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Original Article

Intercalibration of counting methods for *Ostreopsis* spp. blooms in the Mediterranean Sea

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ABSTRACT

This paper describes the adoption and validation of two innovative methods for the automated count of *Ostreopsis* spp. concentration in sea water: a molecular assay based on RT-qPCR and an opto-electronic device implementing automatic recognition algorithms. The proposed approaches were tested on samples coming from different locations along the Mediterranean Sea and compared with the standard counting method based on microscopy observation by a taxonomy expert. The results demonstrate the effectiveness of both automatic approaches which provide a valuable tool, mostly cost and time effective, for the establishment of wide pan-Mediterranean monitoring strategies of *Ostreopsis* spp. blooms. Moreover, the two automatic methods demonstrated the ability to discriminate for the presence of a different but similar species, *O. fattorussoi*, for which new species-specific qPCR primers were developed.

1. Introduction

Over the last decade, episodes of exceptional blooms of microalgae *Ostreopsis* spp., have been reported repeatedly along the coasts of the Mediterranean Sea. *Ostreopsis* is a potentially toxic benthic dinoflagellate that, when reaching high concentration during its blooms, may cause dangerous side effects to human health and marine organisms, therefore being deleterious for commercial activities, tourism, fishery and aquaculture. *Ostreopsis* proliferates in shallow and sheltered waters, usually characterized by low hydrodynamics and by rocky bottoms covered by macroalgal vegetation, forming mucilaginous aggregates (Honsell et al., 2011; Giussani et al., 2015). In presence of wave motion, these dinoflagellates can be resuspended in the water column, giving rise to flocculates and sometimes to surface foam, which can be conveyed in marine aerosols (Ciminiello et al., 2014). Traces of putative palytoxin, among the most powerful non peptidic marine toxins known to date, and ovatoxins are found in *Ostreopsis* samples (Ciminiello et al., 2006, 2012), and *Ostreopsis* blooms were associated to mass death of benthic organisms (sea urchins, limpets and barnacles) (Amzil et al., 2012; Shears and Ross, 2009) and intoxication to humans,

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characterized by irritation of the upper airways, coughing, rhinorrhoea, shortness of breath, muscle and joint pain, fever and irritation of the mucous membranes of the eye and skin (Gallitelli et al., 2005; Durando et al., 2007; Ciminiello et al., 2012).

Notwithstanding the Mediterranean scale of the emerging phenomenon, still little has been produced in terms of guidelines for managing risks *associated* to blooms in the Mediterranean countries. As far as *Ostreopsis* spp. is concerned, no European or international official thresholds in the water (planktonic concentrations) or on macroalgae (benthic concentrations) have been proposed, yet. Since almost all Italian coastal regions are affected by *O.* cf. *ovata* blooms and being Italy one of the Mediterranean countries that earlier experienced some of the most noxious events (Ciminiello et al., 2006), in early 2007 a manual on the management of the risk associated to *O.* cf. *ovata* blooms along the Italian coasts was produced by a group of experts put in charge by the Ministry of Health, formed by the Italian Central Health Institute, Regional Environmental Agencies and scientists. These guidelines were recently updated (Funari et al., 2014, 2015), differentiating thresholds according to environmental conditions: cells concentration between 10,000 and 30,000 cells/L produces a shift from routine phase to alert phase in case of environmental conditions considered favourable to *O.* cf. *ovata* proliferation, while a shift to the emergency phase is set at 30,000 cells/L, only in case of conditions that are favourable to bloom events and to aerosol production. Similarly, in France, since 2007, the French National Health Agency (Direction General de la Santé) indicated a "Warning" threshold at 4000 cell/L and an "Alert" threshold at 30,000 cell/L (Tichadou et al., 2010), since 2009, thresholds have been increased respectively to 30,000 cell/L (Warning) and 100,000 cell/L (Alert). All these thresholds refer to concentrations in the water column. In Spain, the Catalan Water Agency (ACA) setted the threshold at ~100,000 cells/g of macroalgae (fresh weight) or ~10,000 cells/L.

Direct count of algal abundance has been so far the commonly used methodology in accounting for bloom events. Nevertheless, microalgae identification and counting require a great deal of taxonomic expertise, in addition to being time consuming and impractical for processing a large number of samples in the monitoring activities, such as those performed during the summer period for satisfying the sanitary regulatory requirements for bathing waters. The long processing time implies that these operations are carried out at a frequency that may not be appropriate to promptly generate early warning for bathing water associated and productive activities.

In the framework of the ENPI-CBCMED M3-HABs Project, standardized protocols for sampling and counting, together with innovative counting techniques, were produced and tested in a cross-Mediterranean perspective, in order to improve monitoring design. In the Mediterranean Sea, *Ostreopsis* spp. can include various *Ostreopsis* species including *O.* cf. *ovata*, *O.* cf. *siamensis* and the recently described *O. fattorussoi*, which was found in the eastern Mediterranean coastal waters (Lebanon and Cyprus) (Penna et al., 2010, 2014; Accoroni et al., 2016). Generally, all *Ostreopsis* species are extremely difficult to be identified by optical microscopy; meanwhile, they can be detected by PCR and qPCR based assays using species-specific primers (Battocchi et al., 2010; Perini et al., 2011; Casabianca et al., 2014).

Different protocols relying on traditional optical microscopy and two different approaches (automatic opto-electronic and molecular qPCR counting methods) were carried out on the same environmental samples (benthic and planktonic). Results were compared and intercalibrated also with the traditional optical counting method in order to assess reliability of automated identification and different counting methods.

2. Materials and methods

2.1. Study area and sampling procedure

A total of 70 macroalgae (all from the same macrolgal species, *Halopteris scoparia* (Linnaeus) Sauvageau) and 60 seawater samples were collected in triplicate at various Mediterranean sites for the intercalibration exercise: Salammbô (Tunisia), Batroun (Lebanon), Nice and Rochambeau (France), Genoa (Liguria, Italy), Alghero (Sardinia, Italy), Ancona (Marche, Italy) and Llavaneres (Spain). Samples were fixed with 1% acidic lugol solution, and stored at +4 °C until counting analyses by microscopy, opto-electronic and qPCR systems.

Samples were collected during summer of 2014 and 2015. To avoid counting variations associated to differences in the sampling protocol between the different research groups involved in the intercalibration activities, a common procedure was agreed at the end of 2014 in the framework of M3-HABs project and applied at all sampling stations during 2015 campaign. The data collected during both sampling seasons were used in the present work and the agreed and shared protocol used for the 2015 sampling season is reported below.

The sampling frequency was adapted to seasonal variations of *Ostreopsis* blooms in each surveyed Mediterranean area. In the northern Mediterranean Sea, blooms are often occurring between July and August, extending also in September–October. Monitoring of such blooms was performed from June to October, with a sampling frequency of at least once per week, increased to every 2–3 days in the blooming period. In the eastern Mediterranean Sea, samples were collected weekly in the blooming period and monthly all over the year. For each station, water samples (1 L) were collected (indicated as planktonic throughout this paper) before benthic ones (0.5 L), in order to avoid a mechanical re-suspension of benthic cells that might artificially increase planktonic concentrations. Benthic substrates were sampled at 0.5 m depth and planktonic samples were collected at 20 cm above the substrate (corresponding to 0.3 m depth). In order to ensure replication, at least three stations were considered for each site, spaced apart approximately 10 m. The list of sampling stations with the total number of collected replicates is reported in Table 1.

The sampling of benthic substrates was performed by collecting seawater samples and macrophytes (the brown alga *Halopteris scoparia*), using the protocol reported in Mangialajo et al. (2011). In short, isolation of epiphytic cells from macroalgal benthic samples was performed by vigorously shaking (for 10 s) the macrophyte samples, then rinsing with 100 mL of filtered seawater (0.2 µm) and shaking two times. Counting was performed on 1 mL, using the Sedgewick Rafter chamber and cells abundances are reported as number of Ostreopsis per gram of macroalgae, after oven drying at 60 °C the macroalgal sample. For water samples, the Utermöhl method was used, where a sub-sample (in the standardized protocol for *Ostreopsis*: 50 mL) is poured in a cylinder/chamber complex and left to settle for typically 24 h before observation at the inverted microscope. Counting is performed on the whole surface of the chamber or across transects, in order to count a minimum number of 200 cells per sample. Concentrations are reported as cells/L.

2.2. Sample conservation

Environmental samples were fixed using 1% (vol/vol) of acidic Lugol solution (final concentration). Samples were collected at different phases of the bloom (development, peak and decline), in order to improve the intercalibration of counting methods at different cell concentration levels. The samples were processed for the three different counting methods (optical and opto-electronic microscopy, and molecular qPCR assay) in less than one month in order to avoid any deterioration of samples.

Table 1
List of sampling stations. For each station, the LSU gene copy number cell^{-1} determined by qPCR assay from environmental samples are indicated. Only for Batroun, Lebanon the gene copy number cell^{-1} was determined on ITS-5.8S gene. The number of studied samples (collected and analysed in triplicate by all three methods) divided by year was also reported. France samples from Nice and Rochambeau have different rDNA calibration but they were counted and analysed together.

Sampling station	rDNA gene copy number cell^{-1} (± S.D.)	Collected samples			
		Plankton		Benthic	
		2014	2015	2014	2015
Genova, Liguria, Italy	4851 ± 116	3	11	5	9
Llavaneres, Spain	1974 ± 144	n.a.	5	6	4
Rochambeau, France	22269 ± 7406	8	10	14	10
Nice, France	46458 ± 2394	//	//	//	//
Alghero, Sardinia, Italy	94723 ± 10202	n.a.	6	n.a.	6
Salammbo, Tunisia	2009 ± 175	n.a.	5	n.a.	6
Ancona, Marche, Italy	41788 ± 3466	n.a.	5	n.a.	5
Batroun, Lebanon	8333 ± 4616	n.a.	5	n.a.	5

2.3. Manual counting [MAN]

Macroalgal benthic samples were counted using a Sedgewick Rafter Counting chamber (1 mL) and a classical optical microscope. For water samples, the Utermöhl method was used, where a sub-sample (in the standardized protocol for *Ostreopsis*: 50 mL) is poured in a cylinder/chamber complex and left to settle for typically 24 h before observation at the inverted microscope. Counting is performed on the whole surface of the chamber or, in case of high concentration, along two orthogonal transects (cross-like).

2.4. Molecular counting method [MOL]

2.4.1. Sample processing

A volume ranging from 1 to 50 mL and from 1 to 10 mL of water and benthic samples, respectively, were lysed as described in Casabianca et al. (2014). Briefly, samples were centrifuged at 4000 rpm for 10 min or at 12,000 rpm for 10 min at room temperature; the pellets were washed with 1 mL filtered artificial seawater, then centrifuged at 11,000 rpm for 15 min and stored at −80 °C or directly processed. Pellets were resuspended and lysed using a freeze/thaw cycle protocol in 500 µL of lysis buffer (10 mM Tris–HCl pH 8.3, 50 mM KCl, 0.5% Nonidet P40, 0.5% Tween 20, 2.5 mM $CaCl_2$, 0.1 mg/mL Proteinase K). The suspension was incubated at 55 °C for 3 h and vortexed every 20 min. Samples were then incubated at 100 °C for 5 min, to inactivate the proteinase K and centrifuged at 12,000 rpm for 1 min to precipitate cell debris. The supernatants, or crude extracts, were transferred into new tubes, and were diluted at 1:10 for the qPCR experiments.

2.4.2. PCR primer design and specificity

Species-specific primers for the Mediterranean *O. fattorussoi* were designed based on 5.8S-ITS rDNA sequences of *O. fattorussoi* L1000 (accession no. LT220223), and L1020 (accession no. LT220224) from Batroun, Lebanon, and *O. fattorussoi* C1005 (accession no. LT220222) and C1012 (accession no. LN875554) from Vassiliko Bay, Cyprus. The multiple sequence alignments were constructed using ClustalX2 ver. 2.0 (Larkin et al., 2007). Primers were designed using Primer-BLAST (Ye et al., 2012). The species-specific primers for the amplification of 104 bp (Tm = 78 °C) fragment of the 5.8S-ITS rDNA gene were: *O. fattorussoi* str clade sp. F 5ˆ′-TAAATGAAGGGTGCAGCCAAT-3ˆ′; *O. fattorussoi* str clade sp. R 5ˆ′-GTACCAAGGTGATACCCAAGT-3ˆ′. The species-specificity of the primers was first examined *in silico* using BLAST, and then, tested in qPCR on purified genomic DNA. The total genomic DNA was extracted as described in Penna et al. (2010) from cultured *Ostreopsis* spp. strains and other dinoflagellate genera, including also *Ostreopsis* strains collected in different geographical areas, as well as environmental samples previously checked by light microscopy for the presence and/or absence of the *Ostreopsis* spp.

2.4.3. Standard curves for the Ostreopsis spp. quantification

Site-specific environmental standard curves for *O.* cf. *ovata* and *O. fattorussoi* LSU rDNA (LSU-STD) and 5.8S-ITS rDNA (ITS-STD), respectively, were constructed as described in Casabianca et al. (2014). Moreover, a plasmid standard curve for *O.* cf. *ovata* was obtained as described in Perini et al. (2011), while the standard curve for *O. fattorussoi* was constructed, from the purified PCR product generated from the DNA of *O. fattorussoi* L1000 following the method described in Penna et al. (4238).

2.4.4. The qPCR assay of Ostreopsis spp.

The qPCR assay of *O.* cf. *ovata* and *O. fattorussoi* was performed in a final volume of 25 µL using the Hot-Rescue Real-time PCR Kit SG (Diatheva, Fano, Italy), primers at a final concentration of 300 nM, 0.5 U of Hot- Rescue Taq DNA polymerase, and 2 µL undiluted and 1:10 diluted crude extracts of environmental samples. Amplification reactions were carried out using a Step-one Real-time PCR System (Applied Biosystem, Foster City, CA, USA). The thermal cycling conditions consisted of 10 min at 95 °C, followed by 40 cycles at 95 °C for 15 s and 40 cycles at 60 °C. The *O.* cf. *siamensis* qPCR targeting the LSU rDNA genes assay was performed as previously described by Casabianca et al. (2014).

2.4.5. PCR data analysis

For qPCR assay, acquisition of data and subsequent analyses were carried out using StepOne software v. 2.3. A dissociation curve was generated after each amplification run to check for amplicon specificity and primer dimers. The automatically generated standard curves were accepted when the slope was between 3.38 and 3.32 (98–100% efficiency) and the correlation coefficient was at least 0.97.

2.5. Optical recognition and counting method [OPR]

The Optical Recognition Method (OPR) is composed of three main components: a dedicated microscopy platform, an XYZ motorized stage and a custom software implementing a specialized identification algorithm. The system was previously described in detail (Sbrana et al., 2017) and the main components are here recalled.

The hardware platform is based on a custom microscopy design (VacuumFAB srl, Milano, Italy), featuring a 10X plan-apo objective in transmission mode. The XY stage is motorized through a commercial stepper motor controller (Phytron Gmbh, Munich, Germany) connected to the PC via USB. Image acquisition is performed using a DMK 23G274 GigE camera (The Imaging Source, Bremen, Germany) exploiting a fast ethernet connection to a dedicated adapter on the PC. A mechanical interface to the commercial K-frame was developed to house both Utermhöl and Sedgewick-Rafter deposition chambers.

All the peripherals of the system (CCD camera, motors controller,

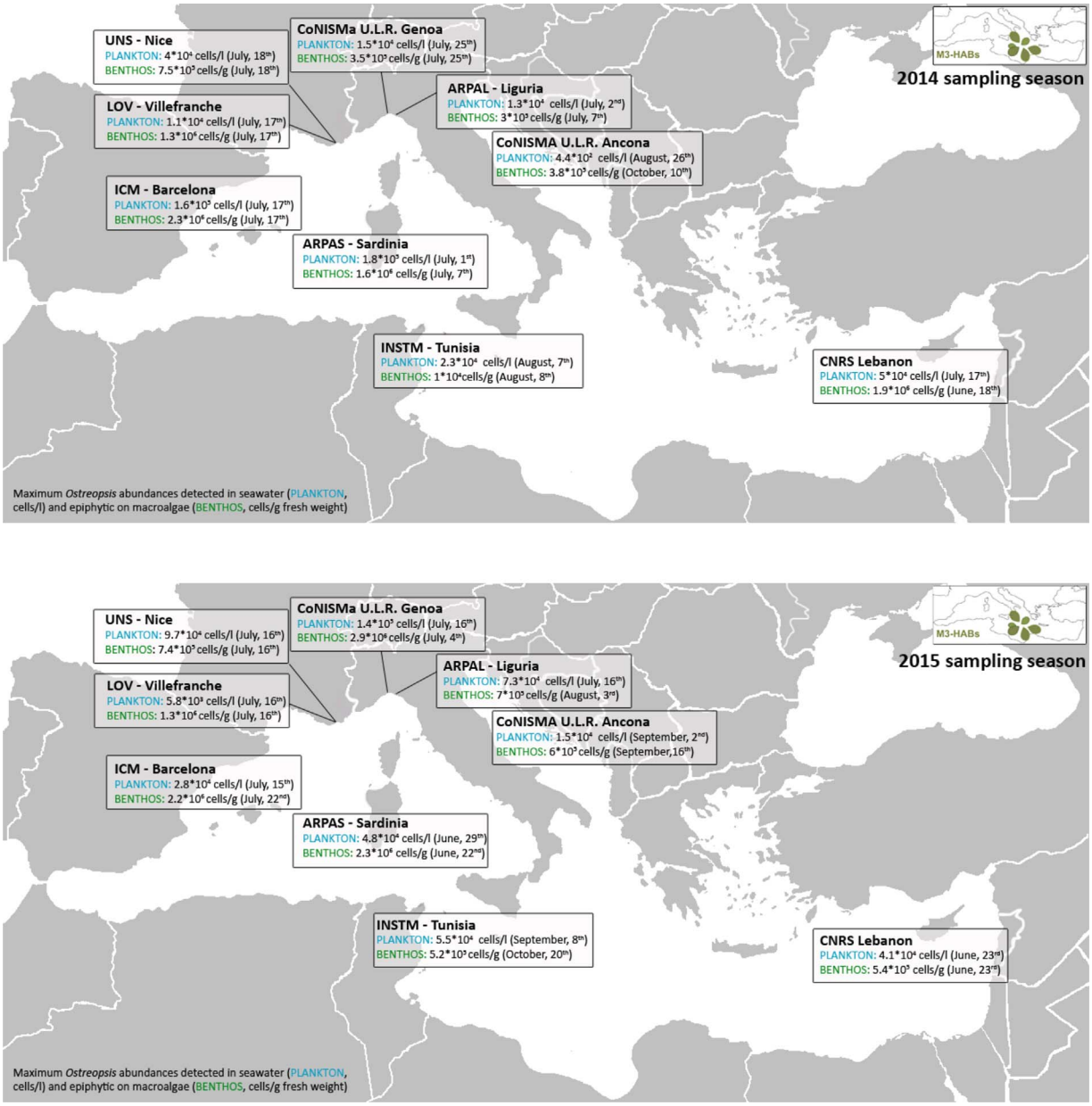

Fig. 1. Maximum abundance of *Ostreopsis* cells in seawater and on macroalgae during 2014 (a) and 2015 (b) summer season in the sampled locations along the Mediterranean Sea.

illumination LED) are connected to a PC and driven by a custom software developed in Labview (National Instruments, TX, USA). The software takes care of all the duties of the acquisition procedure: initialization, calibration, raster motion of the sample over the objective and image acquisition. In counting mode, each image is analyzed by a dedicated procedure to obtain the number of spotted *Ostreopsis* cells inside the field of view.

The software architecture is composed by a set of modules operating on digital images provided by the acquisition engine and computing, as final output, the number of cells in each image.

The automatic cell recognition in digital microscopy images is one of the most "classical" problems in image-based pattern recognition. The solution is often customized for specific types of cells to be counted, but a few aspects are common to every approach. They can be summarized as a list of step to be performed:

- to extract all interesting "objects" from the image (segmentation);
- to evaluate a vector of descriptors (features) for each object;
- to classify each vector in order to identify only those belonging to a given set of species.

The aim of image segmentation is to select all "interesting" objects inside the image, given some basic conditions to be met by them. The result is a set of objects that could be interpreted as cells.

Feature extraction computes, for each object, a set of descriptors allowing a good discrimination between the "true" cells and "false" ones. Here the "true" class is composed only by the *O. ovata* specimens, while the "false" one is made by all other items visible inside the images, keeping attention to the fact that some of them may be confusing. The feature set should be as large as needed to capture all important aspects of the problem.

The last step deals with pattern classification, that means to find the most probable answer to the question whether the current object, described by its feature vector, belongs to the *O. ovata* class or not. This problem can be solved with many different algorithms, based on different statistical classification algorithms trained over a dataset of supervised vectors. Expert supervision is essential to define a set of known mapping between some feature vectors and the corresponding correct

class.

In the OPR system, specific choices have been made in terms of algorithms and parameters, leading to a trained machine able to deal with the intercalibration tests in a fully autonomous way.

2.6. Statistical analysis

In order to quantify the agreement of the automatic methods to the manual one, we calculated the Pearson's correlation coefficient ρ. This parameter provides a quantitative measure of the linear dependence between two set of measures A and B. If set B comes from a certified instrument (in the specific case, the manual count) and the other set (A) comes from a new instrument (any of the automated methods), the Pearson's coefficient is a measure of how well the new method A could be calibrated to be in full agreement with the certified one, B. In linear correlation analysis, the ρ is closely related with the coefficient of determination R^2 or, in other words, ρ^2 represents per percentage of the variance of B which is explained by A. In mathematical terms, ρ is defined as:

$$\rho(A, B) = \frac{\mathrm{cov}(A, B)}{\sigma_A \sigma_B} = \frac{\sum_{i=1}^{n} (x_i - \bar{x})(y_i - \bar{y}))}{\sqrt{\sum_{i=1}^{n} (x_i - \bar{x})^2} \sqrt{\sum_{i=1}^{n} (y_i - \bar{y})^2}}$$

where σ indicates the standard deviation of the dataset and cov is the covariance and the last step follows from the definition of covariance and standard deviation for a sample of n measurements. The Pearson's coefficient ranges between -1 and $+1$, with 0 indicating no correlation. We highlighted values of ρ larger than 0.5, being considered to provide a satisfactory agreement. Additionally, for the cumulative graph of Fig. 4, we also provided the p value, roughly indicating the probability to find an uncorrelated system producing datasets that have a Pearson's correlation at least as extreme as the one computed from these datasets. The use of Pearson's statistical test while comparing an automatic counter with a reference one obtained with careful manual inspection of each sample, is motivated by the fact that automatic counters are based, in several cases, on a statistical decision test. For instance, imaging systems select “objects” inside images and decide if they are or not members of a given biological class (target). This type of automatic decision presents two type of errors (false positives and false negatives), whose rates can be evaluated after experimental tests.

3. Results

3.1. Quantification of Ostreopsis spp. in environmental samples

Environmental samples of macroalgae and surface seawater collected at the four Mediterranean coastal sites during the summers of 2014 and 2015 were analysed by standard light and opto-electronic microscopy and qPCR assay. Data are reported in Fig. 1a and b, where maximum concentrations in seawater and on macroalgae are displayed as well as the time of the year when they were recorded for each summer period (data from traditional optical microscopy).

During the two consecutive summer seasons, concentrations in the water and on macroalgae followed seasonal trends (with higher values reached earlier along the Lebanese coasts), in July in the western Mediterranean basin, and later, in August-October, in Tunisia and Northern Adriatic Sea. Highest concentrations were measured along the Ligurian coast both in the water (in Chiavari, $1.4 \cdot 10^5$ cells/L on July 16th, 2015), and on macroalgae (in Genova Quarto, $2.9 \cdot 10^6$ cells/g on July 4th, 2015).

3.2. Ostreopsis fattorussoi qPCR specificity assay

The species-specificity of primers for *O. fattorussoi*, designed to target 5.8S-ITS rDNA region, was checked as reported in Material and Methods. Primers were highly specific for this species as positive PCR amplification was obtained only with strains of *O. fattorussoi* from eastern Mediterranean (Crete, Cyprus and Lebanon). Further, positive PCR amplifications were obtained in plankton and benthic samples containing target species-specific cells of *O. fattorussoi*. Negative results by qPCR assay were achieved on environmental samples where the *Ostreopsis* spp. was not observed by light microscopy or on environmental samples containing *O.* cf. *ovata* (data not shown).

3.3. Curve characterization of qPCR assay

The environmental samples were analysed for the presence of the *O.* cf. *ovata*, *O.* cf. *siamensis* and *O. fattorussoi*. The *O.* cf. *ovata* was found in all benthic and water samples with exception of Batroun locality (Lebanon), where only the *O. fattorussoi* was found. No PCR amplification were obtained for the presence of *O.* cf. *siamensis* (data not shown).

The seven different site-specific LSU-STD and one 5.8-ITS STD curves for *O.* cf. *ovata* and *O. fattorussoi*, respectively, showed a PCR efficiency of 98–100%, a linear correlation of 5 $\log_{10}$(R^2 = 0.97–0.99) and a quantification limit of 0.0008 cells per PCR reaction. The reproducibility analysed as CV_{Ct} mean inter-assay variability of different environmental standard curves was 0.6% (range 8–0.0008 cells).

The mean pLSUO for *O.* cf. *ovata* and pITSO for *O. fattorussoi* standard curves (R^2 = 0.99) showed 96% and 102% efficiency ($y = -3.42x + 34.76$ and $y = -3.27 + 33.39$, respectively) with a linear correlation of 5 log linear range and a sensitivity of 2 copies/reaction (Ct mean = 33.09 ± 0.09 and 36.98 ± 0.08 for pLSUO and pITSO, respectively). The reproducibility was assayed using the pLSUO and pITSO curves and the CV_{Ct} mean values were 2.5% and 2.6%, respectively, while, the CV_{Cn} mean values were 32% and 37%, respectively.

The *O.* cf. *ovata* LSU rDNA and *O. fattorussoi* ITS-5.8S rDNA copy numbers per cell in environmental samples are presented in Table 1.

3.4. Intercalibration

The correlations between automatic methods (optical and molecular) and the standard manual counting are reported in Fig. 2 for planktonic samples and Fig. 3 for benthic samples. Only sites for which at least 3 samples were collected and counted with all methods, for summer 2014 and 2015, are reported. Single dots represent the average of 3 replicates of the same measurement for each method; the size of the dots was chosen to match the typical repetition incertitude of about 10–15%. The Pearson's correlation coefficient is reported for each dataset, to provide a numerical evaluation of the correlation between the methods. From the point of view of the environmental managing authority, the effectiveness of automatic methods with respect to the manual one is more critical near the alert threshold, in a situation in which the population is starting to grow, and for which a correct estimate is crucial for managing the bloom and forecasting any potentially upcoming criticality. In turn, the analysis of the results was focused in a region we called *sensible region*, defined taking ± 1 decade around the threshold concentration (see Section 4). This region is highlighted in Figs. 2–4 as a white central band. All statistical parameters were calculated taking into account only points lying inside this confidence region, neglecting very high and very low concentrations.

The overall agreement between counting methods is shown in Fig. 4 in which all planktonic and benthic data for all sites are plotted together (panels A and B). Statistical Pearson's correlation coefficient was calculated using only data points lying inside the sensible region (see Section 3.3), indicated in white. Overall, more than 50 different measurements were recorded and plotted in Fig. 4, allowing to associate a meaningful p-value to the proposed linear regression. Panels C and D of the same figure provide a closer look to the region around the alert threshold (10^4 cells/L for plankton and 10^5 cells/g for benthos, see discussion). The coloured areas provide a visual information about the

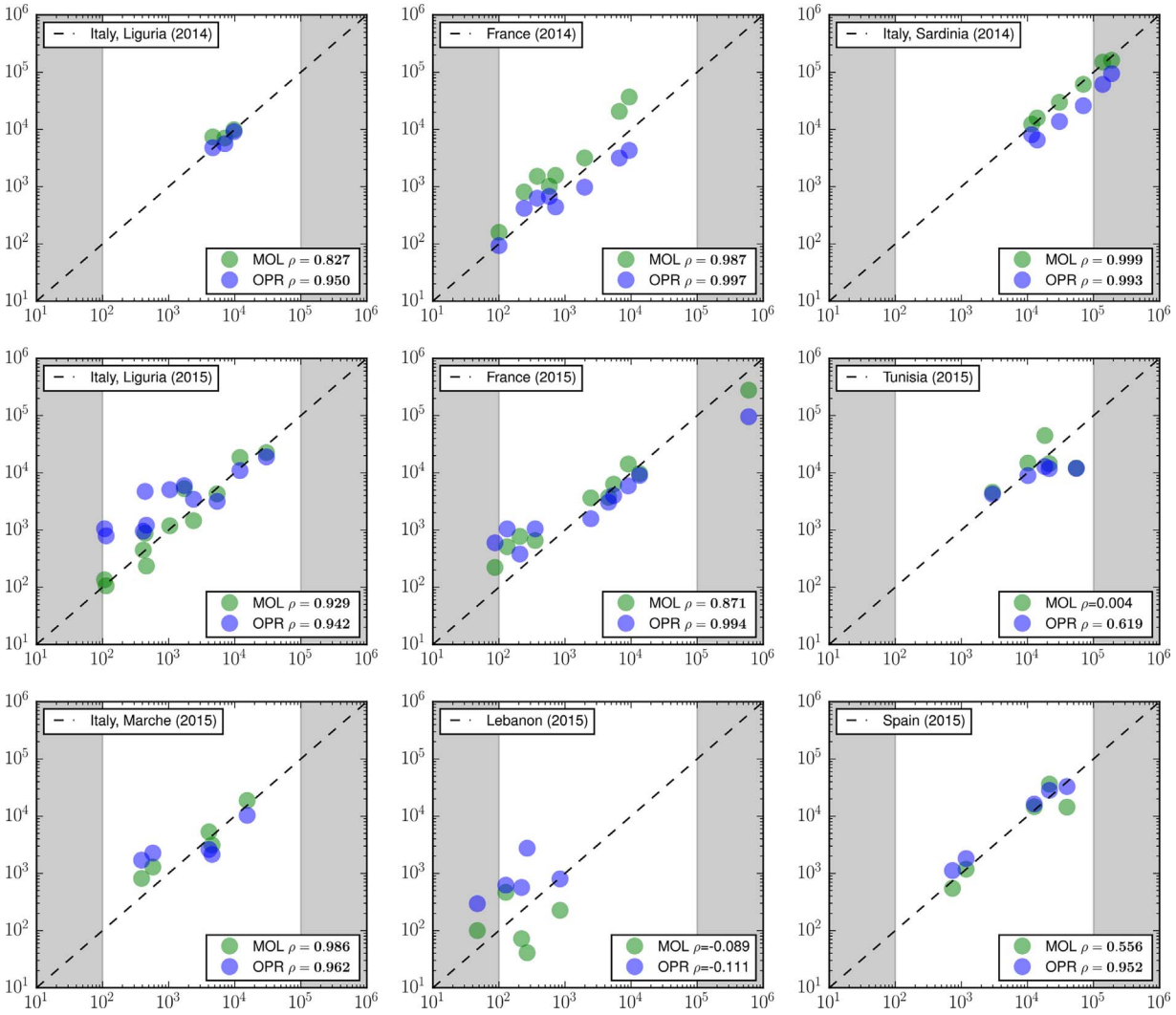

Fig. 2. Comparison of automatic methods versus manual counting for planktonic samples. Blue and green dots represent optical recognition [OPR] and molecular [MOL] qPCR counts, respectively. Only values lying inside the white region (sensible region, in white, see text) were considered for the statistical analysis. The dashed line represents the bisector, to which points should align in case of perfect identity of the methods. For each sampling period and location (indicated in the legend) relevant statistical values are reported (see Section 2). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

effectiveness of automatic methods with respect to the manual one (taken as a reference), in providing reliable alerts. In particular, dots found in the yellow area are associated to false alarms (points for which manual count is lower than threshold while the automatic one is above), while the red area hosts critical points, for which manual count would trigger an alert and the automatic one would not.

4. Discussion and conclusions

Manual counting methods, although their wide application, are time consuming and require a high degree of taxonomical expertise. The lack of fast and automatic counting approaches is nowadays hindering the possibility to set up wide sampling designs with relevant temporal and spatial scales for providing reliable alerts. This issue is particularly stringent for *Ostreopsis* species for which the correct identification is often challenging, due to the extreme variability of morphological features. Moreover, accurate species-specific identification and enumeration are crucial to address the connection to the toxicity of the sample which has been shown to depend on several biological and environmental factors (Tartaglione et al., 2015). As a consequence a strong need for innovative approaches is felt in the perspective of risk management of *Ostreopsis* blooms.

In this study, we report the development of two advanced procedures, specifically a new qPCR assay for specific *O. fattorussoi* quantification and an automatic opto-electronic procedure. *O. fattorussoi* was described recently in the eastern Mediterranean Sea, and it showed to produce low toxin amounts (0.28–0.94 pg/cell) (Accoroni et al., 2016; Tartaglione et al., 2015), respect to the *O.* cf. *ovata* (14–75 pg/cell in field samples and 10–200 pg/cell in culture) (Accoroni et al., 2011; Funari et al., 2015). The species-specific primers were designed on the ITS-5.8S rDNA sequence. An environmental standard curve for *O. fattorussoi* was generated for Batroun (Lebanon) sampling site, as well as for the other Mediterranean sampling sites using LSU rDNA standard curves for the estimation of *O.* cf. *ovata* abundance (Casabianca et al., 2014). The similar efficiencies of environmental and plasmid standard curves allowed the estimation of rDNA copy number per *O. fattorussoi* and *O.* cf. *ovata* cell in each sampling site. The reproducibility, analysed as CV_{Ct} mean inter assay variability, confirmed the reliability and accuracy of the technical set-up over time and over a low range of quantification. In the environmental samples from all Mediterranean coastal sites, *O.* cf. *ovata* was detected with the exception of Lebanese site where only *O. fattorussoi* was found. Finally, *Ostreopsis* spp. abundance was determined by qPCR assay and compared with optical recognition and manual counting methods.

In order to test the comparability of the three methods, during the

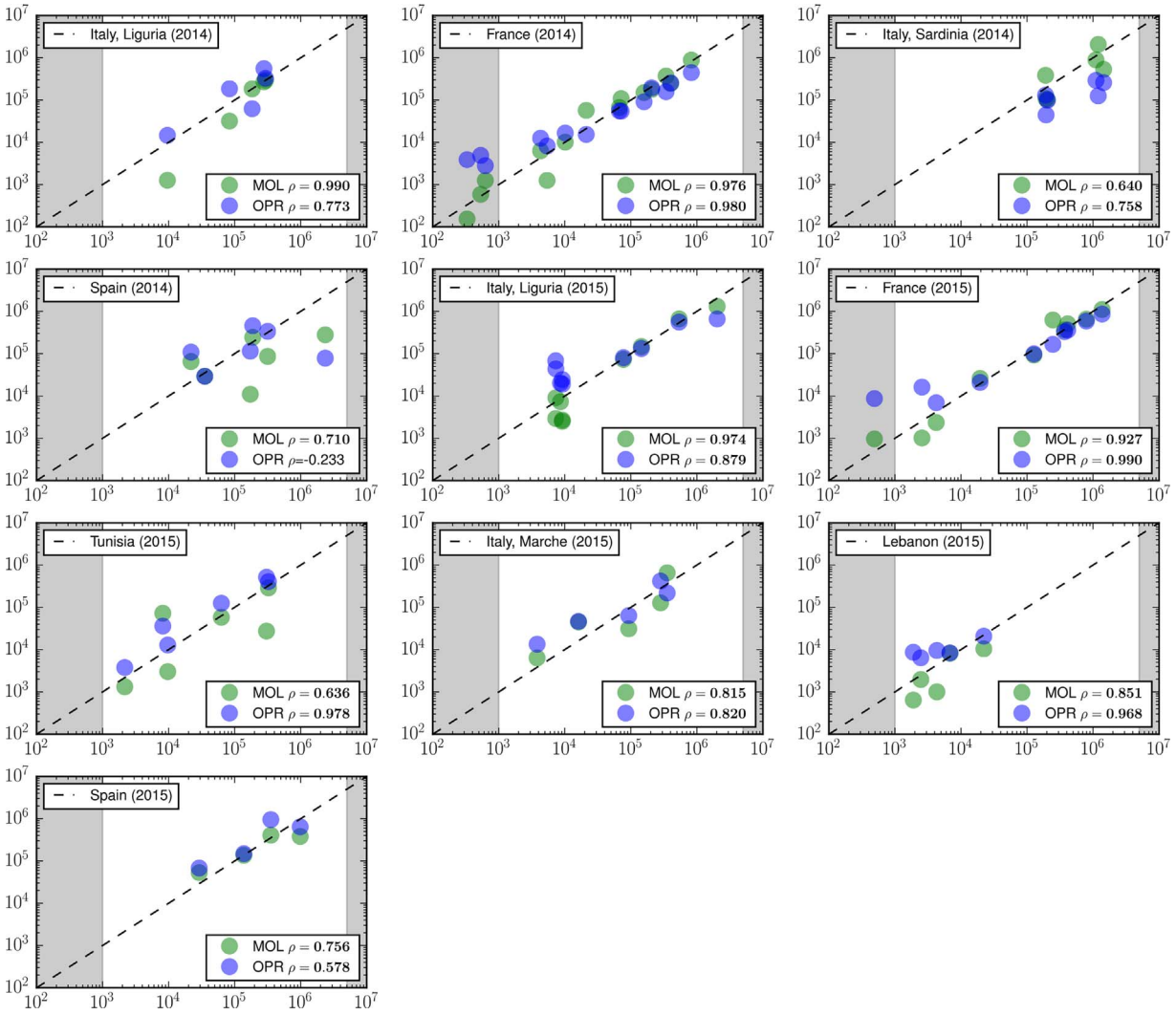

Fig. 3. Comparison of automatic methods versus manual counting for benthic samples. Blue and green dots represent optical recognition [OPR] and molecular [MOL] qPCR counts, respectively. Only values lying inside the white region (sensible region, in white, see text) were considered for the statistical analysis. The dashed line represents the bisector, to which points should align in case of perfect identity of the methods. For each sampling period and location (indicated in the legend) relevant statistical values are reported (see Section 2). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

sampling campaigns 2014 and 2015, a common protocol was agreed among all the participant partners, taking particular care for steps potentially affecting automatic methods. In particular, classical sampling methods require a fixation of the samples using chemical preservatives, typically formaldehyde or Lugol at various concentrations. While the fixation agent is normally not relevant for standard optical microscopy identification, it plays a crucial role for automatic methods (different preservatives provide strongly different optical images and may significantly alter the sample).

The results of the sampling campaigns 2014 and 2015 are reported in Figs. 2 and 3, presenting the data separate for each sampling site. Alternative counting procedures tested in the present study of *Ostreopsis* spp. are, to a large extend, in good agreement with manual results, providing Pearson's coefficients higher than 0.5 in almost all situations, for both planktonic and benthic samples. Interestingly, this good agreement over the Mediterranean Sea was obtained with the MOL method, which intrinsically requires a site-specific calibration, but also with the OPR method that was initially calibrated on a set of samples from the Liguria Region and the obtained template was directly applied for the rest of the analysis. In principle, the calibration of the OPR method could also be repeated with a site-specific perspective, possibly providing an even larger agreement with the local MAN counting, but the results of Figs. 2 and 3 already suggest the robustness of both approaches against regional variations.

Looking in detail at Fig. 2, it appears that one site, Lebanon, is showing a very low correlation for plankton sampling using either OPR or MOL methods. In the case of OPR this discrepancy is expected because all samples (including Lebanese ones) were analysed using the same template, designed for *O. ovata*, which is not effective in *O. fattorussoi* identification. Moreover, the low correlation for the MOL method is probably due to the highest variability of the rDNA copy number cell^{-1} in the *O. fattorussoi* determined from environmental water samples.

A general view of the results is reported in Fig. 4A (plankton) and B (benthos). The region for lower concentration (under the lower threshold of the sensitive region) is clearly characterized by a larger error. The deviation from the expected line (black dashed) is smaller and almost constant inside the sensitive region, while it starts increasing again above the higher threshold.

Overall, the MOL method presented a tendency to slightly over-estimate the count, while the OPR system typically under-estimated the guess. This general trend is clear inside the confidence region where OPR measurements are systematically lower than the MAN count, while MOL values are higher. This behavior is associated to the different nature of the approaches. In fact, OPR is based on a recognition algorithm, explicitly tuned to avoid counting debris or large aggregates,

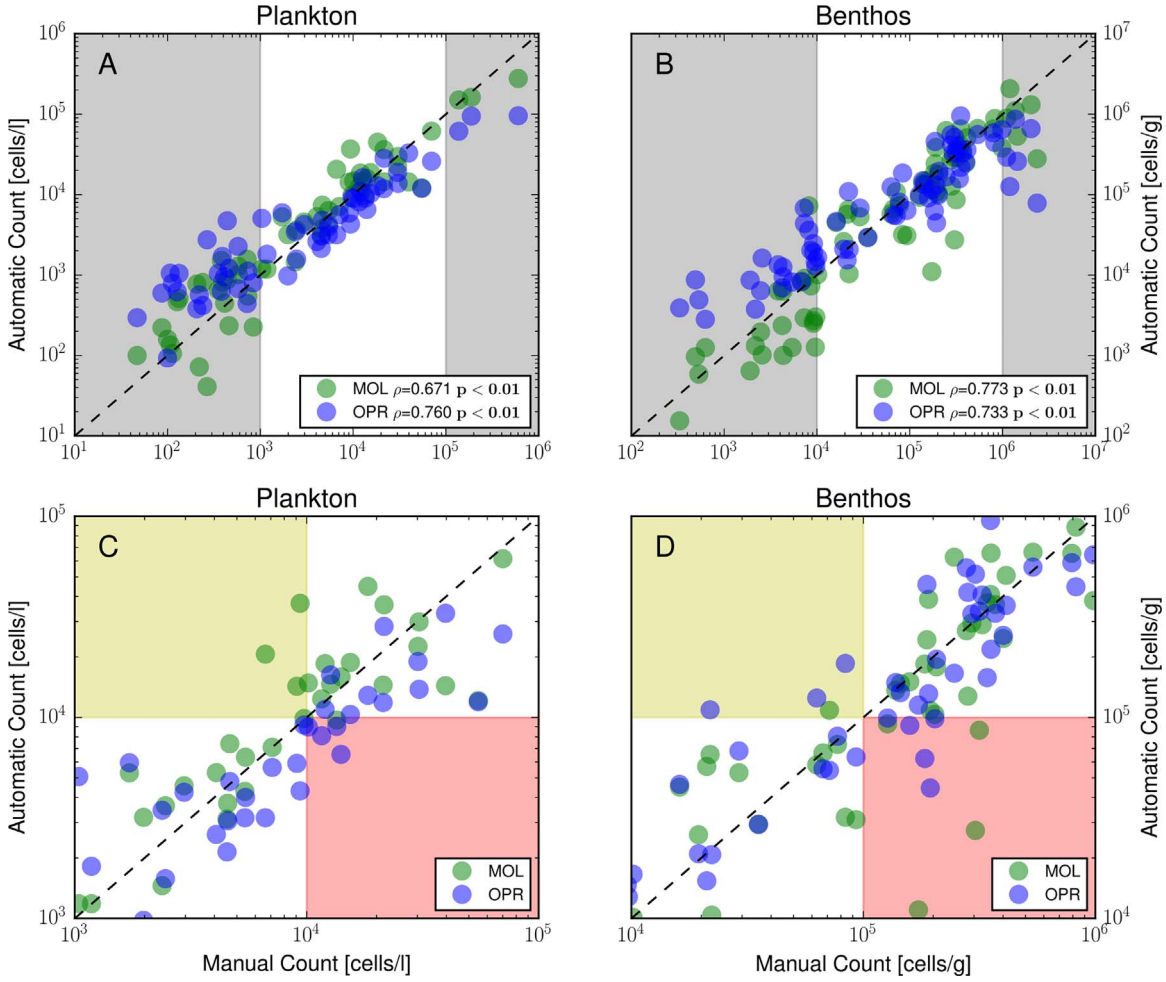

Fig. 4. Overall comparison of automatic methods versus manual counting for water (left) and benthic (right) samples. Optical recognition (OPR, blue) and molecular (MOL, green) counts are reported as a function of the corresponding manual count. Only values lying inside the sensible region (white region, see text) were considered for the statistical analysis. The dashed line represents the bisector, to which points should align in case of perfect identity of the methods. All sampling points are plotted together for both years of activity (2014 and 2015), planktonic (panel A) and benthic (panel B). For each plot, relevant statistical values are indicated. A zoom of the two plots around the threshold concentration is reported in panels C and D, in which the red area corresponds to missed alert events and the yellow area to false alarms (see text for details). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

leading to a slightly lower number of spotted cells. Instead, the MOL approach is based on the evaluation of the molecular content of the sample, regardless of the state of the organisms, thus including in the count the contribution of broken cells which would have been neglected by a manual count. In principle, given the systematic nature of this disagreement, it could be eliminated performing an *a posteriori* normalization of the results.

In a management perspective, we decided to particularly focus on the agreement of the two automatic methods with the manual one in detecting when thresholds are exceeded (Fig. 4C and D), herein set at 10^4 cells/L for planktonic samples (according to governmental guidelines (Funari et al., 2014, 2015)) and 10^5 cells/g of macroalga (according to Spanish regulation and authors experience) for benthic ones. The colored areas provide visual information about errors in providing alerts: samples falling in the red area are undetected alerts (compared to MAN), while those falling in the yellow areas refer to false alarms (compared to MAN).

In conclusion, both MOL and OPR methods proved to be robust and reliable in a risk management perspective, with a fundamental agreement in alert detection potential compared to MAN, providing a strong potential for application and uptake from relevant stakeholders in charge of the institutional monitoring.

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