0.73	107.29	5.41 ± 0.26	26.5	39.8	33.7
04/12/2013	SB	0.69	40.72	0.29	0.64	65.52	20.60 ± 2.93	17.1	25.3	57.5
07/04/2014	NB	2.18	17.47	0.16	0.16	124.47	5.75 ± 0.29	15.9	14.6	69.5
02/04/2014	SB	1.85	93.22	0.30	0.00	–	5.06 ± 0.41	39.5	11.9	48.7
11/09/2014	NB	0.37	0.03	0.00	0.03	13.33	3.78 ± 0.06	7.5	9.2	83.3
09/09/2014	SB	1.24	0.11	0.09	0.11	13.06	3.62 ± 0.11	0.1	11.9	88.0

DIN dissolved inorganic nitrogen, *DIP* dissolved inorganic phosphorus

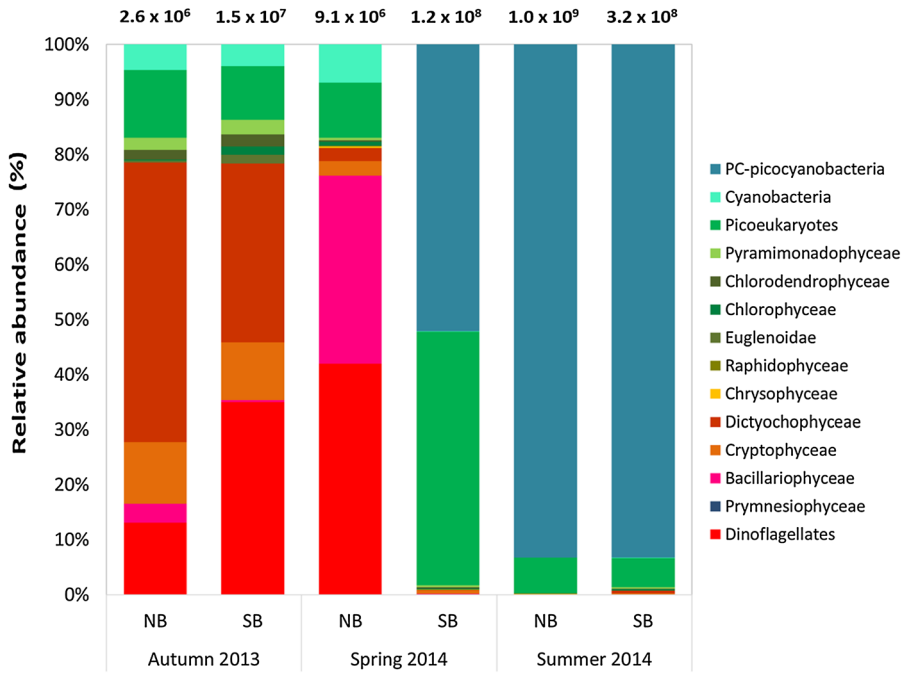

Fig. 2 Relative percentage of the main phytoplankton groups in the two stations (NB and SB) at the three sampling periods. The total abundance (cell l^{-1}) is specified on the top

represented by *H. minima*, *P. cordatum*, and *Heterocapsa niei* (Loeblich III) Morrill & Loeblich III, 1981 (Fig. 3A). Blooms of *H. minima* occurred at SB at the three sampling periods (from 1.1 × 10^5 cells l^{-1} in spring to 3.7 × 10^6 cells l^{-1} in autumn) and at NB in spring (2.2 × 10^6 cells l^{-1}), associated with high abundance of *H. niei* (1.9 × 10^5 cells l^{-1}). At NB in autumn and summer, protists > 10 µm in size were dominated by *P. cordatum* (1.7 × 10^5 cells l^{-1} and 9.4 × 10^4 cells l^{-1}, respectively). At SB, the highest abundances and proportions of +Chl *a* > 20 µm in size in autumn and spring were caused by *M. rubrum* blooms (Fig. 3C). In summer, +Chl *a* protists > 20 µm in size included mainly *Gymnodinium* sp., *Gonyaulax* sp., and *M. rubrum* at both stations (Fig. 3C).

In both size classes, −Chl *a* protists were low in numbers, and even not detected in spring at NB

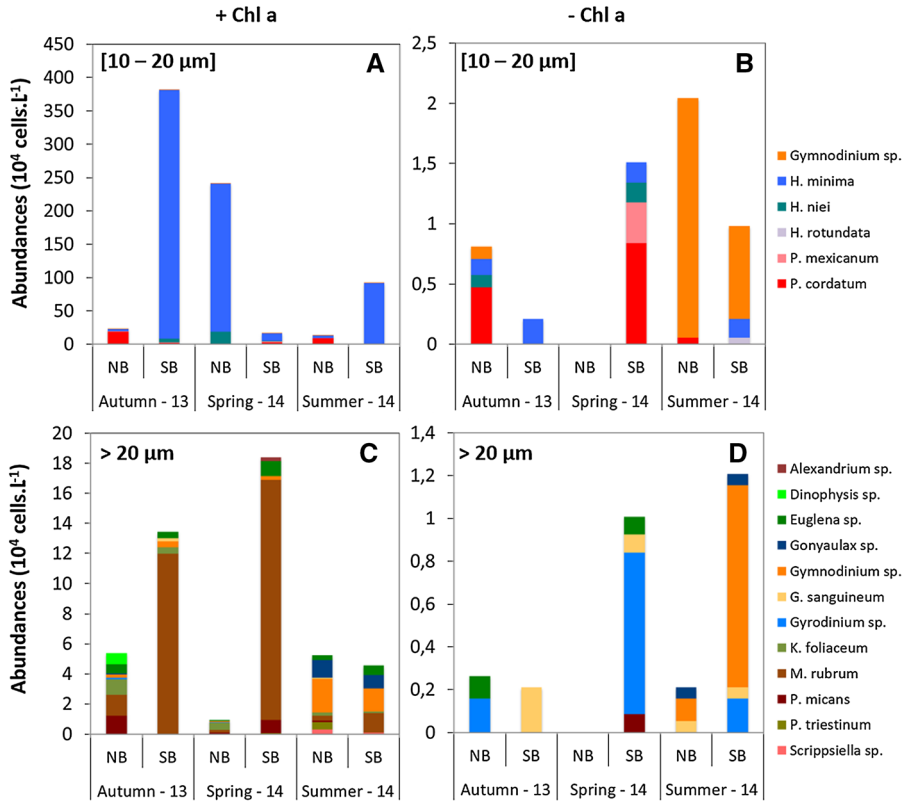

Fig. 3 Abundances of the main potentially mixotrophic protists that contain Chl *a* (+Chl *a*) in the 10 to 20 µm size class (**A**) and the > 20 µm size class (**C**), or without Chl *a* (−Chl *a*) in the 10 to 20 µm size class (**B**) and the > 20 µm size class (**D**) at the NB and SB stations of Biguglia lagoon and for the three periods (autumn 2013, spring and summer 2014). Note differences in scales among the four panels

(Fig. 3B–D). *Heterocapsa minima* and *P. cordatum* (10–20 µm size fraction) and *Gyrodinium* sp. and *Gymnodinium sanguineum* (> 20 µm size fraction) were dominant in autumn and spring (Fig. 3B–D). In summer, *Gymnodinium* sp. was dominant at both fractions (Fig. 3B–D).

Nutrient enrichment/limitation bioassays: abundance of potentially mixotrophic protists

The changes in total Chl *a* concentration in undiluted water samples from the two stations after 24 h of incubation (T24) with the four treatments (without enrichment, WE; full enrichment, FE; enrichment without N, -N; and enrichment without P, -P) are presented in Fig. 4 as percent changes, i.e. 100 * (Chl a_{T24} − Chl a_{T0})/Chl a_{T0}.

The final added concentration of DIN was 20 µM, with different compositions used in the three different seasons assuming a better simulation of the conditions prevailing during the season (i.e., 10/10, 20/0 and 0/20 of NO_3^-/NH_4^+ for autumn, spring and summer, respectively; see "Methods"). The FE enrichment did not always induce an increase of Chl *a* concentration between T0 and T24 (Fig. 4). In autumn, Chl *a* concentration was reduced by 32% at NB and by 23% at SB at T24 (Fig. 4A). In spring, Chl *a* concentration significantly increased by 33% at NB, while it decreased by 10% at SB (Fig. 4B). Conversely, in summer, Chl *a* concentration significantly increased by more than three times at NB and four times at SB (non-parametric ANOVAs and post hoc Kruskal test, P value < 0.05) (Fig. 4C).

In the -P enrichment, Chl *a* concentration at SB decreased after the 24 h incubation in autumn and spring, whereas at NB it increased in autumn (by 12%) and decreased in spring (non-parametric ANOVA, P value < 0.05) (Fig. 4A, B). In summer, Chl

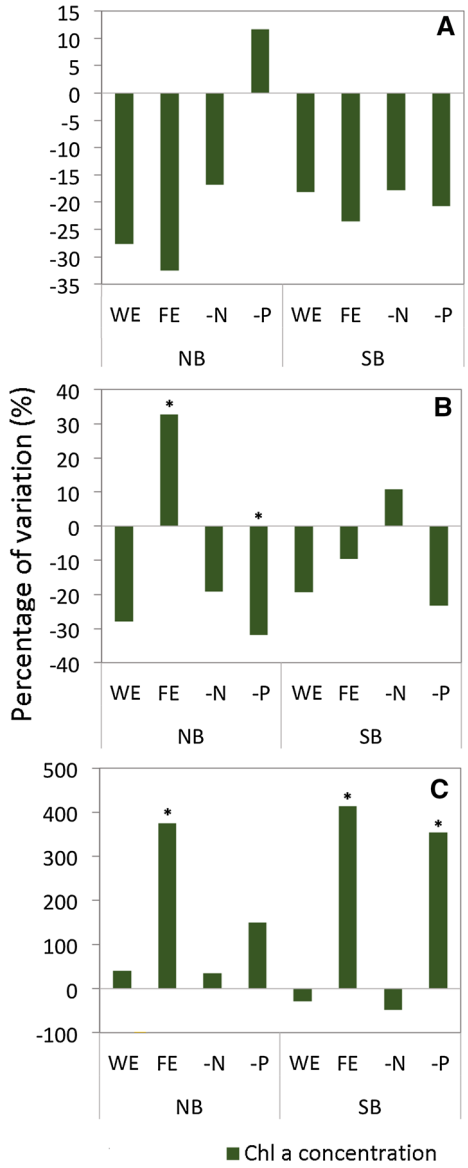

Fig. 4 Percentages of increase (positive values) or decrease (negative values) of Chl *a* concentrations during 24 h of in situ incubation in bottles (100 * (Chl a_{T24} − Chl a_{T0})/ Chl a_{T0}): without enrichment (WE), full enrichment (FE), enrichment without N (-N), enrichment without P (-P) at the NB and SB stations, respectively, of Biguglia lagoon. Panels represent experimental results for autumn 2013 (**A**), spring 2014 (**B**), and summer 2014 (**C**), respectively. Note differences in scales among the three panels

a concentration increased by 150% at NB and by 350% at SB, respectively.

Analysis of the abundance variations of potentially mixotrophic protists > 10 µm in size (+Chl *a* and −Chl *a*) between T0 and T24 (Fig. 5) revealed that after 24 h, +Chl *a* protists between 10 and 20 µm in size were generally more abundant than the larger +Chl *a* protists (> 20 µm). This occurred in all treatments (WE, FE, -N, and -P) at both stations and all sampling times, except at SB in spring where +Chl *a* > 20 µm outnumbered smaller organisms in the FE treatment.

In autumn at NB, only the abundance of +Chl *a* protists > 20 µm in size was significantly reduced by 61% in the -P enrichment (two-ways ANOVA, P value < 0.05, Fig. 5A). However, abundance variations were very high in all incubation conditions and at all seasons. Moreover, in the WE condition, the abundances of −Chl *a* protists between 10 and 20 µm in size were reduced by 81%, and those > 20 µm in size by 50% (Fig. 5A). At SB, the abundance of +Chl *a* protists (both size classes) was significantly decreased after the incubation with the WE treatment (from 38% for the 10 to 20 µm in size to 68% for the > 20 µm in size) (Fig. 5A). Conversely, the abundance of −Chl *a* protists between 10 and 20 µm in size significantly increased in the WE, -N and -P enrichments (two-way ANOVA, P value < 0.05) (Fig. 5A). Specifically, after incubation with the -P treatment, abundance of −Chl *a* protists between 10 and 20 µm in size increased by 13,550% and those > 20 µm in size by 50% (Fig. 5A).

In spring, the abundance of +Chl *a* protists between 10 and 20 µm in size at NB significantly decreased after 24 h incubation with FE, -N and -P (two-way ANOVA, P value < 0.05) (Fig. 5B). Moreover, −Chl *a* protists (both size classes) appeared after 24 h incubation with the four treatments. At SB, the abundance of −Chl *a* and +Chl *a* protists > 20 µm in size did not significantly change after 24 h incubation (all conditions), whereas abundance of +Chl *a* protists between 10 and 20 µm in size decreased upon incubation with the four treatments. Abundance of −Chl *a* protists between 10 and 20 µm in size decreased with the WE and FE treatments whereas it increased with -N and -P treatments (Fig. 5B).

In summer, protists abundance at NB was not significantly affected by any of the four experimental treatments (Fig. 5C), although +Chl *a* protists between 10 and 20 µm in size increased by 12% (WE) and by 47% (-N), while −Chl *a* protists between 10 and 20 µm in size decreased by 50% in the FE enrichment. At SB, the abundance of +Chl *a* protists between 10 and 20 µm in size significantly increased

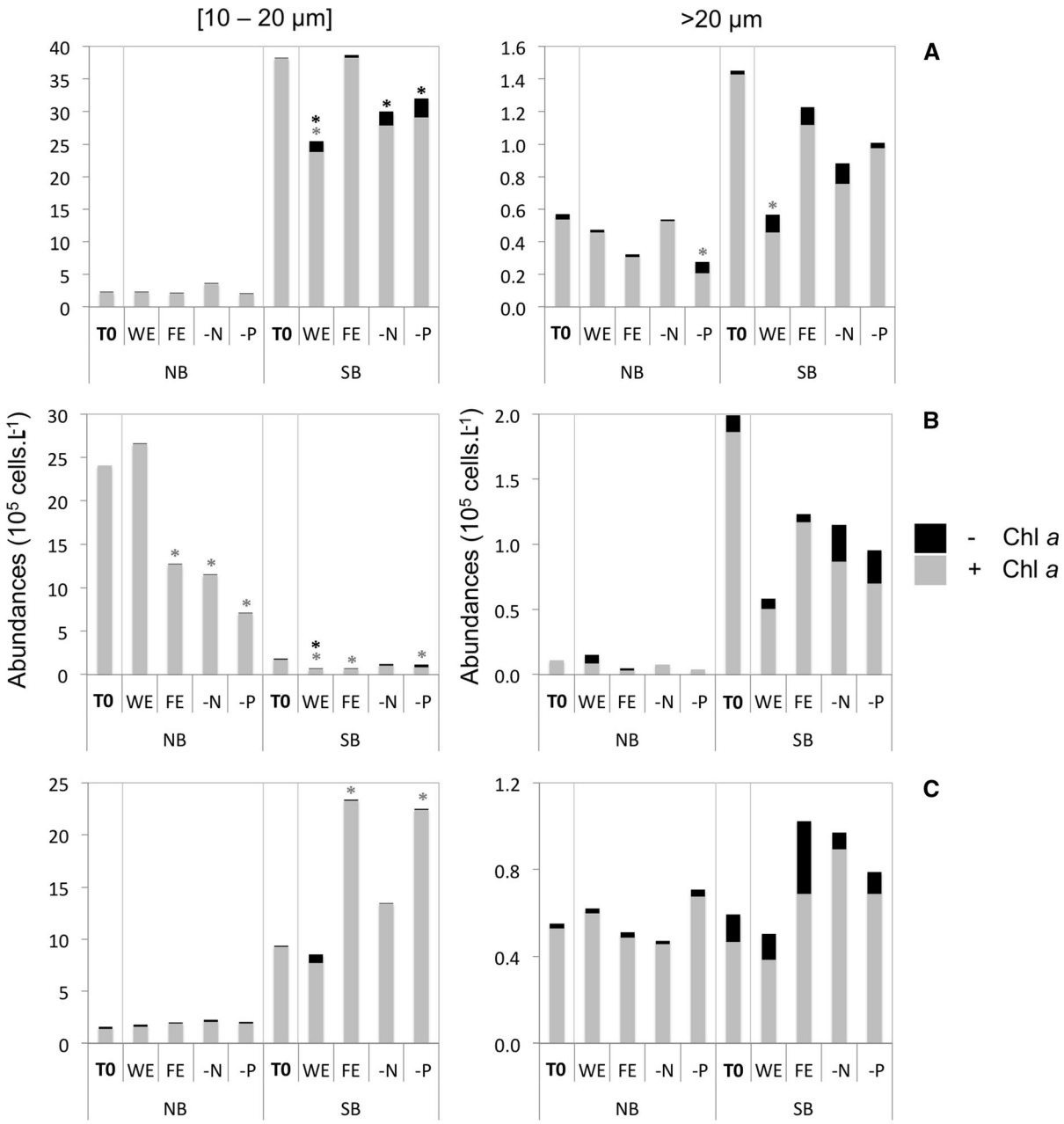

Fig. 5 Abundances of potentially mixotrophic protists containing Chl *a* (+Chl *a*, light gray bars) or without Chl *a* (−Chl *a*, black bars) in the 10 to 20 µm size class (left panels) and in the > 20 µm size class (right panels) at the NB and SB stations, respectively, of Biguglia lagoon. Results for the different experiments are represented for autumn 2013 (Top panels, **A**), spring 2014 (Middle panels, **B**), and summer 2014 (Bottom panels **C**). Different notations along abscissa relate to before the start of the incubation start at T0 (T0), and at T24 i.e. after 24 h incubation, respectively, for without enrichment (WE), full enrichment (FE), enrichment without N (-N) and enrichment without P (-P). Asterisks indicate a significant difference of the value with respect to T0 (gray asterisks, abundance differences for +Chl *a* protists; black asterisks, abundance difference for −Chl *a* protists). Note differences in scales among the six panels

by 151% with the FE and by 141% with the -P treatment (two-ways ANOVA, P-value < 0.05) (Fig. 5C). The abundance of −Chl *a* protists between 10 and 20 µm in size increased by 744% with the WE treatment, but this change was not significant (Fig. 5C).

Nutrient enrichment/limitation bioassays: trophic strategy

Comparison of Chl *a* concentrations with and without enrichment highlighted different responses, e.g. single N or P-limitation, negative response, absence of response, or differential co-limitation by N and P. Analysis of the average responses to factorial addition of N and/or P (Fig. 6) confirmed that incubation with the FE (with N and P) did not always lead to an increase of the phytoplankton biomass compared with

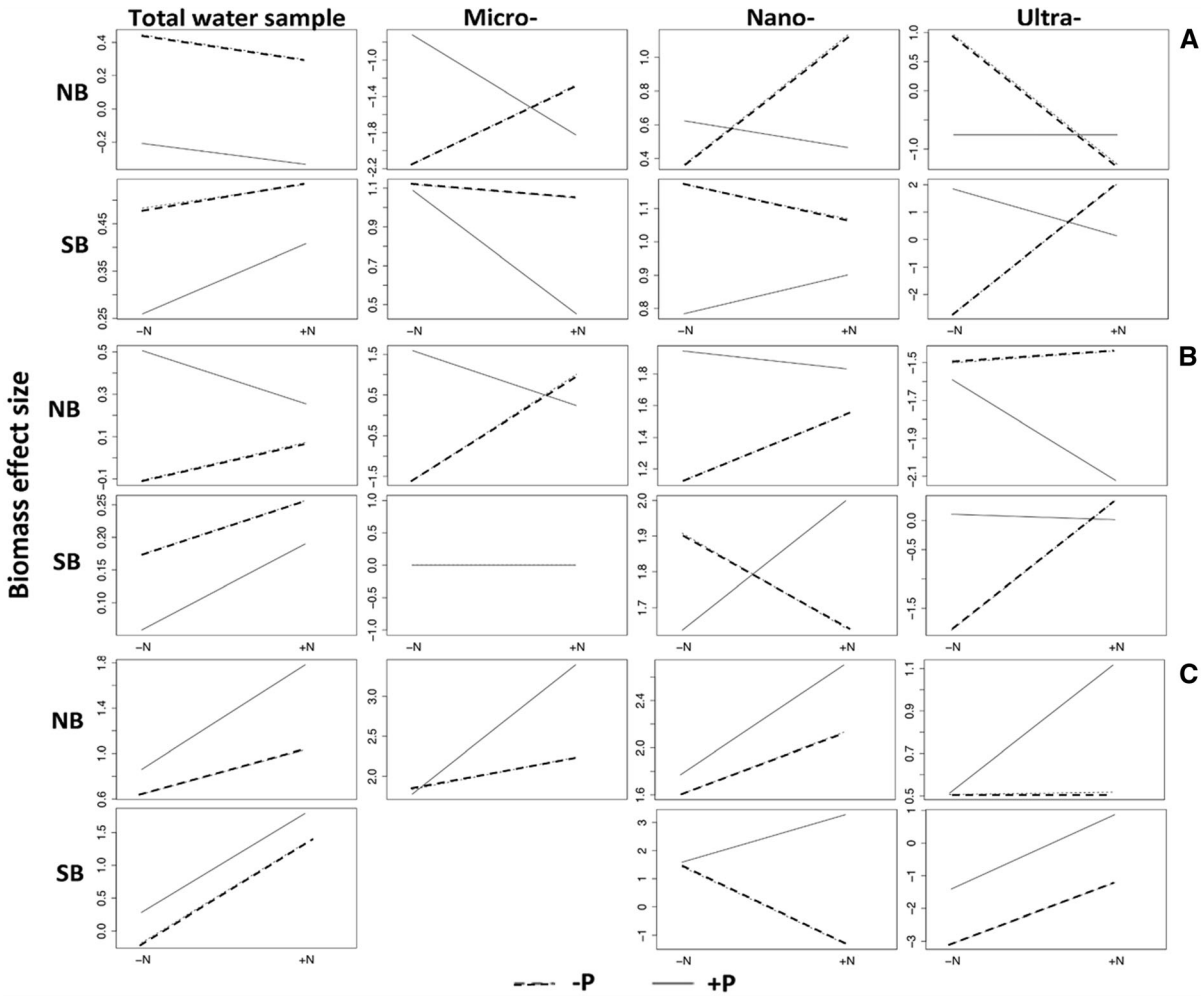

Fig. 6 Interaction plots describing the responses of all phytoplankton (total water sample), micro-, nano- and ultra-phytoplankton to factorial addition of enrichment with N and/or P at the two NB and SB stations of Biguglia lagoon in **A** autumn 2013, **B** spring, and **C** summer 2014. Dashed line represents N addition (without enrichment − enrichment minus P), the solid line represents P addition (enrichment minus N − full enrichment). The *Y*-axis represents the biomass responses to the factorial addition of N and/or P relative to the bottles without enrichment. Trends allow hypothesizing about the co-limitation type (simultaneous, independent, serial and synergistic limitation), the negative response, and the absence of response to nutrient addition (Harpole et al., 2011). Note the scale differences

the enrichments without N and/or P. For example, for the bioassay performed at NB in autumn, the total phytoplankton and the micro- and ultraphytoplankton biomasses were reduced upon exposure to FE compared with the absence of enrichment (Fig. 6A). As several 24 h incubations led to biomass loss and to negative growth rates, we decided to focus only on the positive responses.

In autumn, only the size classes containing high abundances of potentially mixotrophic species showed positive growth rates with the four treatments. Specifically, at NB, nanophytoplankton displayed the highest growth rates upon incubation with FE, -N, and -P compared with WE, indicating a sub-additive, independent co-limitation by N and P (Fig. 6A). At SB, the growth of total phytoplankton, micro- and nanophytoplankton were stimulated by all four conditions. Conversely, ultraphytoplankton lost biomass in the WE treatment, leading to the inability to calculate a positive μ_0 and suggesting a strong nutrient limitation. For total phytoplankton and nanophytoplankton, the interaction plots highlighted a single N-limitation (Fig. 6A), whereas for ultraphytoplankton the higher growth rates with -N and -P compared with FE indicated an independent co-limitation by N and P (Fig. 6A).

In spring, at NB, only nanophytoplankton that contained high abundances of potentially mixotrophic *H. minima* dinoflagellates showed a positive response in the four treatments, particularly with -N rather than WE, and with FE and -P, indicating a single P limitation (Fig. 6B). For the total phytoplankton and the microphytoplankton fraction, the three enrichments allowed a release of the growth limitation observed without enrichment ($\mu_0 < 0$). Total phytoplankton also showed a single P limitation, while microphytoplankton displayed independent co-limitation by N and P (Fig. 6B). At SB, total phytoplankton showed a single N-limitation, while nanophytoplankton was simultaneously co-limited by N and P, and ultraphytoplankton displayed a sub-additive independent co-limitation by N and P (Fig. 6B).

In summer, the responses of the phytoplankton communities to the different enrichments indicated a strong nutrient limitation at both stations. At NB, total phytoplankton and nanophytoplankton displayed a super-additive independent co-limitation by N and P, while microphytoplankton and ultraphytoplankton showed a serial limitation by N and P (Fig. 6C). At SB, total phytoplankton showed an additive independent co-limitation by N and P. As the microphytoplankton biomass at T0 was too low to be estimated, its growth rate and limitation could not be calculated. Nanophytoplankton displayed a serial limitation by N and P, and ultraphytoplankton was simultaneously co-limited by N and P.

Analysis of the potential biomass production supported by internal, external and recycled N and P pools reflects the percentage of their use by total phytoplankton. The three size classes showed that overall, phytoplankton in Biguglia lagoon mainly used internal N and P resources to cope with the experimentally induced limitations during the three periods (negative coefficients were not taken into account; absence of bars in Fig. 7).

In autumn, phytoplankton at NB mainly used recycled N resources in conditions of N limitation. However, micro- and nanophytoplankton mainly used internal N resources (Fig. 7). At SB, phytoplankton used recycled and internal N pools, whereas the three fractions mainly used internal N resources (Fig. 7). In P-limiting conditions, phytoplankton at NB used all three P pools to cope with the P-limitation, while micro- and ultraphytoplankton only used recycled P and nanophytoplankton its internal P resources (Fig. 7). At SB, each of the three size classes of phytoplankton used internal P resources to cope with P limitation (Fig. 7).

In spring, phytoplankton and the different fractions at both stations only used internal N resources to cope with N limitation (Fig. 7). To cope with P limitation, total phytoplankton and nanophytoplankton at NB mainly used their internal P resources, while microphytoplankton relied on recycled and external P resources (90% and 10%, respectively) (Fig. 7). At SB, total phytoplankton and nanophytoplankton mainly used internal P resources to cope with P limitation, and ultraphytoplankton external P pools (Fig. 7).

In summer, to cope with N limitation, total, nano- and ultraphytoplankton at NB mainly used the internal and external N pools, while microphytoplankton only used internal N resources (Fig. 7). At SB, total and ultraphytoplankton only used external N pools, and nanophytoplankton only internal N resources. To cope with P limitation, total, micro- and ultraphytoplankton at NB relied on internal N pools, and nanophytoplankton on all three pools (55% of internal, and 22%

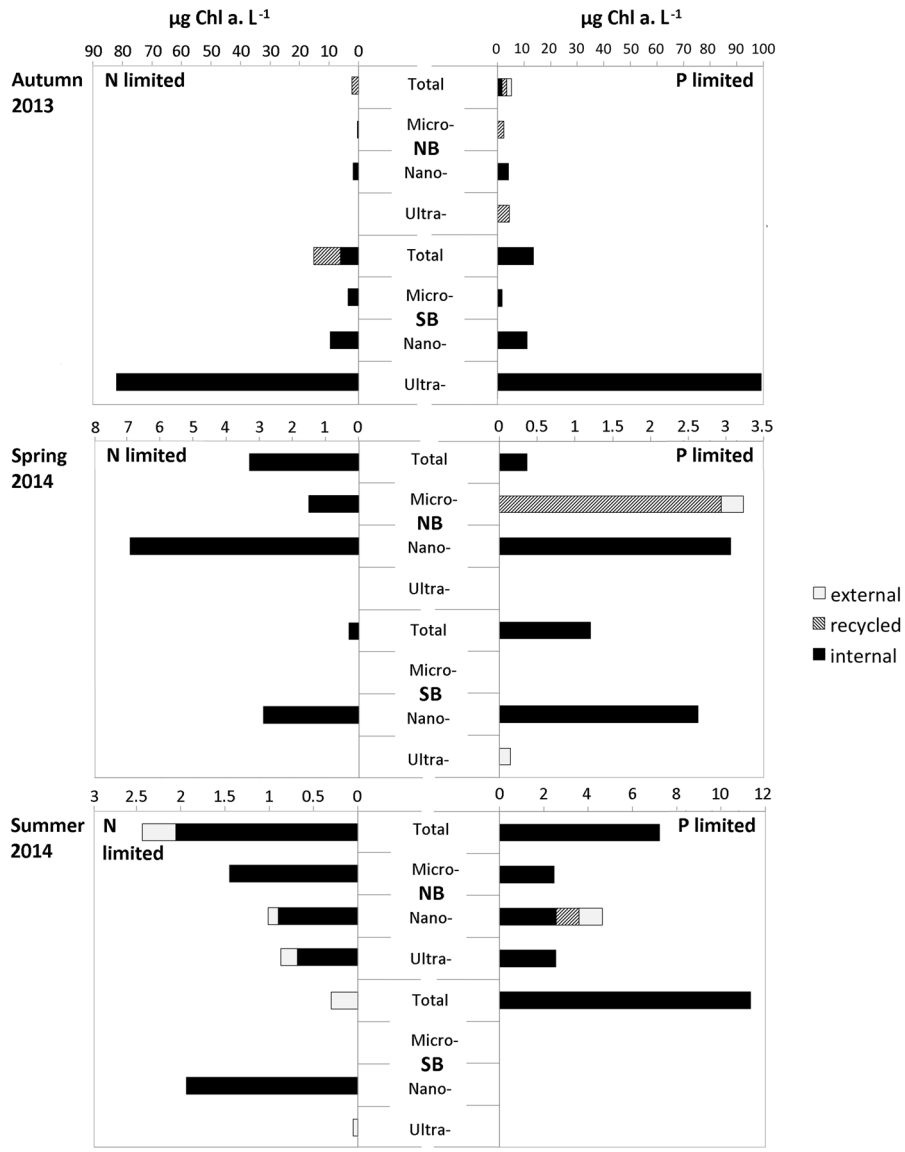

Fig. 7 Estimated contribution of internal (black bars), external (light gray bars) and recycled (dark gray bars) N (left panels) and P (right panels) pools to the potential increments in Chl *a* stocks during the 24 h incubation without N (left panels) or P (right panels) enrichment for the total phytoplankton (total) and the micro-, nano- and ultraphytoplankton at the two stations of Biguglia lagoon in autumn 2013, spring 2014 and summer 2014. Note the scale differences

of recycled and external pools) (Fig. 7). At SB, total phytoplankton only used the internal P pool to cope with the experimentally induced P limitation.

Discussion

Mediterranean coastal lagoons display a high diversity of ecosystem functioning, directly linked to the intrinsic seasonal variability and to anthropogenic pressures. These pressures affect the delivery of freshwater discharge and nutrient load that influence the composition and activity of planktonic primary producers (Paerl et al., 2014). Our experimental study focused on the functional responses of autotrophic plankton communities to nutrient availability changes, and on their adaptive strategies in adverse conditions.

In autumn, despite the high concentrations of dissolved inorganic nutrients (especially NO_3^-), autotrophic planktonic protists showed an independent co-limitation by N and P. However, because of the unbalanced N:P ratios (much higher than the Redfield ratio) caused by the elevated DIN concentration we expected to a strong P limitation. Different reasons might explain the unexpected co-limitation. First, the ambient nutrient N:P ratio does not necessarily reflect the actual concentration of available nutrients, because plankton communities may have already consumed part of these nutrients (Leruste et al., 2016). Second, communities are composed of multiple species with different resource requirements, and specific adaptations to limiting resources. The Redfield ratio is a generalization and many species have different N:P requirements. This niche differentiation can lead to species limited by different nutrients, causing a N and P co-limitation for the total community (Harpole et al., 2011; Burson et al., 2016). Moreover, as many factors affect nutrient limitation, subtle changes in nutrient supply, community composition, and biogeochemical cycling can modify the nutrient availability and thus the nutrient limitation (Paerl et al., 2014).

To cope with limiting resources, autotrophic plankton species can adjust their strategy of nutrient acquisition and uptake using different pools of resources. Many planktonic groups (e.g., diatoms and dinoflagellates) can use their internal N and P reserves in response to nutrient depletion (Andersen et al., 1991). Indeed, the bioassay results for samples collected in autumn showed that internal N and P were the main resources used by autotrophic plankton of Biguglia lagoon. Moreover, dinoflagellates and flagellates, including potentially mixotrophic species, dominated the communities of the two stations. In addition, only the size classes containing high abundances of potentially mixotrophic species (i.e., nanophytoplankton at NB, micro- and nanophytoplankton at SB) showed positive growth rates after incubation with the four treatments.

The decreased abundance of potentially mixotrophic +Chl *a* protists, especially at SB station, and the increase of −Chl *a* protists highlight that several cells lost their Chl *a* content during the bioassay. This suggests that incubation with the four treatments strongly affected the health of these cells that lost their photosynthetic abilities. Alternatively, limiting conditions could have induced phagotrophy rather than photosynthesis in *H. minima* and *P. cordatum*. Indeed, N and P limiting conditions can induce mixotrophy in some dinoflagellates species (Johnson, 2015). Moreover, in the Seto Inland Sea of Japan, an increase of the TN:TP ratio promoted blooms of potentially mixotrophic dinoflagellates, such as *Alexandrium tamarense* and *Gymnodinium catenatum* that used dissolved organic P to cope with the increasing P limitation (Yamamoto, 2003). Therefore, in our study, cells that lost Chl *a* could have obtained their nutrients from ingesting organic forms or preys. The absence of the use of external or recycled nutrient observed during experimentally induced N and P limitations supports this hypothesis and could explain the observed co-limitation by N and P rather than by P alone (Burson et al., 2016). In the presence of unbalanced N:P ratios, potentially mixotrophic species might outcompete strict autotrophic cells, although the latter have a higher growth rate (Mitra et al., 2016). This could also explain the occurrence of dinoflagellate blooms, although nanoflagellates (e.g., *A. radians*) and picoeukaryotes present in the community have higher growth rates and higher affinity for dissolved inorganic nutrient uptake (Reynolds, 2006).

The Chl *a* concentration decrease after 24 h incubation observed in almost all treatments suggests a strong limitation of autotrophic planktonic protist communities, and a strong predation on these autotrophic organisms. The g:μ_{max} ratio can be used as a proxy of primary production consumed by species of higher trophic levels (Fig. 8) (Calbet and Landry, 2004). In autumn, its value indicated a transfer of

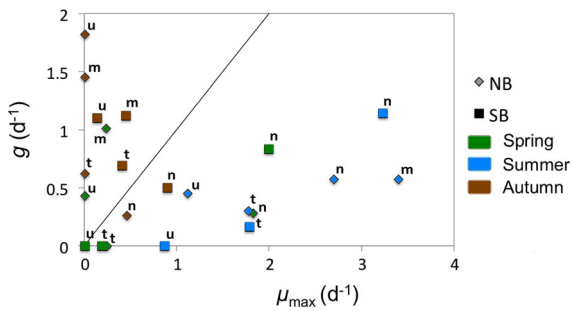

Fig. 8 Mortality rates (g) as a function of the maximum growth rates (μ_{max}) of autotrophic planktonic communities at the NB and SB stations [total phytoplankton (t), micro- (m), nano- (n), and ultraphytoplankton (u)] in bioassays performed in autumn 2013, spring and summer 2014. The line indicates the g:μ_{max} ratio = 1

biomass to higher trophic levels ($g{:}\mu_{max} > 1$) at both stations. This high consumption of primary production may also corroborate the hypothesis of the importance of the phagotrophic abilities of mixotrophic dinoflagellates species. Indeed, at both stations, nanophytoplankton rich in *H. minima* and *P. cordatum* (two potentially mixotrophic species) displayed $g{:}\mu_{max}$ ratios < 1, indicating that this fraction accumulated biomass after incubation with FE. We could hypothesize that they used their phagotrophic abilities because this size class did not rely on the external and recycled N and P pools.

In spring, the community composition was different at the two stations, with a dominance of nanoplanktonic dinoflagellates and diatoms at NB and of PC-picocyanobacteria at SB. This difference in community composition could reflect contrasting environmental characteristics between the two sub-basins, such as freshwater inputs (Leruste et al., 2019b). Their functional responses to the nutrient availability also were different. The single P limitation of the total community and of the nanophytoplankton fraction at NB was coherent with the high N:P ratio observed. This result could be explained by (i) the strong affinity of diatoms for nitrate that was the main N form at the sampling time, and (ii) the potential use of phagotrophy or osmotrophy by *H. minima* rather than strict photoautotrophy. As observed in autumn, this hypothesis could also explain the decreased abundance of potentially mixotrophic +Chl *a* protists during the bioassay, suggesting that the limited number of these less competitive taxa, compared for example with diatoms, might have promoted the use of organic nutrient resources. The $g{:}\mu_{max}$ ratio for this period showed a difference between ultraphytoplankton (mainly picoeukaryotes at NB and PC-picocyanobacteria at SB) that were consumed by higher trophic level species ($g{:}\mu_{max} > 1$), and nanophytoplankton that accumulated biomass ($g{:}\mu_{max} < 1$) (Fig. 8). This also supports the mixotrophy of nanophytoplankton that probably grazed on ultraphytoplankton (Garrido et al., 2016).

In spring at SB and in summer at both stations, communities were largely dominated by blooming PC-picocyanobacteria (more than 10^8 cells l^{-1}). The bloom of PC-picocyanobacteria during summer suggests that internal nutrient loading from the sediments may play a critical role in cyanobacterial bloom development (Glibert et al., 2010). In the SB community in spring, only PC-picocyanobacteria used external P resources under the P-limiting conditions reflecting their strong P uptake efficiency. This may explain why this community was co-limited by N and P or limited only by N rather than by P. Moreover, the ratio $g{:}\mu_{max} < 1$ for ultraphytoplankton at both stations indicates that NH_4^+ and PO_4^{3-} enrichments led to biomass accumulation despite the potential high grazing pressure on this class size (Collos et al., 2009; Śliwińska-Wilczewska et al., 2018) (Fig. 8). The dense bloom of PC-picocyanobacteria reduced oxygen availability, increased water turbidity, and coincided with high abundances of *Gonyaulax* sp. (10^4 cells l^{-1}) at both stations. As several mixotrophic species graze on the cyanobacterium *Synechococcus* sp., PC-picocyanobacteria blooms may have directly benefited to mixotrophic species that could cope with the increased light limitation and nutrient depletion by consuming PC-picocyanobacteria to sustain their carbon requirement (Collos et al., 2009; Flynn et al., 2018).

Mixotrophy represents a metabolic duality that is difficult to characterize due to its complexity. However, recent studies have proposed to categorize mixotrophs in constitutive mixotrophs (CMs) with stable plastids, and non-constitutive mixotrophs (NCMs) that lack plastids but can host endosymbiotic algae or steal plastids from their preys. As in our experiments, all observed morphotypes and taxa included at least one fraction of +Chl *a* cells, they might be classified as strict photo-autotrophic organisms (PAs), or as CMs. They could also correspond to one of the NCM categories (generalist, plastid specialist, or endosymbiotic specialist) if they found enough preys to keep operating plastids during the 24 h incubation. However, our results do not allow classifying them with certainty. For example, even species that always contained Chl *a* could not be strictly classified as PAs or CMs. For instance, *M. rubrum* and *Dinophysis* sp. cells all contained Chl *a*, but they correspond to plastid specialized NCMs (Mitra et al., 2016). Moreover, in several taxa, such as *H. minima*, some cells lost their Chl *a* content during the incubation. These taxa (with + and −Chl *a* cells) could correspond to NCMs. Nevertheless, this is, to the best of our knowledge, the first report showing the potential mixotrophic behavior of *H. minima*, although this adapting trophic regime has been observed for other species of this genus (Millette et al., 2017; Leles et al., 2018). Defining the kind of mixotrophy of these

organisms is fundamental because this has important implications for the whole community behavior. Indeed, each group displays different interactions and dynamics in the trophic food web. These contrasting mixotrophic strategies imply different ecological impacts through their need or removal of preys, and their competitiveness according to the resource availability. For example, belonging to the CM group would imply a higher competitiveness compared with NCMs, and the potential ability to cause important blooms in favorable conditions (Mitra et al., 2016).

Conclusions

One-time bioassays at three different seasons gave only a snapshot of the phytoplankton responses to nutrient availability in Biguglia lagoon. Nevertheless, they validated our hypothesis stated in the introduction, namely that co-limitation by N and P induced a development of potentially mixotrophic dinoflagellates, suggesting the involvement of alternative trophic pathways for their maintenance in the lagoon. We document (i) that the diversity of planktonic protist communities and (ii) that the use strategies for N or P resources varied among seasons, and (iii) that these mixotrophic strategies play a significant role in the development of potentially harmful bloom in the lagoons.

Our experiments have increased knowledge about the seasonal variability of these responses. We particularly highlighted two bloom types that have adverse effects on Biguglia lagoon health. The bloom of PC-picocyanobacteria during summer indicate that internal nutrient loading from the sediments plays a critical role in cyanobacterial bloom development in Biguglia lagoon. This emphasizes the importance of reducing nutrient stocks to prevent blooms of potentially harmful cyanobacteria and other species, such as mixotrophic species favored by high prey abundance.

In autumn and spring, the high abundance of potentially mixotrophic dinoflagellates brings questions about the choice of ecological restoration measures to mitigate the risk of potentially harmful bloom in these seasons. As mixotrophic species can use both inorganic and organic nutrient resources, a reduction of these nutrients is necessary. However, a reduction of prey abundance can further favor mixotrophic and potentially harmful species that benefit from the increase of inorganic nutrient limitation to outcompete the strict photo-autotrophic and phago-heterotrophic species.

This study raises many questions that need closer consideration. Our results suggest that the abundance of mixotrophic dinoflagellate species is increasing in Biguglia lagoon due to several synergistic factors, such as unbalanced N:P ratio due to high N inputs, internal nutrient stocks, and the seasonal presence of high prey abundance. Therefore, we need to identify the driver(s) of mixotrophy for these species, and their relationship with organic nutrient stocks and their potential preys. Identifying the mixotroph category of the observed taxa is also essential because their role in the ecosystem functioning would also be different.

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