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Monitoring toxic *Ostreopsis* cf. *ovata* in recreational waters using a qPCR based assay

Silvia Casabianca [a], Federico Perini [a], Anna Casabianca [a], Cecilia Battocchi [a], Valentina Giussani [b], Mariachiara Chiantore [b], Antonella Penna [a,c,*]

[a] Section of Environmental Biology, Dep. of Biomolecular Sciences, University of Urbino, Viale Trieste 296, 61121 Pesaro, Italy
[b] Department of Earth, Environment and Life Sciences, University of Genoa, Corso Europa 26, 16132 Genoa, Italy
[c] ISMAR CNR, Largo Fiera della Pesca, 60125 Ancona, Italy

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ABSTRACT

Ostreopsis sp. is a toxic marine benthic dinoflagellate that causes high biomass blooms, posing a threat to human health, marine biota and aquaculture activities, and negatively impacting coastal seawater quality. Species-specific identification and enumeration is fundamental because it can allow the implementation of all the necessary preventive measures to properly manage *Ostreopsis* spp. bloom events in recreational waters and aquaculture farms. The aim of this study was to apply a rapid and sensitive qPCR method to quantify *Ostreopsis* cf. *ovata* abundance in environmental samples collected from Mediterranean coastal sites and to develop site-specific environmental standard curves. Similar PCR efficiencies of plasmid and environmental standard curves allowed us to estimate the LSU rDNA copy number per cell. Moreover, we assessed the effectiveness of mitochondrial COI and cob genes as alternative molecular markers to ribosomal genes in qPCR assays for *Ostreopsis* spp. quantification.

1. Introduction

The benthic dinoflagellate *Ostreopsis* sp. is an epi-benthic microalga. Some species, including *Ostreopsis* cf. *ovata*, can produce potent non-protein toxins such as palytoxin-like compounds and various ovatoxins. *Ostreopsis* species typically proliferate in shallow and sheltered waters, with low hydro-dynamism associated with various substrata such as macrophytes, invertebrate shells, rocks and sands, although it can also be planktonic (Shears and Ross, 2009; Selina and Orlova, 2010; Mangialajo et al., 2011). Massive blooms of *Ostreopsis* spp. have a regular occurrence in tropical and temperate regions worldwide (Penna et al., 2010; Totti et al., 2010; Parsons et al., 2012), but recently, *O.* cf. *ovata* blooms in the Mediterranean Sea have become increasingly frequent and invasive. These blooms are deadly for benthic biota and noxious to humans, causing respiratory syndromes in people exposed to marine aerosols during recreational and work activities (Durando et al., 2007; Deeds and Schwartz, 2010; Ramos and Vasconcelos, 2010; Tubaro et al., 2011; Crinelli et al., 2012; Gorbi et al., 2012; Privitera et al., 2012; Vila et al., 2012). Furthermore, the toxic algal cells can be transferred to higher levels in the food chain, namely filter feeding organisms or fish that accumulate cells and palytoxin-like compounds. This could have negative impacts on mariculture and fisheries leading to the prohibition of seafood harvesting and consumption (Taniyama et al., 2002; Aligizaki et al., 2011; Amzil et al., 2012). Currently, the morphological identification of *Ostreopsis* species in environmental samples is highly uncertain because of the species' morphological variability, whereas genetic differentiation between species or clades, such as *O.* cf. *ovata*, is considered reliable (Battocchi et al., 2010; Laza-Martinez et al., 2011; Sato et al., 2011). Since not all *Ostreopsis* species are toxic, their correct taxonomical identification is a critical issue for coastal management (Ciminiello et al., 2013).

Toxic microalgal monitoring programs are implemented systematically in many countries all over the world to assess the potential for bloom formation, guarantee the protection of human health, and ensure water quality, aquaculture and other economic activities related to coastal waters (Andersen et al., 2003; McGillicuddy et al., 2005; Miraglia et al., 2009; Hallegraeff, 2010). Coastal and recreational seawaters are currently monitored using standard optical microscopy methods to assess phytoplanktonic or phytobenthic specific taxa and their relative abundance. These methods are widely accepted by various countries monitoring programs (Godhe et al., 2007; Karlson et al., 2010). However, the traditional light microscopy identification and quantification

* Corresponding author at: Section of Environmental Biology, Dep. of Biomolecular Sciences, University of Urbino, Viale Trieste 296, 61121 Pesaro, Italy. Tel.: +39 0722 304908; fax: +39 0722 304902.
E-mail address: antonella.penna@uniurb.it (A. Penna).

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methods can be difficult to implement, particularly over the long term because they require a great deal of taxonomic expertise to identify species, in addition to being time consuming and impractical for processing a large number of samples. Moreover, a high degree of morphological similarity between closely related species, lack of diagnostic morphological features, as well as the simultaneous occurrences of toxic and non-toxic population within a single species, as in the case of the genus *Ostreopsis*, make this kind of monitoring even more challenging (Accoroni et al., 2012; GEOHAB, 2012).

Molecular technological advances in real time quantitative PCR (qPCR) make it possible to identify toxic algae more rapidly and accurately. These molecular methods, mostly based on SYBR Green, Taqman and Molecular Beacon technologies, directly quantify various HAB (harmful algal bloom) microalgal species in different environmental matrices, such as seawater, sediments, hard and soft substrata and marine aerosol (Erdner et al., 2010; Delaney et al., 2011; Perini et al., 2011; Zamor et al., 2012; Vandersea et al., 2012; Casabianca et al., 2013). A limitation of HAB species quantification stems from the use of target molecular marker. The challenges with using a marker is its copy number stability within a target species, and ensuring specificity (Garneau et al., 2011; Murray et al., 2011; Penna and Galluzzi, 2013). In fact, intra-species variability in ribosomal gene copy numbers and the effect of the growth phase within *Alexandrium* spp. have already been shown and they can compromise the accuracy of qPCR counts of target cells in field samples (Galluzzi et al., 2010).

In our previous study (Perini et al., 2011), we demonstrated the copy number variability of the ribosomal LSU gene in *O.* cf. *ovata* cultured isolates. Due to this potential variation in the rRNA gene copy number, the strategy of using a standard curve generated with DNA from cultured isolates to quantify environmental samples was not feasible. Instead, an environmental standard curve generated from a DNA scalar dilution of pooled cell samples collected during a bloom of toxic *O.* cf. *ovata* in a Mediterranean coastal site was developed. This allowed for normalization of *O.* cf. *ovata* copy number variability, thereby obtaining an accurate and rapid quantification of cells in field samples.

In this study, the seawater affected by *Ostreopsis* spp. blooms at various Mediterranean beaches was monitored during the summer months using a qPCR assay. Environmental standard curves for specific geographical sites were generated to quantify cell abundance and determine the species-specific identification of *Ostreopsis* in field samples. The standard curves were created based on the amplification of target ribosomal LSU sequences. Furthermore, mitochondrial COI and cob genes were tested as potential molecular markers for *Ostreopsis* species quantification in marine samples.

2. Methods and materials

2.1. Cultured strains of O. cf. ovata

The *O.* cf. *ovata* strains were isolated by micropipetting from recreational waters of various Mediterranean beaches during the summer of 2010. Specifically, the following strains were used: CBA1733 (Ancona, Adriatic Sea, Italy); CBA1823 (Taormina, Ionian Sea, Italy), CBA1410 (Genova, Tyrrhenian Sea, Italy), CBA1554 (Villefranche-sur-mer; Tyrrhenian Sea, France), CBA1689 (Llavaneres, Catalan Sea, Spain) and CBA1761 (Aggelochori, Aegean Sea, Greece). Clonal cultures were established and maintained in F/4 —Si medium (Guillard, 1975) at a temperature of 21 ± 1 °C. Light was provided by cool-white fluorescent bulbs (photon flux of 100 $\mu E\ m^{-2}\ s^{-1}$) with a standard 14:10 light–dark cycle. Culture subsamples, each containing 5×10^3 cells, were harvested by centrifugation (4000 rpm for 10 min) after 7, 13 and 26 days of growth, corresponding to the exponential, early and late stationary phase, respectively. Pellets were washed twice with filtered artificial seawater and stored at −80 °C for subsequent molecular analyses.

2.2. Environmental samples

A total of 18 macroalgae and 16 seawater samples were collected during the period from June to October of 2011, 2012 and 2013 at different Mediterranean beaches (Fig. 1). Macroalgae samples were collected at a depth of 20–40 cm and treated following the protocol described by Totti et al. (2010). Surface seawater (50–250 ml) samples were collected using polyethylene bottles. Macroalgae wash seawater, as well as surface seawater samples, were fixed with acid Lugol's solution or neutralized formalin (0.8% final concentration) and stored at +4 °C until molecular analyses. At the Bari sampling site, seawater (100 ml) sampling was carried out using the syringe method according to the method of Ungaro et al. (2010). Seawater samples were then fixed with acid Lugol's solution and stored at +4 °C until molecular analyses.

2.3. Microscopy analyses

Ostreopsis sp. was counted using an inverted microscope (Zeiss Axiovert 40 CFL) equipped with phase contrast, at 200× or 400× magnification. Subsamples of macroalgae wash seawater (1–5 ml), surface seawater (10–25 ml), syringe seawater (1–2 ml), as well as *O.* cf. *ovata* cultured strains (2 ml), were settled in counting chambers according to Utermöhl's method (Utermöhl, 1958). Counting was performed on the whole sedimentation chamber. *Ostreopsis* spp. abundance was expressed as cells g^{-1} fw (fresh weight) for macroalgae, cells l^{-1} for the water column and cells ml^{-1} for cultured strains.

2.4. Molecular analyses

2.4.1. Environmental and culture sample processing

A volume of 1 ml of macroalgae wash seawater and syringe samples and 10 ml of surface seawater samples were lysed according to the lysis protocol described in detail by Casabianca et al. (2013). Briefly, macroalgae wash seawater and syringe, and seawater subsamples were concentrated by centrifugation at 12,000 rpm for 10 min and at 4000 rpm for 10 min at room temperature, respectively; the obtained 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 of both cultures and field samples 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 and 1:100 for the qPCR experiments.

2.4.2. PCR primer design and specificity

Genus-specific primers for *Ostreopsis* spp. were designed based on *Ostreopsis* COI and cob sequences (Penna, submitted for publication). The multiple sequence alignments were constructed using ClustalX2 ver. 2.0 (Larkin et al., 2007). Primers were designed using Oligo 6.65. The genus-specific primers for the amplification of the 205 bp (Tm = 80 °C) fragment of the COI gene were: COI-F 5′-CTTATCAGGAGCTTTTCTCTTGG-3′; COI-R 5′-GTTGCCAAGGATT ATTTTATGTAA-3′; while the primers for the 245 bp (Tm = 80 °C) fragment of the cob gene were Cob-F 5′-TGGAATTCTTCTTGG AATCTTG-3′; and Cob-R 5′-GAACATAAAAAAGATGGAGAATTC-3′.

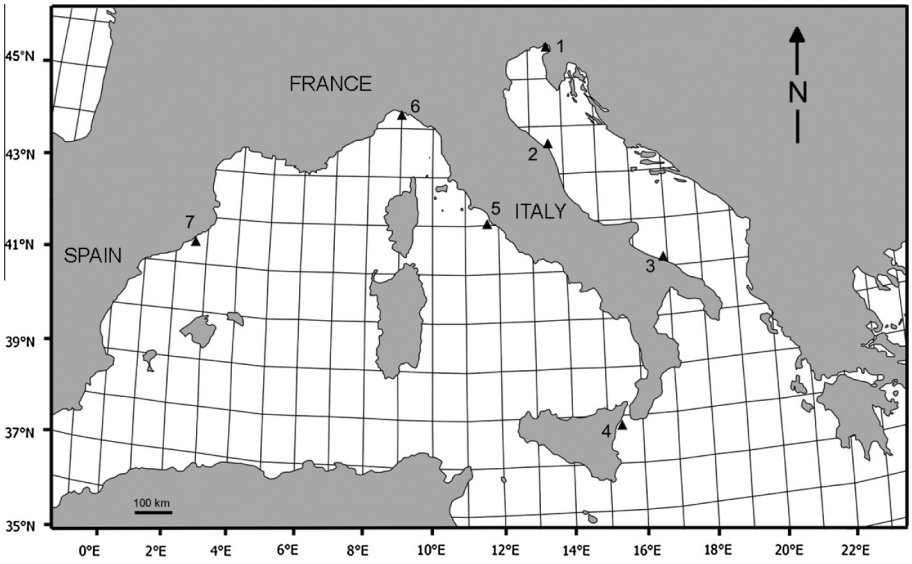

Fig. 1. Location of sampling stations in the Mediterranean Sea. (1) Trieste, NW Adriatic Sea, Italy. (2) Ancona (Passetto–Portonovo), NW Adriatic Sea, Italy. (3) Bari, SW Adriatic Sea, Italy. (4) Taormina, Ionian Sea, Italy. (5) Civitavecchia, Tyrrhenian Sea, Italy. (6) Genova, Tyrrhenian Sea, Italy. (7) Llavaneres, Catalan Sea, Spain.

The genus-specificity of the two sets of primers targeting the mitochondrial genes was first examined *in silico* using BLAST and then tested on purified DNA of cultured *Ostreopsis* spp. and other dinoflagellate genera, as well as environmental samples, which were checked for the presence/absence of *Ostreopsis* spp. by light microscopy observations before the qPCR experiments.

The species-specific primers for *O.* cf. *ovata* were used for the amplification of 204 bp of LSU rDNA (Perini et al., 2011), while species-specific primers for *Ostreopsis* cf. *siamensis* were used to amplify a 100 bp LSU rDNA fragment (Casabianca et al., 2013).

2.4.3. Environmental curves

A total of seven site-specific LSU rDNA environmental standard curves (LSU-STD) of ten fold serial dilution in the 8 to 0.0008 cellular range were constructed. Each standard was obtained amplifying the 204 bp fragment from a mixed *O.* cf. *ovata* crude extract of a 2000 cell pool from macroalgae sub-samples ($n = 4$) harvested at each Mediterranean Sea sampling site during the summers of 2011 and 2013 (also see Fig. 1).

2.4.4. COI, cob and LSU rDNA plasmid standard curves

The COI and cob genus-specific fragments (205 bp and 245 bp, respectively) were cloned into the pCR2.1 vector (Invitrogen, Carlsbad, CA, USA) and the derived pCOI and pCob plasmids were purified using the Qiaprep Miniprep Kit (Qiagen, Valencia, CA, USA). Plasmid concentrations were measured using a Qubit fluorometer (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions.

The plasmid standard curve for pCOI and pCob was obtained amplifying the specific fragment from 10-fold scalar dilution with copy number ranging from 10^5 to 2×10^0 molecules.

The plasmid standard curve (pLSUO) for the ribosomal LSU gene was generated as described above.

2.4.5. The qPCR assay of Ostreopsis spp.

The qPCR assay of the genus *Ostreopsis*, targeting COI and cob genes, 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 200 nM, 0.5 U of Hot- Rescue Taq DNA polymerase, and 2 µl undiluted, 1:10 and 1:100 diluted crude culture extracts. 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 or 61 °C for 1 min, for COI and cob, respectively. Standard curves for pCOI and pCob were included in each PCR run. A total of six *O.* cf. *ovata* isolates were analysed to estimate the copy number per cell of COI and cob genes at different growth phases. The qPCR experiments were carried out using 20, 2, and 0.2 cells obtained by 10-fold serial dilution of crude extract per sampling day.

The *O.* cf. *ovata* and *O.* cf. *siamensis* qPCR targeting the LSU rDNA genes assays were performed as previously described by Perini et al. (2011) and Casabianca et al. (2013).

2.5. Data analysis

Acquisition of qPCR data and subsequent analyses were carried out using StepOne software v. 2.1. 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 (R^2) was at least 0.97.

The LSU rDNA PCR results of environmental samples that showed a low copy number or no amplification were further analysed and, subsequently classified. Briefly, (i) if the Ct value obtained was lower than the Ct value from 0.0008 cells of the LSU-STD curve, the sample was quantifiable; (ii) if the Ct value was higher than 0.0008 cells, the sample was positive for the presence of *O.* cf. *ovata,* but not quantifiable being below the quantification limit; (iii) if no amplification was obtained, the sample was checked for the presence of inhibitors by adding 0.008 cells to 1:10 and 1:100 crude extract dilutions. If the Ct values corresponded to those obtained from 0.008 cells of LSU-STD environmental curves, the samples were not inhibited and classified as not detected. *O.* cf. *ovata* abundance on macroalgae was normalized to cells g^{-1} fw, while *O.* cf. *ovata* abundance in surface seawater was normalized to cells l^{-1}. Cells collected with the syringe method were also expressed as cells l^{-1}.

Statistical analyses were performed with non parametric Mann–Whitney, Kruskal Wallis, and Spearman correlation tests using PAST ver. 2.0.8 with a $p < 0.05$ determining significance.

3. Results

3.1. COI and cob genus-specificity assay

The genus-specificity of the two primer sets designed to target the COI and cob *Ostreopsis* sp. genes was checked: (a) *in silico* using BLAST, which showed that they were highly specific for this genus; (b) on cultured isolates of *Ostreopsis* spp. and other dinoflagellate species obtaining positive PCR amplification only with *Ostreopsis* spp. cultures; (c) on crude extracts from macrophyte and seawater samples containing target cells obtaining positive results by PCR amplification; (d) on environmental samples where the *Ostreopsis* spp. have never been observed by light microscopy. These samples yielded negative results by PCR assay.

3.2. LSU-STD, pCOI, pCob and pLSUO curve characterization

The seven different site-specific LSU-STD curves 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 (Table 1). The reproducibility analysed as CV_{Ct} mean inter-assay variability of different environmental standard curves was 0.6% (range 8–0.0008 cells).

The pCOI and pCob standard curves were validated. They showed 100% (n = 8 experiments) efficiency ($y = -3.33x + 32.55$ and $y = -3.32x + 32.93$ for COI and cob, respectively) with a linear correlation of 5 orders of magnitude (R^2 mean = 0.99 and 0.98, respectively) and a sensitivity of 2 copies/reaction (Ct mean 31.15 ± 0.62 and 31.86 ± 0.9 for COI and cob, respectively).

The mean pLSUO standard curve (R^2 = 0.98) showed 100% efficiency ($y = -3.32x + 34.43$, n = 8 experiments) with a linear correlation of 5 log linear range and a sensitivity of 2 copies/reaction (Ct mean = 33.09 ± 0.09).

The reproducibility was also assayed using the pCOI, pCob and pLSUO curves and the CV_{Ct} mean value were 2.5%, 2.5% and 1.5% (1×10^5–2×10^0 molecules), respectively, while, the CV_{Cn} mean values were 32%, 39%, and 25%, respectively.

3.3. Estimation of COI, cob and LSU rDNA gene copy number in O. cf. ovata cultures

The mean of COI, cob and LSU copy number per cell of six *O.* cf. *ovata* cultured isolates was calculated by plotting the Ct values obtained against the corresponding plasmid standard curve, taking into account the dilution factors of the crude extracts. The quantitative analysis showed that the three genes had different median copy number per cell among every growth phase (Hc = 41.38, $p < 0.0001$; Hc = 35.08, $p < 0.0001$; Hc = 30.18, $p < 0.0001$ for COI, cob and LSU, respectively) (Fig. 2). Moreover, the Kruskal–Wallis test rejected the null hypothesis of equal medians among COI, cob and LSU copy number per cell within each growth phase (Hc = 46.05, $p < 0.0001$, Hc = 38.83, $p < 0.0001$ and Hc = 47.37, $p < 0.0001$ at the 7th, 13th and 26th sampling day). However, a posteriori pairwise Mann–Whitney test showed that there was no significant difference between COI and cob (Mann–Whitney' U = 217, P = 0.5958; U = 203, P = 0.3956; U = 282, P = 0.9097 at 7th, 13th and 26th day of growth, respectively).

3.4. Estimation of the LSU rDNA in O. cf. ovata from environmental samples

The *O.* cf. *ovata* LSU rDNA copy numbers per cell in environmental samples were different at each of the seven sampled Mediterranean coastal sites (Table 1). These values calculated from different mean Ct cell^{-1}, showed significant differences (Hc = 30.03, $p < 0.0001$, Kruskal–Wallis test). The pairwise a posteriori comparison of the different copy number per cell showed that the copy number per cell calculated at both the Bari and Taormina sites was not significantly different from that which was calculated at the Genova site (Mann–Whitney' U = 5, P = 0.1658 and U = 12, P = 0.3785, for Bari and Taormina versus Genova, respectively).

3.5. Quantification of O. cf. ovata in environmental samples

Environmental samples of macroalgae and surface seawater collected at the seven Mediterranean coastal sites during the summer of 2011 and 2013 were analysed by both qPCR assay, using LSU environmental standard curves, and light microscopy. A greater abundance of *O.* cf. *ovata* was found in sample n. 14 ($1.18 \times 10^6 \pm 6.33 \times 10^5$ cells g^{-1}fw); minimum abundance was found in sample n. 18 (772 ± 41 cells g^{-1}fw) by qPCR. *O.* cf. *ovata* cells were detected in all macroalgal samples analysed with the exception of samples n. 1 and 2 collected at Llavaneres (Spain) in June 2011 (Table 2).

The abundance of *O.* cf. *ovata* recorded in seawater samples was generally lower than cell concentrations on macroalgae samples, with the exception of the highest value observed in sample n. 32 ($1.90 \times 10^6 \pm 8.45 \times 10^5$ cells l^{-1}). No cells were found in sample n. 20. Furthermore, the PCR reaction of sample n. 23, in which no *Ostreopsis* cells were found by microscopy resulted in a positive reaction containing 1106 ± 426 cells l^{-1} (Table 3).

There was a significant positive correlation between cell densities on macroalgal samples and in water column (n = 16, Spearman r = 0.8386, $p < 0.0001$). Furthermore, a significant correlation was found between *O.* cf. *ovata* abundance determined by light microscopy and qPCR assays (n = 16, Spearman r = 0.9808, $p < 0.0001$ and n = 15, Spearman r = 0.9263, $p < 0.0001$ for macroalgae and surface seawater samples, respectively).

All environmental samples (macroalgae and seawater) were analysed for the presence of *O.* cf. *siamensis*. The qPCR amplification reactions yielded negative results (no amplification).

Table 1
LSU-STD environmental standard curve, mean Ct cell^{-1} and LSU gene copy number cell^{-1} determined by qPCR assay from the macroalgal samples collected in 2011–2013 at different Mediterranean coastal sites.

Sampling locality	Mean LSU-STD curve	Mean Ct cell^{-1}	LSU copy No.[a] (cell^{-1} ± SD)
Llavaneres (Catalan Sea, Spain)	$y = -3.3113x + 22.921$	23.42 ± 0.5	2137 ± 190[b]
Civitavecchia (Tyrrhenian Sea, Italy)	$y = -3.3253x + 22.337$	22.33 ± 0.14	4429 ± 424
Trieste Canovella (Adriatic Sea, Italy)	$y = -3.3409x + 24.979$	24.99 ± 0.09	699 ± 41
Passetto–Portonovo (Adriatic Sea, Italy)	$y = -3.3759x + 18.861$	18.87 ± 0.03	48,617 ± 908
Genova (Tyrrhenian Sea, Italy)	$y = -3.3626x + 23.026$	23.02 ± 0.11	2736 ± 204
Bari (Adriatic Sea, Italy)	$y = -3.3438x + 22.906$	22.92 ± 0.13	2929 ± 168
Taormina (Ionian Sea, Italy)	$y = -3.359x + 23.072$	23.06 ± 0.06	2649 ± 108

[a] Mean LSU gene copy number calculated in triplicates ± standard deviation (SD). These data were obtained by plotting the Ct value per cell against pLSUO plasmid standard as the two curves showed the same PCR efficiencies ($\Delta S < 0.1$).
[b] From Casabianca et al. (2013).

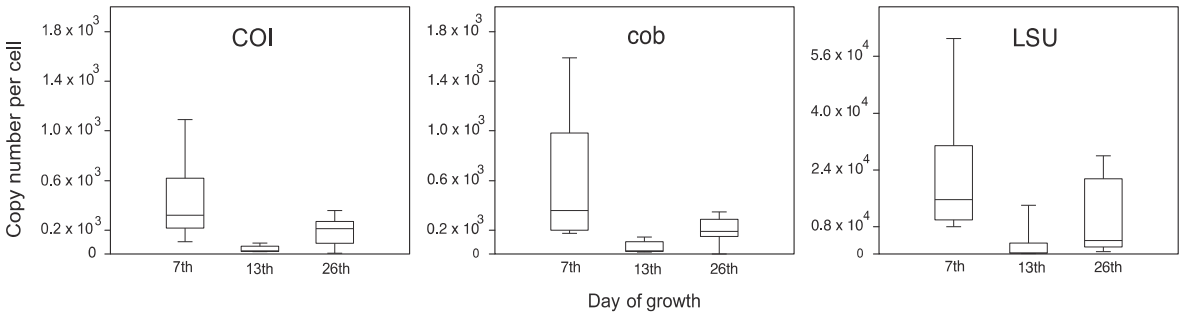

Fig. 2. Copy number per cell content of mitochondrial COI and cob genes, and ribosomal LSU gene in six different cultured isolates of *Ostreopsis* cf. *ovata* in various growth phases. The gene copy number was determined by qPCR assay.

Table 2
QPCR assay and microscopy analysis of *Ostreopsis* cf. *ovata* abundance from macroalgae samples collected in 2011 and 2013 at various Mediterranean coastal sites.

Sample No.	Locality	Sampling date	Abundance[a] (cells g^{-1}fw ± SD)	
			qPCR	Microscopy
1	Llavaneres (Spain)	22.06.2011	n.d.[b]	n.d.
2	Llavaneres (Spain)	22.06.2011	n.d.	n.d.
3	Llavaneres (Spain)	21.07.2011	98,677 ± 1091	169,120 ± 2039
4	Llavaneres (Spain)	22.07.2011	342,459 ± 43,691	484,086 ± 3913
5	Civitavecchia15888 (Italy)	03.10.2013	49,484 ± 3688	36,548 ± 2673
6	Civitavecchia15892 (Italy)	03.10.2013	5408 ± 115	6793 ± 1708
7	Trieste Canovella 1 (Italy)	16.09.2013	212,797 ± 66,631	241,067 ± 28,706
8	Trieste Canovella 2 (Italy)	16.09.2013	208,733 ± 23,336	240,816 ± 6136
9	Trieste Canovella 3 (Italy)	16.09.2013	265,722 ± 16,939	239,811 ± 34,194
10	Ancona, Portonovo (Italy)	10.09.2013	850 ± 232	1173 ± 774
11	Ancona, Passetto 1 (Italy)	10.09.2013	415,062 ± 29,170	417,572 ± 15,025
12	Ancona, Passetto 2 (Italy)	10.09.2013	673,835 ± 42,287	689,281 ± 40,586
13	Genova a (Italy)	22.07.2013	473,598 ± 30,455	455,607 ± 71,592
14	Genova c (Italy)	26.07.2013	1,180,043 ± 63,326	1,143,652 ± 125,136
15	Genova e (Italy)	31.07.2013	661,039 ± 10,950	656,814 ± 9676
16	Genova g (Italy)	02.08.2013	965,409 ± 79,188	925,962 ± 97,907
17	Taormina St.1 (Italy)	13.09.2012	2286 ± 111	1734 ± 270
18	Taormina St. 2 (Italy)	13.09.2012	772 ± 41	713 ± 59

[a] Mean abundance determined in triplicates ± standard deviation (SD).
[b] Not detected.

Table 3
QPCR assay and microscopy analysis of *Ostreopsis* cf. *ovata* abundance in seawater samples collected in 2011 and 2013 at various Mediterranean coastal areas.

Sample No.	Locality	Sampling date	Abundance[a] (cells l^{-1} ± SD)	
			qPCR	Microscopy
19	Civitavecchia 15,887 (Italy)	03.10.2013	1832 ± 90	1260 ± 85
20	Llavaneres (Spain)	22.06.2011	n.d.[b]	n.d.
21	Llavaneres (Spain)	20.07.2011	56,320 ± 10,478	97,667 ± 12,503
22	Llavaneres (Spain)	22.07.2011	28,405 ± 5226	66,000 ± 5657
23	Ancona, Portonovo (Italy)	10.09.2013	1106 ± 426	n.d.
24	Ancona, Passetto (Italy)	10.09.2013	92,600 ± 8414	98,400 ± 19,819
25	Genova b (Italy)	22.07.2013	44,353 ± 4590	14,500 ± 2121
26	Genova d (Italy)	26.07.2013	17,000 ± 1044	23,500 ± 707
27	Genova f (Italy)	31.07.2013	58,000 ± 7071	29,778 ± 5441
28	Genova h (Italy)	02.08.2013	17,500 ± 707	11,776 ± 1275
29[c]	Bari, Trullo 1 (Italy)	18.09.2013	115,750 ± 19,363	142,000 ± 19,305
30[c]	Bari, Trullo 2 (Italy)	18.09.2013	135,250 ± 27,476	154,000 ± 26,969
31[c]	Bari, S. Spirito 1 (Italy)	19.09.2013	1,077,265 ± 34,479	542,250 ± 148,572
32[c]	Bari, S. Spirito 2 (Italy)	19.09.2013	1,907,088 ± 84,540	1,198,750 ± 226,729
33[c]	Bari, Giovinazzo 1 (Italy)	19.09.2013	357,255 ± 24,375	131,000 ± 26,255
34[c]	Bari, Giovinazzo 2 (Italy)	19.09.2013	841,710 ± 39,675	256,250 ± 25,617

[a] Mean abundance determined in triplicates ± standard deviation (SD).
[b] Not detected.
[c] Samples collected with the syringe method, cell abundance is expressed as cells l^{-1}.

3.6. Evaluation of PCR inhibitors by spiking experiments

Ten-fold crude extract serial dilutions of each field sample were analyzed for the presence of potential inhibitors by evaluating the amplification efficiency. The PCR results of samples with a ΔCt ranging from 3.3 to 3.4 were accepted. All dilutions were used for quantification as they fell within this range (data not shown). Moreover, samples with no amplification products (samples n. 1, 2 and 20 from the Llavaneres site) were checked for the putative presence of PCR inhibitors by adding 0.008 cells from crude extract of Llavaneres LSU-STD curve to the 1:10 and 1:100 dilutions. As the Ct mean value of the 0.008 spiked cells (29.78 ± 0.62) was not significantly different from the Ct relative to the 0.008 cells of the LSU-STD curve (29.83 ± 0.40) (Mann–Whitney' $U = 18$, $P = 0.9383$), negative amplification of environmental samples was actually due to the absence of target cells and not to the presence of PCR inhibitors.

4. Discussion

Monitoring programs of *Ostreopsis* spp. events provide insight into the dynamics of these marine microbes and can help us to develop new strategies to mitigate their impacts on human health, economic activities and ecosystem functioning.

The correct identification of various *Ostreopsis* species is very difficult using current light or epifluorescence microscopy methods because the species' morphological and morphometric ambiguities call for a high level of taxonomic expertise (Selina et al., 2014). Moreover, the quantification of distinct *Ostreopsis* species in field samples containing mixed species using light microscopy is fairly inaccurate, not to mention time consuming. Accurate species-specific identification and quantification is important because individual genetic species can produce different toxins, which pose potential risk to humans and other marine organisms (Parsons et al., 2012). Molecular technologies based on PCR and qPCR were developed for the identification and enumeration of *Ostreopsis* spp. in various matrices in marine coastal environments. PCR-based methods have proved to be a fast, specific, and sensitive way to confirm taxonomic identity at species level by amplifying target genes (Battocchi et al., 2010; Perini et al., 2011; Casabianca et al., 2013).

The aim of the present study was to monitor recreational water quality in various Mediterranean coastal areas affected by the toxic dinoflagellate *O.* cf. *ovata* by performing qPCR. In particular, we sought to accurately and rapidly quantify the *O.* cf. *ovata* abundance on macroalgae and in surface seawater samples collected at several sampling stations during the summers of 2011 and 2013.

In previous studies, we reported the development of a qPCR assay, which proved to be specific, sensitive and rapid for *O.* cf. *ovata* identification and enumeration in field samples from the Conero Riviera (northern Adriatic Sea) and Sant Andreu de Llavaneres Beach (northwestern Mediterranean Sea). This molecular method was based on species-specific primers targeting the LSU rRNA gene. Due to significant variations in the LSU rDNA gene copy number among cells in cultured isolates of *O.* cf. *ovata*, it was not possible to develop a quantification method based exclusively on a plasmid standard curve or DNA standard curve derived from cultured samples. Hence, the strategy was based on the construction of a standard curve using a pool of DNA from environmental samples in order to normalize the variability of the LSU copy number among cells in *O.* cf. *ovata* to obtain the amounts in environmental matrices (Perini et al., 2011; Casabianca et al., 2013).

In addition, in this study, a qPCR assay based on COI and cob mitochondrial genes was developed to investigate the potential of mitochondrial genes as new DNA markers for the quantification of *O.* cf. *ovata* abundance. Genus-specific primers targeting *Ostreopsis* COI and cob were designed against nearly identical sequences of mitochondrial genes (98%) from different *Ostreopsis* species. Insufficient sequence variability made it impossible to design species-specific oligonucleotide primers within the genus *Ostreopsis* (data not shown). The qPCR was applied to *O.* cf. *ovata* cultured isolates from different Mediterranean sampling sites. We found significant variability in mtDNA copy number per cell of the *O.* cf. *ovata* in the various culture growth phases, as well as significant variations in rDNA copy number in the same cultured samples. The latter finding confirmed previous data on rDNA amounts per cell variation in *O.* cf. *ovata* cultures (Perini et al., 2011). Moreover, in the present study, a significant decrease in mtDNA and rDNA amounts was evident in *O.* cf. *ovata* cells after the exponential phase. This is probably due to a decrease in metabolic activity in the stationary phase (Thessen et al., 2009). It is well known that the transition from exponential growth to the stationary phase results in numerous changes in microbial cell morphology and physical and biochemical properties, including RNA degradation, a decrease in ATP content, expression of some novel "starvation" genes and elevated resistance of stationary cells to different kinds of stress (Kaprelyantst and Kell, 1993). However, the fact that the cells of a stationary-phase population contain different quantities of genetic material, i.e., different gene copies, should be considered when discussing genome instability (Nyström, 2004).

Significant differences were found among COI, cob and LSU gene copy numbers per cell in the exponential, stationary and late stationary growth phases. However, there was no significant difference between COI and cob copy numbers per cell, probably because both genes code for mitochondrial respiratory chain proteins (Nash et al., 2008; Lin et al., 2009).

Since the two mitochondrial genes did not show stability and species-specificity, it was not possible to apply a qPCR assay for *O.* cf. *ovata* quantification in environmental samples. Therefore, the standardized method of qPCR based on LSU rDNA environmental standard curve was applied in order to monitor the quality of recreational waters in several Mediterranean coastal areas affected by toxic blooms or the occurrence of *O.* cf. *ovata*. Based on a preliminary screening by qPCR of *O.* cf. *ovata* in environmental samples using 1030 copy number per cell (Perini et al., 2011), there was no correlation between qPCR and microscopy abundance (data not shown). We also observed a temporal variability, as was the case at the Passetto–Portonovo site (Conero Riviera) between 2009 (Perini et al., 2011) and 2013. Thus, in order to normalize the LSU rDNA variability of *O.* cf. *ovata*, an environmental standard curve was generated for each sampling site. The similar efficiencies of environmental and plasmid standard curves allowed the accurate estimation of rDNA copy number per *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 natural samples, *O.* cf. *ovata* populations showed different copy numbers per cell in the various Mediterranean coastal sampling sites with the exception of the Bari and Taormina populations, which showed no significant differences from that of Genova. It is likely that this finding is due to the variability of this ribosomal marker as explained above (Penna and Galluzzi, 2013).

The qPCR assay for the estimation of *O.* cf. *ovata* abundance was applied to field samples collected during the summer of 2011 and 2013 from several Mediterranean coastal areas. A total of 34 environmental samples were analysed and *O.* cf. *ovata* cells were quantified. The accuracy and reliability of the molecular method was confirmed by the significant correlation between *O.* cf. *ovata* abundance determinations by qPCR and light microscopy. Samples showing no amplification were checked for the presence of

inhibitors, and spiking experiments demonstrated the absence of any substance that could affect amplification reactions. The qPCR assay and microscopy counting yielded different results for seawater sample n. 23. In particular, 1106 ± 426 *O.* cf. *ovata* cells l^{-1} were counted by qPCR while no cells were recovered by microscopy highlighting the higher sensitivity of the molecular assay compared to microscopy. The latter is dependent on the sedimentation volume, resuspension matter and cell community abundance. The factors, individually or in combination, could adversely affect the microscopy counting.

In the present study, COI and cob mitochondrial genes were explored as potential candidates for *O.* cf. *ovata* quantification in environmental samples. However, due to the demonstrated variability and lack of species-specificity of these mitochondrial genes, the LSU rDNA gene remains the target molecule of choice to accurately quantify *Ostreopsis* spp. abundance in natural samples by qPCR methods. We therefore developed and optimized a system based on the generation of environmental site-specific standard curves. This approach was validated in several Mediterranean coastal areas where we rapidly obtained the correct quantification of *O.* cf. *ovata* in field. Hence, after the generation of a site-specific standard curve for the quantification of toxic *O.* cf. *ovata* abundance, the method could be applied in recreational water quality monitoring programs.

5. Conclusions

This investigation has provided evidence for the specificity and sensitivity of qPCR for the monitoring of *O.* cf. *ovata* in environmental samples at Mediterranean coastal sites affected by toxic blooms. The qPCR approach was effective in assessing beach water quality during the survey activity of the study period, and proved to be a powerful tool for rapid and efficient quantification of toxic *O.* cf. *ovata* cells. Hence, we propose the implementation of qPCR technology, based on site-specific environmental standard curves, in the monitoring and management of harmful *Ostreopsis* blooms.

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