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Evaluation of FluoroProbe® performance for the phytoplankton-based assessment of the ecological status of Mediterranean coastal lagoons

Marie Garrido · Philippe Cecchi  · Nathalie Malet · Béatrice Bec · Franck Torre · Vanina Pasqualini

Abstract The European Water Framework Directive and several other legislations worldwide have selected phytoplankton for monitoring the ecological status of surface waters. This assessment is a complicated task in coastal lagoons due to their intrinsic variability, prompting moves to use real-time measurements. Here, we tested the ability of the submersible spectrofluorometer FluoroProbe® to accurately estimate the phytoplankton biomass and to efficiently discriminate spectral groups in Mediterranean coastal lagoons, by using sub-surface water samples ($n = 107$) collected at Biguglia lagoon (Corsica) in different environmental situations (salinity and trophic state) from

March 2012 to December 2014. We compared the estimates of biomass and phytoplankton group composition obtained with the FluoroProbe® (in situ and lab measurements) with the spectrofluorimetrically measured biomass and HPLC-derived quantifications of pigment concentrations. FluoroProbe® provided good estimates of the total phytoplankton biomass (particularly, the lab measurements). The FluoroProbe® data were significantly correlated with the HPLC results, except for the in situ measurements of very weak concentrations of blue-green and red algae. Our findings indicate that factory-calibrated FluoroProbe® is an efficient and easy-to-use real-time phytoplankton monitoring tool in coastal lagoons, especially as an early warning system for the detection of potentially harmful algal blooms. Practical instructions dedicated to non-specialist field operators are provided. A simple and efficient method for discarding in situ measurement outliers is also proposed.

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Introduction

Mediterranean coastal lagoons are increasingly exposed to nutrient enrichment, mainly driven by urbanization, tourism, and agricultural activities (Justic et al. 1995; Flo et al. 2011). Nutrient enrichment disturbs the ecosystem metabolism and the structure of the native aquatic communities (Pasqualini et al. 2017),

intensifies the eutrophication of coastal waters (Nixon 1995; Glibert 2017), and favors the occurrence of harmful algal blooms (Collos et al. 2004; Spatharis et al. 2007; Smayda 2008; Heisler et al. 2008; Cecchi et al. 2016; Glibert and Burford 2017). In response to the degradation of water resources, the European Union Water Framework Directive 2000/60/EC (EU-WFD) was put in place with the aim of maintaining and improving water quality. According to the EU-WFD, the phytoplankton metrics required for defining and classifying the ecological status of transitional waters are biomass (chlorophyll *a*), community structure (composition and species abundances), and algal bloom frequency and intensity.

Chlorophyll *a* concentration is an integrative measure of the phytoplankton community responses to nutrient enrichment (Giovanardi et al. 2018) and is generally quantified using a spectrofluorometer (e.g., Neveux and Lantoiné 1993). However, with such deferred lab methods, the diagnosis is not immediately available. Moreover, they are intrusive (use of solvent) and labor-intensive. The eutrophication-driven increase in chlorophyll *a* is always accompanied by changes in the phytoplankton community structure (Bec et al. 2011). The Utermöhl method (Utermöhl 1958; CEN EN 15204 2006), which is based on traditional cell counts with an inverted microscope, is the only method that allows the taxonomical resolution at the species level for the largest species. This method remains widely used for the routine monitoring of potentially toxic taxa. However, taxonomic identification requires experienced analysts (Vuorio et al. 2007), is time-consuming, and is debated for cells smaller than $\sim 10 \mu\text{m}$. Pigment analysis by high-performance liquid chromatography (HPLC) is an alternative option that provides a highly reproducible and relatively rapid bulk estimate of the major phytoplankton groups present in a sample (Wright and Jeffrey 1997; Schlüter et al. 2014; Leruste et al. 2016) by detecting diagnostic marker pigments, known as accessory pigments (Vidussi et al. 2001; Johnsen and Sakshaug 2007; Marty et al. 2008; Bel-Hassen et al. 2009). All the phytoplankton cell sizes of a sample are considered, including picophytoplankton and mesoplankton (Leruste et al. 2015). However, the lack of pigment specificity for some groups led to couple HPLC with optical microscopy to identify dominant and sub-dominant species (Havskum et al. 2004). Flow cytometry was first used to analyze the abundant picophytoplankton cells (Marie et al. 1997; Jacquet et al. 1998; Bec et al. 2005), and technical developments

have extended the cell size range up to hundreds of microns. However, this method does not provide detailed insights on the sample taxonomic composition. FlowCAM theoretically allows combining flow cytometry capabilities and microscopy accuracy, but significant methodological improvements are still required (See et al. 2005; Álvarez et al. 2011, 2014; Romero-Martínez et al. 2017). Recently, molecular techniques (metabarcoding) have been developed for inventorying taxa in environmental samples, and they can efficiently identify rare, unknown, or hidden microorganisms (Grzebyk et al. 2017). However, even promising, such approaches are not yet adapted to frequent and rapid monitoring because they are still in development and lack standardized procedures, they remain time-consuming and expensive, and will require inter-calibration efforts with classical methods. Moreover, they remain very sensitive to laboratory protocols and statistical analysis strategies (Eckford-Soper et al. 2018) and require better supplemented DNA reference libraries (Rivera et al. 2018; Hering et al. 2018). Beutler et al. (2002) designed a multi-wavelength probe called FluoroProbe® (BBE-Moldaenke, GmbH). This is a submersible spectrofluorometer that can assess chlorophyll biomass and distinguish the main phytoplankton classes by using the autofluorescence properties of pigment-containing microorganisms. FluoroProbe® contains five light-emitting diodes (450, 525, 570, 590, and 610 nm) for excitation of the accessory pigments associated with the photosystem-II antenna system of phytoplankton and one 370 nm diode for excitation and subsequent subtraction of the fluorescence from chromophoric dissolved organic matter (CDOM). This technology is now widely applied in very different environments by scientists and freshwater resource managers for lab and in situ studies of phytoplankton communities (Rolland et al. 2010; Catherine et al. 2012, 2016; Švanys et al. 2014; Patidar et al. 2015; Maloufi et al. 2016; Morgan-Kiss et al. 2016; Poxleitner et al. 2016; Silva et al. 2016; Wang et al. 2016; Blottière et al. 2017; Cyr 2017; Karpowicz and Ejsmont-Karabin 2017; Giling et al. 2017; Teufel et al. 2017). FluoroProbe® main advantage is the immediacy of the performed measurements, although it does not give the same level of taxonomic precision as microscopy-based methods, and may significantly underestimate the biomass when phytoplankton communities are very dense (i.e., chlorophyll *a* $> 250 \mu\text{g L}^{-1}$, Wang et al. 2016), or in samples with very low biomasses (Bradie et al. 2018). Moreover, the relationships between

fluorescence, chlorophyll *a* value, and the proportion of the different phytoplankton groups could be disturbed by strong variability linked to the phytoplankton community structure, environmental conditions, physiological status, quenching, and irradiance history of individual algal cells, making the conversion to phytoplankton biomass sometimes imprecise (Lawrenz et al. 2010; MacIntyre et al. 2010; Catherine et al. 2012; Escoffier et al. 2015; Blottière et al. 2017). The quality of the provided estimates is also strongly dependent on the instrument calibration (factory or home-made settings), which is generally performed using laboratory-grown phytoplankton strains that might not exactly reflect the environmental conditions of natural samples (Harrison et al. 2016; Silva et al. 2016). Nevertheless, MacIntyre et al. (2010) reported that the relationships between fluorescence intensity and chlorophyll *a* are robust when the fluorescence variation range is large enough, and when there is qualitative agreement between the phytoplankton classes defined by their spectral fluorescence signatures and by other analytical methods. Few studies have used FluoroProbe® measurements for the analysis/monitoring of coastal and transitional waters. See et al. (2005) found that in the Gulf of Mexico, diatom and dinoflagellate contributions to the whole phytoplankton biomass were comparable when using FluoroProbe® and HPLC. Similar results were reported by Liu et al. (2012) by comparing FluoroProbe® estimates, HPLC results, and microscope observations in the Yellow Sea, except when the biomass was very low (i.e., $< 1 \mu\text{g L}^{-1}$). van Beusekom et al. (2009) concluded that by combining the FluoroProbe® technology and microscopy, the small-scale plankton dynamics (biomass and taxonomic spectrum) could be fully captured in the Baltic Sea. Richardson et al. (2010) showed that in US estuaries, the estimates of biomass and taxonomic structure of phytoplankton obtained using the Algae Online Analyzer (a spectral fluorometer, like FluoroProbe®) were consistent with those derived from HPLC-measured marker pigments. Houliez et al. (2012) confirmed that FluoroProbe® and flow cytometry allowed obtaining similar *Phaeocystis globosa* annual dynamic estimates in the coastal waters of the eastern English Channel. More recently, Gordon et al. (2016) efficiently documented the temporal changes of phytoplankton assemblages associated with a shift from oligohaline to hyperhaline conditions in the St Lucia Lake (South Africa) using a FluoroProbe®. However, to our knowledge, no study has investigated FluoroProbe® performance for assessing the long-term phytoplankton dynamics in

euryhaline Mediterranean coastal lagoons, which often are characterized by very variable trophic statuses.

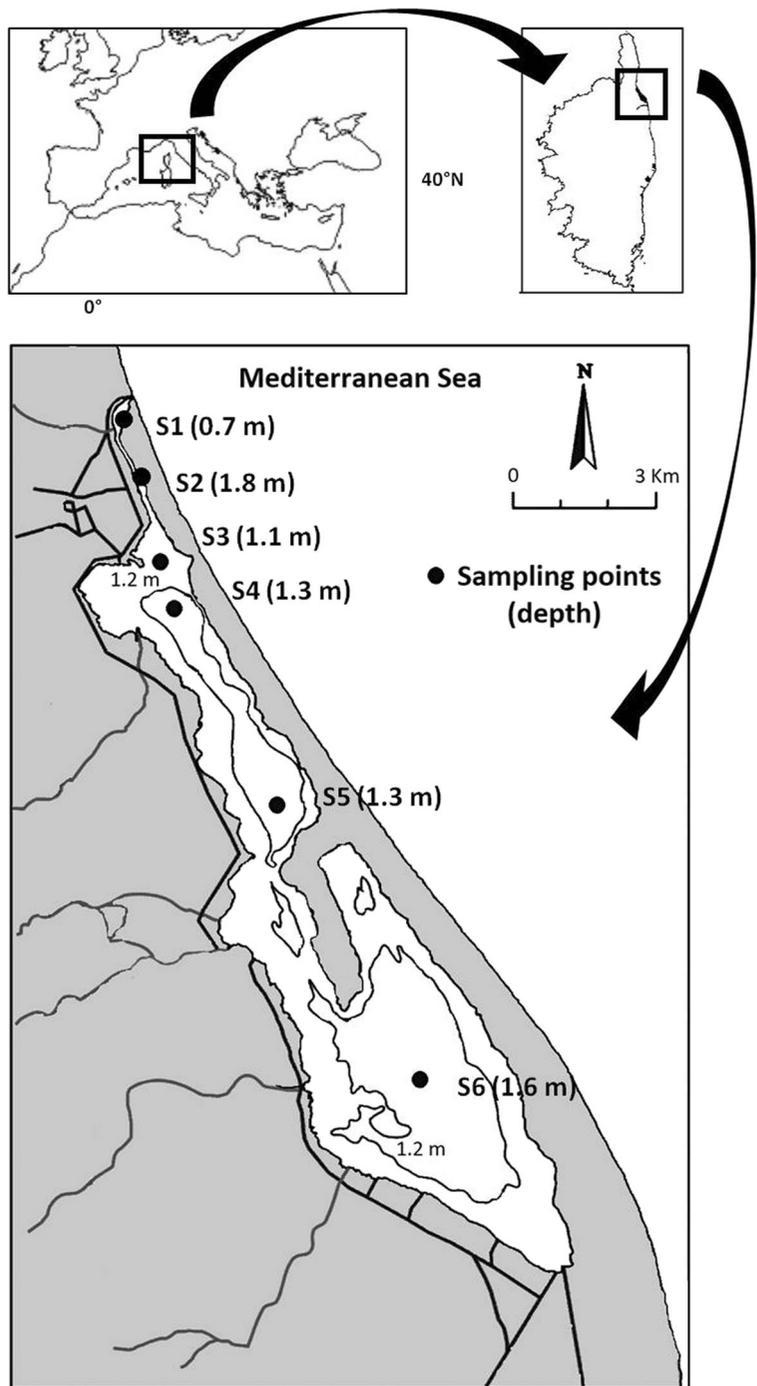
Here, we tested FluoroProbe® ability to estimate the phytoplankton biomass and to discriminate the phytoplankton spectral groups, by using sub-surface water samples ($N = 107$) collected in the Biguglia lagoon (Corsica) in very different environmental situations (salinity and trophic state) over 3 years. For this purpose, we compared FluoroProbe® estimates of total biomass with the spectrofluorimetrically measured chlorophyll *a* values, and the FluoroProbe® in situ and lab estimates of the taxonomic structure with HPLC-based identification of marker pigments of the studied phytoplankton communities. Microscope counts were available only for a small fraction of the samples (see Garrido et al. 2016) and were not included in the study. Moreover, the main end-users of our results (i.e., managers of Mediterranean coastal lagoons) rarely have the required taxonomical expertise. Indeed, this study wants to provide a simple, quick, and efficient tool for non-specialists who need to characterize and monitor the ecological status of the water bodies they supervise.

Materials and methods

Sampling site and sample preparation

Samples were collected in the Biguglia lagoon (Corsica, France, Mediterranean Sea; Fig. 1). This shallow coastal lagoon is a confined ecosystem that is disturbed by growing eutrophication and that exhibits a wide range of environmental conditions and trophic states documented since the 1980s (Bec et al. 2011; Garrido et al. 2013, 2016; Pasqualini et al. 2017). From 2000, a very sharp drop of salinity has been observed in the entire lagoon, associated with a gradual closing of the natural grau (sea side inlet) and a simultaneous increase (controlled or not) in the freshwater input. This hydrological context affected the water quality: accumulation of nutrients supplied by run-off and reduced possibility of dilution by seawater. This situation alerted the public authorities in charge of the management and protection of this ecosystem, and remediation efforts have been undertaken to improve the water quality of the Biguglia lagoon, mainly through the management of water fluxes: artificial openings of the grau in the North and increased freshwater entrance in the South. The effects of such hydrological manipulations have been

Fig. 1 Situation map: the Biguglia lagoon and the sampling points (depths are indicated)



monitored through a long-term survey of the phytoplankton communities.

Grab samples were collected regularly at six different sampling points (Fig. 1), from March 2012 to December 2014. All sampling points were deep enough for the in

situ use of the FluoroProbe®. Water samples ($n = 107$) were collected in a Niskin-like bottle and immediately distributed in opaque 1-L flasks. Two samples were systematically collected at each location, one ≈ 50 cm below the surface and a second ≈ 50 cm above the

sediment (in order to avoid contamination by bottom resuspension), with the aim of tracking the contrasting phytoplankton communities that may inhabit the two horizons (see for example Table 3 in Garrido et al. 2013 and Fig. 7 in Garrido et al. 2016).

Sample preparation and analyses were performed within 2 h after sample collection. For each sample, salinity, temperature, dissolved oxygen, and turbidity were measured in situ with a portable multi-parameter meter (YSI Environmental Monitoring Systems). The concentration of dissolved inorganic nutrients was measured in aliquots filtered through Whatman GF/F filters (0.7 μm nominal pore size) using the methods described by Holmes et al. (1999), Raimbault et al. (1999), and Aminot and K erouel (2007).

FluoroProbe® measurements

Phytoplankton biomass ($\mu\text{g eq. Chl } a \text{ L}^{-1}$) was assessed in situ and at lab using a FluoroProbe®. This device can differentiate between “spectral” groups of phytoplankton on the basis of their relative chlorophyll *a* fluorescence intensity at 680 nm (due to the photosystem-II core pigments; Beutler et al. 2002; Leboulanger et al. 2002; Gregor and Mař alek 2004). The instrument segregates phytoplankton in xanthophyll-containing brown algae (dinoflagellates, diatoms, and chrysophyceae) (525 nm), green algae (chlorophyceae, euglenophyceae, and prasinophyceae) rich in chlorophyll *a* and *b* (450 nm), blue-green algae (phycocyanin-rich cyanobacteria) (590 and 610 nm) and red algae (phycoerythrin-rich cyanobacteria and cryptophyceae) (570 nm; Beutler et al. 2002). The system detects a set of characteristic fingerprints that are used by dedicated software (version 2.2.6) to calculate the relative contributions of each algal class to the global phytoplankton biomass (Beutler et al. 2002). The UV LED (370 nm) that was added to the latest generation of devices is used to measure the fluorescence of CDOM, and their eventual influence is corrected by the software. Given the very contrasting phytoplankton communities potentially associated to the different environmental conditions of our dataset, the FluoroProbe® was used with the factory calibration. The factory fingerprints were obtained by the manufacturer from repeated fluorescence measurements using 35 laboratory cultures that involved several species per spectral algal group (green: *Scenedesmus* sp., *Chlamydomonas* sp., *Monoraphidium* sp., *Chlorella* sp., *Miractinium* sp.; blue: *Microcystis* sp.,

Synechococcus sp., *Aphanizomenon* sp., *Anabaena* sp.; brown: *Cyclotella* sp., *Nitzschia* sp., *Synedra* sp., *Ceratium* sp., *Peridinium* sp.; mixed: *Cryptomonas* sp.). The associated Chl *a* amounts were determined by HPLC. Samples were previously adapted to the measuring light for 90 s and fluorescence measurements were performed at 20 $^{\circ}\text{C}$. For more details, see Beutler et al. (2002). This corresponds also to the simplest way of using this tool by non-specialists.

The in situ measurements using the FluoroProbe® were performed at 50 cm below the surface and at 50 cm above the sediment. In the laboratory, FluoroProbe® measurements were done using 25-mL fractions of each sample placed in the original optical glass cuvette (25 \times 25 \times 70 mm) fitted into the device. Samples were systematically dark-adapted for 20 min before measurements (Beutler et al. 2002). The total chlorophyll *a* concentration and the equivalent concentrations of chlorophyll *a* for each phytoplankton class identified on the basis of the fluorescence signals were calculated with the FluoroProbe® software.

Spectrofluorimetric quantifications

Chlorophyll *a* was quantified using 100 mL of each sample filtered through Whatman GF/F filters (25 mm diameter; 0.7 μm nominal pore size) under gentle vacuum, and immediately stored at -20°C after filtration. The used filters were ground in 90% acetone and extracted in the dark at 4 $^{\circ}\text{C}$ for 24 h. Pigment content was then measured by spectrofluorimetry (Perkin–Elmer LS50b) and calculated according to Neveux and Lantoine (1993).

HPLC analysis

Pigments were analyzed by HPLC as described in Leruste et al. (2015, 2016). In the laboratory, 1 L of each sample was filtered through Whatman GF/F filters (47 mm diameter) under low-vacuum pressure ($< 100 \text{ mmHg}$) and immediately stored at -20°C or at -80°C when possible. Pigments were extracted with 2.5 mL of 100% methanol in the dark at 4 $^{\circ}\text{C}$ for 5 min. Samples were then sonicated 5 times for 10 s (20 W) spaced by 10 s in ice to avoid excessive heating of the extracts. After 10 min in the dark at 4 $^{\circ}\text{C}$, extracts were filtered on cellulose acetate filters (0.45 μm pore size) to remove cell debris. An aliquot of 600 mL of each sample was diluted with 150 mL of Milli-Q water,

and then 150 mL of this dilution was injected in the HPLC system (Waters HPLC Alliance D600). Pigment extracts were analyzed using the method described by Wright and Jeffrey (1997) with a flow rate of 1 mL min⁻¹ and a run duration of 29 min. The HPLC system was calibrated with external standards (DHI Water and Environment, Hørsholm, Denmark). Chromatograms were extracted at 440 nm, and pigments were identified by comparison with a spectral library established from the pigment standard database and by checking the elution order and absorption spectra (Roy et al. 2011), using the software Empower Pro 3. Each peak was checked and the baseline readjusted to minimize errors due to noise. Pigments were then quantified by comparing the peak area with the standard calibration curves (mg L⁻¹).

To establish the correlations between HPLC quantifications and FP results, a series of marker pigments were used: (i) peridinin for dinoflagellates and chrysophyceae, and fucoxanthin for diatoms and chrysophyceae; (ii) chlorophyll *b*, prasinoxanthin, lutein, violaxanthin and neoxanthin for chlorophyceae, euglenophyceae and prasinophyceae; (iii) zeaxanthin for cyanobacteria; and (iv) alloxanthin for cryptophyceae (Vidussi et al. 2001; Marty et al. 2008; Bel-Hassen et al. 2009).

Statistical analyses

Linear regressions were performed to assess the relationship between the phytoplankton biomass values deduced from the in situ and lab FluoroProbe® measurements and those quantified using a spectrofluorometer and HPLC analyses. The Chi² tests and Kruskal-Wallis one-way analyses of variance on ranks were used to compare outliers and ‘regular’ data. Bravais-Pearson correlation coefficients, regression slopes, and the associated hypothesis tests were then carried out to detect (i) correlation and regression slope coefficients different from 0, which indicated a significant linear correlation between measurements, and (ii) regression slope coefficients different from 1, which indicated a lack of quantitative agreement between measurements. Cases where, for example, the FluoroProbe® measurements exceeded the spectrofluorometer or HPLC measurements are illustrated by the location of most observations below the 1:1 regression line. All statistical analyses were done with the R software (R Core Team 2015). The probability threshold value was fixed at $p = 0.01$.

Results

In situ and lab phytoplankton biomass measurements with the FluoroProbe®

The dataset encompassed very different environmental situations (Table 1) that are fully representative of conditions encountered in euryhaline Mediterranean lagoons throughout the year (Souchu et al. 2010; Bec et al. 2011; Leruste et al. 2016; Le Fur et al. 2018), although hypertrophic conditions (Chl *a* > 100 µg L⁻¹), which sometimes occur in such systems during summer (Derolez et al. 2019) were not observed during this survey. The in situ and lab FluoroProbe® values of 15 of the initial 107 water samples were considered a posteriori as outliers (Fig. 2). These 15 outliers did not reveal any specific pattern or peculiarity when compared with ‘regular’ samples. Samples were collected from 2012 to 2014, and the FluoroProbe® measurement distribution of outliers over time was not different compared with that of ‘regular’ data (Chi² = 2.078; $p = 0.149$). Moreover, their presence could not be explained by a seasonal effect, when considering the calendar (Chi² = 0.304; $p = 0.582$) and the hydrological seasons (Cecchi et al. 2016) (Chi² = 1.109; $p = 0.292$). Similarly, the sampling site (Chi² = 2.059; $p = 0.151$) and sampling depth (Chi² = 0.327; $p = 0.567$) also did not influence the occurrence of outliers. Finally, comparison of the environmental conditions (temperature, salinity, turbidity, nutrient concentrations) and of the phytoplankton biomass and the contribution of the four different spectral groups that constituted this biomass did not highlight any significant difference between outliers and ‘regular’ samples ($p > 0.05$; Kruskal-Wallis one-way ANOVAs on ranks). This suggests that there is a

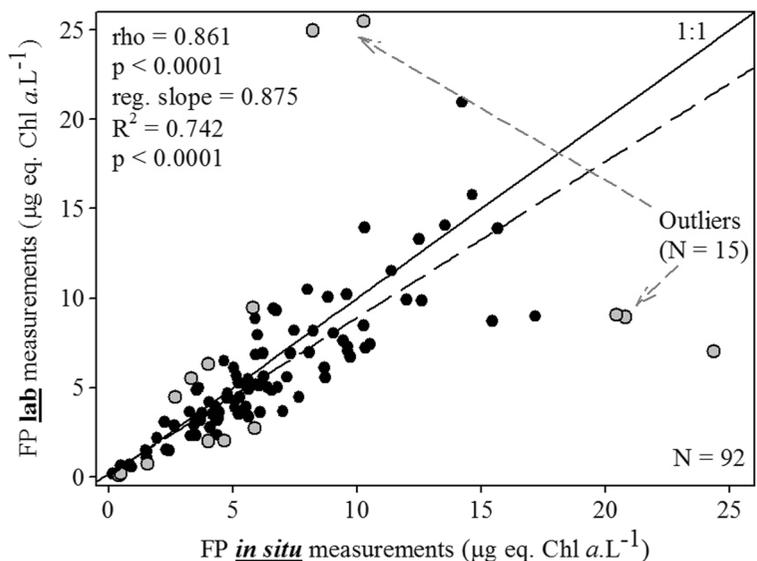
Table 1 Environmental characteristics of the phytoplankton samples considered in the study

	<i>N</i>	Mean	Min	Max
Temperature (°C)	137	18.5	6.9	30.1
Turbidity (NTU)	131	3.7	<LoD	35.4
Salinity	137	13.9	2.0	38.3
Dissolved oxygen (%)	116	91.2	39.9	167.1
Ammonium (µM)	93	5.3	<LoD	48.2
Nitrate (µM)	93	8.1	<LoD	93.2
Phosphates (µM)	84	0.2	<LoD	1.3
Chl <i>a</i> (µg L ⁻¹ , spectrofluorometer)	137	6.3	0.1	42.2

<LoD below limit of detection

stochastic, but real risk of misleading FluoroProbe® measurements (with a frequency of 3 measurements in 20). It is not possible for a non-specialist to immediately identify outliers, whereas they are easily graphically identifiable a posteriori. Therefore, we defined an empirical, but simple and efficient method for discarding such data. After performing the in situ and lab FluoroProbe® measurements of one sample, if the difference between the obtained values is higher than 50% of the in situ measurement, then the data should be discarded. After outlier elimination, the in situ and lab FluoroProbe® measurements of chlorophyll *a* were closely correlated (Bravais-Pearson's $\rho = 0.861$; regression slope = 0.875; $p < 0.0001$ in both cases, $N = 92$; Fig. 2), and the slope coefficient was not statistically different from 1 ($p = 0.023$). The relationships between the chlorophyll *a* concentrations provided by lab FluoroProbe® measurements and the spectrofluorimetric analyses were highly significant (Fig. 3a). The Bravais-Pearson's ρ (0.863) and regression slope (0.913) coefficients were significantly different from 0 ($p < 0.0001$ in both cases), while the regression slope coefficient was not different from 1 ($p = 0.124$). Similar results were obtained for the in situ FluoroProbe® measurements ($\rho = 0.739$), although a few points were more dispersed, as indicated by the lower value of the regression slope (0.793; Fig. 3b), with a coefficient that was significantly different from 1 ($p = 0.008$). Overall, the in situ FluoroProbe® measurements tended to slightly, but systematically overestimate the concentrations of phytoplankton biomass.

Fig. 2 Relationship between lab and in situ FluoroProbe® measurements. Influential outliers (gray dots; $n = 15$ out of 107 samples) were not included in the statistical analyses. The solid line corresponds to the 1:1 regression line and the dashed line corresponds to the trend line from simple linear regression

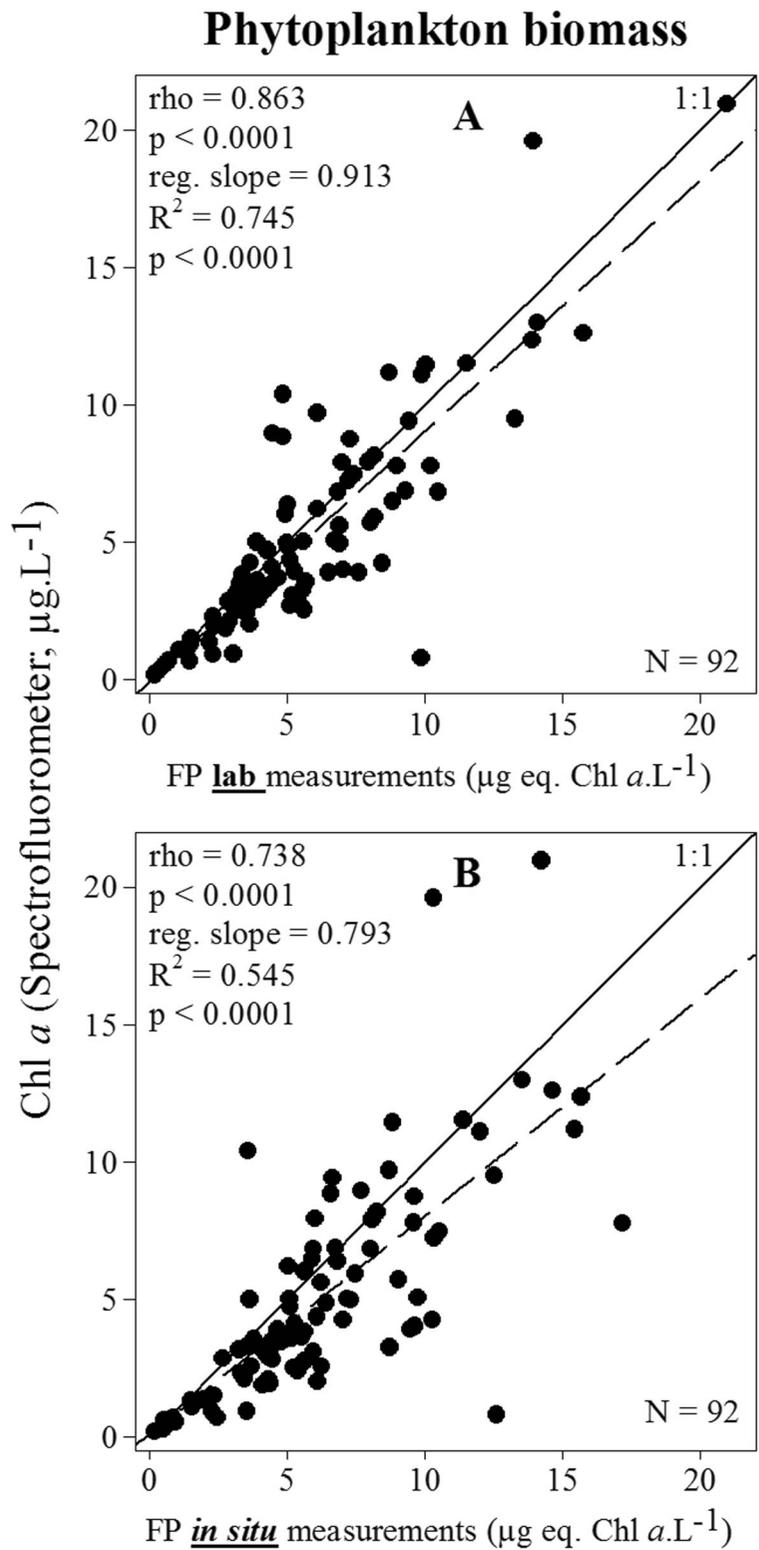


In agreement, in the 15 outliers, the lab FluoroProbe® measurements were closely correlated with the spectrofluorimetric and HPLC quantifications of chlorophyll *a* concentration (Spearman rank order correlation coefficients: 0.807 and 0.868, respectively; $p < 0.0001$ for both), but not the in situ FluoroProbe® measurements (Spearman rank order correlation coefficients: 0.404 and 0.611, $p = 0.131$ and 0.015, respectively).

Composition of phytoplankton assemblages

To evaluate FluoroProbe® ability to describe the composition of phytoplankton assemblages, we compared the relationships between the biomass of each of the four discriminated groups calculated by FluoroProbe® and the HPLC-measured concentrations of the accessory pigments specific to each group. For brown algae, the correlations between HPLC (peridinin and fucoxanthin) and FluoroProbe® data (diatoms, dinoflagellates, and chrysophyceae) were significant (Fig. 4a, b). Both Bravais-Pearson's correlation coefficients (0.774 and 0.700 for lab and in situ FluoroProbe® measurements, respectively) and linear correlation coefficients (0.599 and 0.490 for lab and in situ measurements, respectively) were higher for lab measurements. The slope coefficients were different from 0 and also from 1 ($p < 0.0001$ in both cases). Both approaches correctly described the main variations of brown algae contribution to the whole biomass, although in situ FluoroProbe® data appeared more scattered than the lab values. We observed exactly the

Fig. 3 Relationship between spectrofluorimetric measurements and lab (a) and in situ (b) FluoroProbe® assessments of the phytoplankton biomass. The solid line corresponds to the 1:1 regression line and the dashed line corresponds to the trend line from simple linear regression



same trends after excluding the dot corresponding to the highest biomass value (Bravais-Pearson's correlation coefficients: 0.692 and 0.667 for lab and in situ FluoroProbe® measurements, respectively; $p < 0.0001$ for both; linear correlation coefficients: 0.479 and 0.445 for lab and in situ measurements, respectively; $p < 0.0001$ for both). For green algae, also the correlations between HPLC (chlorophyll *b*, prasinoxanthin, lutein, violaxanthin, and neoxanthin) and FluoroProbe® data (chlorophyceae, euglenophyceae, and prasinophyceae) were very significant (Fig. 4c, d) (Bravais-Pearson's rho = 0.554 and 0.558 and linear regression coefficients = 0.307 and 0.311 for lab and in situ measurements, respectively), indicating that the two methods provided equivalent information. The slope coefficients were different from 0 and also from 1 ($p < 0.0001$ for both). For red algae, the correlations between HPLC (alloxanthin) and FluoroProbe® measurements (cryptophyceae) were again significant, with stronger relationships for the lab FluoroProbe® estimates (Bravais-Pearson's rho = 0.533 and 0.489 and linear regression coefficients = 0.285 and 0.240, for lab and in situ measurements, respectively; $p < 0.0001$ in all cases) (Fig. 4e, f). The slope coefficients were statistically different from 0 and also from 1 ($p < 0.0001$ in both cases). However, the exclusion of

the most elevated biomass value from the dataset considerably reduced the strength and significance of the correlations for lab measurements (Bravais-Pearson's correlation coefficient = 0.287 and linear regression coefficient = 0.082) that, nevertheless, remained significant ($p = 0.006$ for both). This was no longer the case for the in situ FluoroProbe® data (Bravais-Pearson's coefficient = 0.219 and linear regression coefficient = 0.048, $p = 0.037$ for both). The dispersion of data associated with the smallest biomasses (i.e., $< 1 \mu\text{g eq. Chl } a \text{ L}^{-1}$) could be largely responsible of this loss of significance. For blue-green algae, the correlations were significant only between HPLC (zeaxanthin) and lab FluoroProbe® measurements (cyanobacteria) (Bravais-Pearson's coefficient = 0.423 and linear regression coefficient = 0.178; $p < 0.0001$ in both cases) (Fig. 4g), but not in situ measurements (Bravais-Pearson's correlation = 0.164, $p = 0.119$ and linear regression coefficients = 0.028, $p = 0.113$) (Fig. 4h). The points were largely dispersed relative to the regression line. Moreover, the concentrations were very low (median concentrations: $0.05 \mu\text{g L}^{-1}$ for HPLC-quantified zeaxanthin and $0.06 \mu\text{g eq. Chl } a \text{ L}^{-1}$ for in situ FluoroProbe®-assessed cyanobacteria), particularly when compared with those of the other taxonomic groups (see concentration distribution for the different

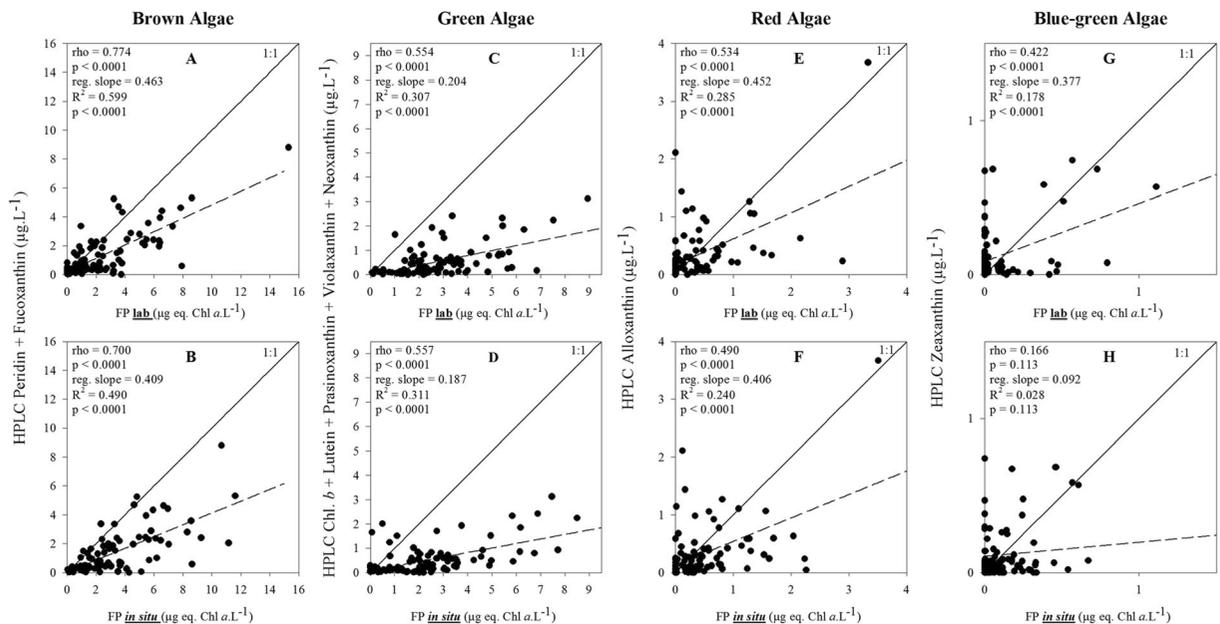


Fig. 4 Relationships between HPLC measurements of specific accessory pigments and lab and in situ FluoroProbe® assessments of the biomass of the different phytoplankton groups ($N = 92$): xanthophyll-containing brown algae (a, b); green algae rich in chlorophyll *a* and *b* (c, d); phycoerythrin-rich red algae (e, f) and

phycocyanin-rich blue-green algae (g, h) ($N = 92$ samples). The scales of the X- and Y-axes are not the same for the different phytoplankton groups. The solid line corresponds to the 1:1 regression line and the dashed line corresponds to the trend line from simple linear regression

groups in Fig. 5), and were close to the detection limits of the instrument. Overall, the relationships were generally more robust with lab than with in situ FluoroProbe® measurements, although FluoroProbe® tended to systematically overestimate phytoplankton biomass.

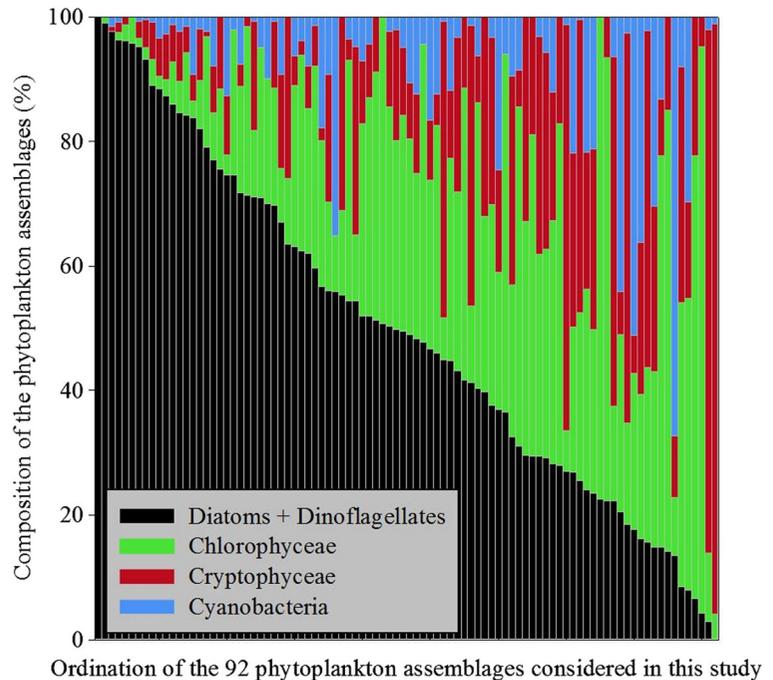
Finally, analysis of the HPLC data on the phytoplankton assemblage composition revealed an important heterogeneity (Fig. 5). The contribution of cyanobacteria varied between 0 and 67%, but remained almost always marginal (median value = 3%). Cyanobacteria were mostly associated with small phytoplankton biomasses, as indicated by the inverse relationship between the percentage of cyanobacteria and the chlorophyll *a* concentration (by spectrofluorometer) (Spearman rank order correlation coefficient = -0.336 , $p = 0.0011$). The contribution of diatoms and dinoflagellates varied from 0 to 100%, with a median value of 49.8%. Diatoms and dinoflagellates were often associated with elevated chlorophyll *a* biomasses (Spearman rank order correlation coefficient = 0.262 , $p = 0.0117$). The contributions of chlorophyceae and cryptophyceae (median values: 26% and 17%, respectively) varied independently of the chlorophyll *a* concentration (Spearman rank order correlation coefficients = -0.0812 and -0.0556 ; $p = 0.441$ and 0.598 , respectively) and were not linked one to the other (Spearman rank order correlation coefficients = 0.101 , $p = 0.338$), or with the abundance of cyanobacteria

(Spearman rank order correlation coefficients = 0.0418 and 0.0574 , $p = 0.692$ and 0.586 , respectively). Conversely, their contributions were strongly and inversely correlated with the variations in diatom and dinoflagellate assemblages (Spearman rank order correlation coefficients = -0.626 and -0.602 for chlorophyceae and cryptophyceae, respectively, $p < 0.0001$ for both).

Discussion

Water quality monitoring in coastal ecosystems is crucial owing to the multitude and magnitude of environmental threats. In France, it is also an obligation registered in the EU-WFD. Phytoplankton taxonomy-based tools are already available, but they require important laboratory efforts and a solid expertise, particularly for the correct identification of organisms. Alternative approaches that exploit the fluorescence properties of algae, such as the spectrofluorometer Fluoroprobe®, are now routinely used in freshwater ecosystems (Catherine et al. 2016; Švanys et al. 2014; Patidar et al. 2015; Maloufi et al. 2016; Morgan-Kiss et al. 2016; Poxleitner et al. 2016; Silva et al. 2016; Wang et al. 2016; Blottière et al. 2017; Cyr 2017; Karpowicz and Ejsmont-Karabin 2017; Giling et al. 2017; Teufel et al. 2017). However, the performances of this instrument have never been

Fig. 5 HPLC-deduced composition of the phytoplankton assemblages of the 92 water samples considered in this study (% of specific accessory pigments)



formally assessed in Mediterranean coastal ecosystems, where both environmental conditions and the composition of phytoplankton assemblages are hugely variable and different from those of freshwater systems. To evaluate Fluoroprobe® performance (phytoplankton biomass quantification and discrimination of the main phytoplankton classes) in such conditions, we used 107 samples collected at six sites in the coastal Biguglia lagoon between March 2012 and December 2014. Our aim was to provide valid outcomes for users who are not specialists in phytoplankton (ecology and taxonomy), but are involved in the regular monitoring and follow-up of Mediterranean coastal aquatic ecosystems.

One of the first problems associated with this a posteriori approach was the presence of 15 outliers among the 107 FluoroProbe® measurements. Beyond their graphical identification, which remains subjective, we defined an empirical and very simple method (i.e., a difference between the in situ and lab measurements higher than 50% of the in situ) to allow the identification of such outliers by field users and managers who are the main end-users targeted by this study. This implies that lab measurements must also be available. We failed to find any element to explain the presence of outliers. The lab, but not the in situ FluoroProbe® measurements were closely correlated with the spectrofluorimetric and HPLC quantifications of chlorophyll *a* in the 15 outliers. This suggests that in situ measurements are primarily responsible for the observed discordances. Indeed, although the samples used for laboratory analyses (i.e., lab FluoroProbe® measurement and HPLC and spectrofluorimetric quantifications) were theoretically collected at the same depth where the in situ measurement was performed, depth discrepancies cannot be excluded. This could lead to measurement differences in the case of vertical heterogeneity in the phytoplankton community repartition. Such heterogeneity was previously observed (but not during the survey) in the Biguglia lagoon, particularly during periods of important water fluxes that may generate the development of a salt wedge (Garrido et al. 2016), and/or near the marine outlet of the lagoon where important vertical gradients might induce significant stratifications of water masses and their phytoplankton communities (Garrido et al. 2013). Coastal lagoons are by definition transitional ecosystems where both marine and freshwater circulate and often create complex hydrodynamic patterns even at short spatial and time scales. The associated patchiness of phytoplankton structures in such ecosystems has been

known for decades (Platt and Denman 1980; Therriault and Platt 1981) and remains a challenging issue when working on discrete samples. Moreover, the existence of very fine vertical microstructures, particularly in the vicinity of important vertical gradients, should also be considered (Viličić et al. 1989), with possible phytoplankton decay and mortality that may considerably modify their chlorophyll content and thus their fluorescence properties. Finally, turbidity maxima regularly occur at the halocline, which may locally disturb the photosynthetic machinery with important consequences on microalgae fluorescence. Within such microlayers, although the composition of phytoplankton assemblages is not directly involved, adaptive physiological strategies, involving particularly photosynthesis and nutrient uptake, allow phytoplankton to cope with contrasting environments, but affect its auto-fluorescence properties. This has been extensively documented in near-surface microlayers, which were not involved here (sub-surface sampling was systematically performed at about 50 cm below the surface). Depending on the vertical organization of the environmental parameters, particularly in the case of strong thermal stratification (i.e., during summer), phytoplankton cells could be confined in water masses with poor or even deleterious conditions for their metabolism. For instance, variations in light exposure might lead to important physiological adaptations. Alternatively, concomitantly, the pigment ratio (amount of pigments and quantity of active chlorophyll per cell) could vary considerably (see the third edition of the reference book of Kirk 2011) and explain the observed discrepancies. However, such ecophysiological mechanisms seem improbable here, because neither the depth where outliers were sampled (i.e., potentially involving light inhibition of sub-surface samples), nor the season of their collection (e.g., potentially corresponding to stratification periods during summer) appeared to affect the outlier occurrence. Lastly, in situ measurements were performed without any sample preparation, whereas lab measurements were systematically done after sample adaptation to the dark for 20 min, as classically recommended (Beutler et al. 2002). Such difference might justify important modifications in the auto-fluorescence properties of phytoplankton, primarily due to changes in the redox states of photosynthetic chain intermediates and, subsequently, of their respective fluorescence fingerprints. This hypothesis cannot be tested because direct in situ dark-adaptation is impossible with the FluoroProbe® device.

Beyond the large variability of environmental conditions regularly encountered in such ecosystem (Table 1), the natural phytoplankton assemblages sampled and used for testing the FluoroProbe® performance revealed also an important heterogeneity (Fig. 5). Therefore, we may assume that almost all possible types of transitional water were encountered at least once during sampling and were incorporated in the analyses. The strong relationships between chlorophyll *a* concentrations provided by the FluoroProbe® measurements and spectrofluorometer analyses are in agreement with literature data on lakes and reservoirs (Gregor and Maršálek 2004; Gregor et al. 2005; Rolland et al. 2010; Catherine et al. 2012) and on marine and transitional waters (See et al. 2005; MacIntyre et al. 2010; Richardson et al. 2010; Houliez et al. 2012, 2017; Ostrowska et al. 2015). Errors in absolute concentration assessments may arise from various causes. Differences in abiotic environment (e.g., light, nutrient concentrations, CDOM; Lawrenz et al. 2010; MacIntyre et al. 2010) and phytoplankton community composition (especially cell size) can affect the fluorescence responses, resulting in variations in the fluorescence-to-chlorophyll *a* ratio (F^{Chl}) that is used to convert the fluorescence data into chlorophyll *a* units. In addition, as the FluoroProbe® method measures the fluorescence emitted by physiologically active cells, the estimated values of chlorophyll *a* concentration are influenced by the cell physiological status (active, senescent, and/or lysed) that can also affect their pigment content (MacIntyre et al. 2010). The correlations between FluoroProbe® measurements and HPLC analyses were systematically strongly significant for brown, red, and green algae (FluoroProbe® overestimation), in line with the literature (Richardson et al. 2010; Rolland et al. 2010; Catherine et al. 2012), but not for cyanobacteria. FluoroProbe® diagnostic efficiency appeared to be lower also for discriminating very weak biomasses of cryptophytes. Diatoms and dinoflagellates are always well represented and often dominant in Mediterranean lagoon waters (Ayadi et al. 2004; Bec et al. 2011; Carić et al. 2011; Garrido et al. 2016), as observed here. Chlorophyceae and cryptophyceae tend to dominate phytoplankton communities in lagoon areas influenced by freshwater inputs (Gregor and Maršálek 2004; Catherine et al. 2012; Garrido et al. 2016). Cyanobacteria are regularly sampled in Mediterranean lagoons, notably in the most degraded (towards hypertrophy) ecosystems (Bec et al. 2005; Chomérat et al. 2007; Pulina et al. 2011). During the survey, the cyanobacteria biomass in the Biguglia lagoon was always very small, close to the

FluoroProbe® limit of detection value, although an impressive summer bloom of cyanobacteria has already been reported in this ecosystem (up to $100 \mu\text{g L}^{-1}$ in 2007, associated with the massive development of the potentially toxic cyanobacteria *Anabaenopsis circularis*; Ifremer data). The annual fluctuations of cyanobacteria abundance within Mediterranean coastal lagoons exhibit a strong seasonality that could be related to water temperature, availability of nutrients and/or irradiance (Agawin et al. 1998; Bec et al. 2005; Chomérat et al. 2007; Armi et al. 2010). The different causes (environmental and physiological) that may induce interferences or excessive biases when assessing in situ cyanobacteria biomasses have been recently discussed by Zamyadi et al. (2016), who concluded that ‘*bbe* [i.e. FluoroProbe®] has the smallest bias’. The environmental risks and health hazards potentially associated with cyanobacteria are directly linked to the importance of their biomass during proliferation events. The World Health Organization (WHO) indicated thresholds below which the risks remain low to moderate (i.e., $50 \mu\text{g Chl } a \text{ L}^{-1}$ or $100,000 \text{ cells L}^{-1}$) (Chorus and Bartram 1999). Within the context of a monitoring network aware of such possible occurrences (as this is the case in the Biguglia lagoon), even relatively imprecise biomass measurements are useful to alert about a possible change in cyanobacteria abundance, prompting the collection of grab samples for microscopic analyses.

The discrepancies between FluoroProbe® assessments and HPLC analyses for some phytoplankton groups could stem from the heterogeneity of such communities and the fact that the probe was factory-calibrated (species and CDOM concentrations; see the Introduction part in Twiss 2011). Correlations could be significantly improved by calibrating in function of the representative species in the region under study (Leboulanger et al. 2002; Richardson et al. 2010; Houliez et al. 2012; Kring et al. 2014) and by factoring in CDOM concentrations (Lawrenz et al. 2010). This would have been difficult here due to the heterogeneity of the many phytoplankton communities studied. However, the ecological situation of the Biguglia lagoon has changed in recent years. The apparition and durable maintenance of harmful dinoflagellates especially the species *Prorocentrum minimum* (Garrido et al. 2016) requires special attention. In this new context, the recalibration of the instrument with a reference specific of this species would be perfectly justified in order to allow the effective monitoring of this noxious invader by managers. However, one of the disadvantages of the

FluoroProbe is that it is able to discriminate only four phytoplankton groups so that the addition of a new fingerprint is only possible if one of the four default fingerprints is disabled. The selection of this fingerprint is not obvious, and the added value of this re-calibration has thus to be perfectly assessed. Comparisons of the FluoroProbe® dataset against HPLC analyses may sometimes be skewed by the non-specificity of some pigments for phytoplankton taxonomic compositions (Richardson et al. 2010). For example, phycoerythrin-rich cyanobacteria are combined with red algae and this leads to weak correlations when comparing results provided by these two methods (Beutler et al. 2002; Echenique-Subiabre et al. 2016). On another note, FluoroProbe® factory calibration may identify haptophytes as mixtures of brown algae and green algae (See et al. 2005; MacIntyre et al. 2010; Richardson et al. 2010; Houliez et al. 2012). However, haptophytes never constitute significant populations within Mediterranean coastal lagoons (cf. Fig. 5 in Leruste et al. 2016), and noticeably in the Biguglia lagoon (Leruste et al. 2019) where this potential bias remains thus negligible.

Conclusions

In Mediterranean coastal ecosystem, FluoroProbe® provided very good estimates of phytoplankton total biomass and good indications of phytoplankton community composition, after the removal of outliers. Therefore, it can be used to obtain information for water management in a timely manner. A clear advantage offered by this instrument is the ability to differentiate phytoplankton assemblages into broad groups, thus making FluoroProbe® suitable for large-scale surveys of lagoon environments using simply the factory calibrations. FluoroProbe® could be a useful phytoplankton monitoring tool, especially as an early warning system for the detection of harmful algal blooms in lagoon waters that constitute an increasing threat to Mediterranean transitional ecosystems (see Fig. 1 in Cecchi et al. 2016). FluoroProbe® could be used for the long-term monitoring of phytoplankton communities as required by the EU-WFD, because it offers higher temporal resolution than the classical HPLC analysis, in real-time and at far lower cost, with a rapid non-destructive measure of their relative abundance. This is a major advantage given the amount of time and expertise required for the microscopy and

HPLC analyses. The limited information obtained for some groups (particularly, cyanobacteria) suggests that FluoroProbe® should be coupled with other methods to strengthen the precision and quality of the observations. Specific calibrations will undoubtedly increase FluoroProbe® diagnostic performances; however, we strongly recommended complementing FluoroProbe® measurements with less frequent sample collections for microscopic analyses to accurately identify the phytoplankton taxa involved in the spatial-temporal dynamics. Ultimately, users need to determine whether FluoroProbe® inherent error is acceptable for their type of research or monitoring (Kring et al. 2014).

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