New Approach Using the Real-Time PCR Method for Estimation of the Toxic Marine Dinoflagellate *Ostreopsis* cf. *ovata* in Marine Environment

Federico Perini¹*, Anna Casabianca¹*, Cecilia Battocchi¹, Stefano Accoroni², Cecilia Totti², Antonella Penna¹*

¹ Department of Biomolecular Sciences, University of Urbino, Pesaro, Italy, ² Department of Marine Sciences, Polytechnic University of Marche, Ancona, Italy

Abstract

**Background:** We describe the development and validation of a new quantitative real time PCR (qrt-PCR) method for the enumeration of the toxic benthic dinoflagellate *Ostreopsis* cf. *ovata* in marine environment. The benthic *Ostreopsis* sp. has a world-wide distribution and is associated during high biomass proliferation with the production of potent palytoxin-like compounds affecting human health and environment. Species-specific identification, which is relevant for the complex of different toxins production, by traditional methods of microscopy is difficult due to the high morphological variability, and thus different morphotypes can be easily misinterpreted.

**Methodology/Findings:** The method is based on the SYBR I Green real-time PCR technology and combines the use of a plasmid standard curve with a “gold standard” created with pooled crude extracts from environmental samples collected during a bloom event of *Ostreopsis* cf. *ovata* in the Mediterranean Sea. Based on their similar PCR efficiencies (95% and 98%, respectively), the exact rDNA copy number per cell was obtained in cultured and environmental samples. Cell lysates were used as the templates to obtain total recovery of DNA. The analytical sensitivity of the PCR was set at two rDNA copy number and 8.0×10⁻⁴ cell per reaction for plasmid and gold standards, respectively; the sensitivity of the assay was of cells g⁻¹ fw or 1⁻¹ in macrophyte and seawater samples, respectively. The reproducibility was determined on the total linear quantification range of both curves confirming the accuracy of the technical set-up in the complete ranges of quantification over time.

**Conclusions/Significance:** We developed a qrt-PCR assay specific, robust and high sample throughput for the absolute quantification of the toxic dinoflagellate *Ostreopsis* cf. *ovata* in the environmental samples. This molecular approach may be considered alternative to traditional microscopy and applied for the monitoring of benthic toxic microagal species in the marine ecosystems.

Introduction

The qrt-PCR is a powerful technique able of accurate and sensitive estimation of microbial species abundance in different environments for applied ecology studies. Several qrt-PCR assays, mostly based on SYBR Green I, Taqman and Molecular Beacon technologies, have been developed for a large number of toxic microagal species quantification [1,2,3]. The method used in these studies is to generate a standard curve using plasmids containing target ribosomal DNA sequences, or genomic DNA extracted from cultured cells or resting stages with known concentrations of the target microbial species. Although ribosomal genes have been the target molecules of choice in the development of the qrt-PCR assays, few of the data reported so far in the literature show that in some microbial taxa the rRNA genes are present as pseudogenes and also organised in extra-chromosomal molecules [4,5]. Given this potential variation in the rRNA gene copy number [6], the strategy of pooling different genomic DNAs derived from several cultured isolates of the same species to generate a standard for quantifying environmental samples may affect the results of the qrt-PCR assay [7]. Moreover, other qrt-PCR assays applied to environmental samples have yielded highly variable results. This may be due to other factors, such as different amplification efficiencies in standard and field samples, and low and unreliable recovery of total DNA extracted using conventional methods [8].

The toxic genus *Ostreopsis* includes various benthic species which have world-wide distribution from tropical to temperate coastal sites and are associated with the production of potent palytoxin-like (PLTX) compounds [9]. In tropical benthic assemblages, *Ostreopsis* spp. co-occurs with other harmful benthic dinoflagellates,
including *Gambierdiscus* spp., responsible for ciguatera [10]. In last
decade, *Ostreopsis* spp. blooms regularly occurred in the warm
temperate coasts of the Mediterranean Sea [11,12,13] and have
recently been associated both with human poisoning by toxic
aerosols [14,15] and with mortality of benthic organisms caused by
water deterioration or by direct toxin intake through the food web
[16]. Taxonomy of the *Ostreopsis* species based only on morpho-
logical characteristics is rather controversial due to the high
morphological variability of both natural populations and cultured
specimens [17] and therefore, species-specific identification by
traditional method of microscopy is extremely difficult. With
respect to the Mediterranean Sea, two *Ostreopsis* species, previously
classified as *O. cf. ovata* and *O. cf. siamensis* by both morpхо-
logical and genetic analyses, have been found together in
bloom events in several coastal areas [18,19]. Of these, the *O. cf.
ovata* genotype seems to predominate and has been found with
greater frequency and abundance in all analysed samples from the
Mediterranean and the rest of the world [20]. Correctly
identifying and quantifying these two *Ostreopsis* species simulta-
neously is therefore crucial, given that distinct species can produce
different toxic compounds with a variety of potential risks to public
health, the environment and the economic activities of tourism
and aquaculture [21].

In this study, we describe the development of the first method
based on qrt-PCR for species-specific identification and enumera-
tion of *Ostreopsis cf. ovata* from macroalgae and surface seawater
samples, which takes into account all the above-mentioned qrt-
PCR problems and biases. The species-specific primers designed
in the LSU (Large Subunit) rRNA gene were first validated in
isolates and then in field samples by qrt-PCR. The new element in this assay is the construction of a standard
curve using crude lysates of pooled samples collected during a
*O. cf. ovata* bloom. We demonstrated that this standard has the same
amplification efficiency of the generated plasmid standard
containing the target LSU gene. By the data comparison of the
two curves we are able to calculate the cell number and LSU gene
copy number per cell of the *O. cf. ovata* in the bloom. The qrt-PCR
was also compared with the traditional microscopy analysis.

This new method proved to be a more accurate and specific
alternative molecular technique to microscopy for investigating the
population dynamics of benthic microbial species.

**Materials and Methods**

**Microalgal cultures**

*Ostreopsis cf. ovata* isolates were obtained from Italian coastal
waters (Mediterranean Sea) during the summers of 2008 and 2009
and are listed as follows: *O. cf. ovata* CBA165 (Pisa, Tyrrhenian
Sea), CBA166 (Trieste, northern Adriatic Sea), CBA1273 (Genoa,
northern Tyrrhenian Sea), CBA1298 (Livorno, Tyrrhenian Sea),
CBA1377 (Bari, southern Adriatic Sea), CBA1346 (Conero
Riviera, central Adriatic Sea). The cultures were maintained in
F/4 medium at 23±1°C. Light was provided by cool-white
fluorescent bulbs (photon flux of 100 μE m⁻² s⁻¹) on a standard
14:10 h light-dark cycle. Culture sub-samples were fixed with
 Lugol’s iodine solution and stored at +4°C until molecular
analysis. *O. cf. siamensis* CNRT15 (Taormina, Ionian Sea) was used
as a control in the species-specific qrt-PCR experiments.

**Environmental samples**

A total of 43 samples of the green macroalga *Ulva rigida* and of
surface seawater were collected at Portonovo (Conero Riviera)
during the period March–November 2009. Samples of *U. rigida*
were harvested at a depth of 20–40 cm and treated according to
[13]. Seawater samples (50 ml) were collected in triplicates using
polyethylene bottles. The macroalgal wash seawater and surface
seawater samples, were fixed with neutralized formalin (0.08% final
concentration) and stored at +4°C until molecular analysis.

**Microscopy analyses**

Subsamples of the macroalgal wash water (10 ml) and the
surface seawater (25–40 ml) samples were settled for 25–40 h in
Utermöhl chambers. *Ostreopsis* spp. were counted on the entire
sedimentation chamber under an inverted microscope (Axiovert
40 CFL and Axiovert 135H, Zeiss or a Leitz DM-II) at 200 or
400× magnification. Abundances in macroalgae and surface
seawater samples were expressed as number of cells per gram of
fresh weight (cells g⁻¹ fw) and number of cells per liter (cells l⁻¹),
respectively. *Ostreopsis* spp. were identified under epifluorescence
microscope after samples were treated with Calcofluor, and
Scanning Electron Microscopy according to [22].

**Lysis of environmental and culture samples**

The final volume of processed environmental sub-samples
(macrophytes and surface seawater), obtained as described above,
was 40 ml. Culture and field sub-samples were concentrated by
centrifugation at 4000×g for 15 min at room temperature. Cell
pellets were carefully washed with 1 ml sterile artificial seawater,
centrifuged at 7000×g for 15 min, and stored at −80°C or
immediately processed. Pellets of cultures and field samples were
resuspended respectively in 500 μl and in 250, 500 or 1000 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 CaCl₂, 0.1 mg ml⁻¹ proteinase K). The
susension was sonicated for 10 sec at 50 W with Ultrasonic
Homogenizer LABSONIC (B. Braun, Biotech International,
Germany) and incubated at 55°C for 3 h by vortexing every
30 min. Finally, the samples were incubated at 100°C for 3 min
to inactivate proteinase K. After centrifugation at 12000×g for 1 min
at room temperature to precipitate cell debris, the supernatants
containing total DNA or crude extracts were transferred into new
tubes and diluted at 1:10 and 1:100 for the qrt-PCR assay or
stored at −80°C and processed within two weeks. The genomic
DNA of the *Ostreopsis* cultures contained in the crude extracts
was quantified after incubation at 55°C and before boiling using a
Qubit fluorometer with a Quant-IT dsDNA HS Assay Kit, as
recommended by the manufacturer (Invitrogen, Carlsbad, CA,
USA).

**Ostreopsis cf. ovata primer design**

The design of the species-specific primers was based on all
*Ostreopsis* and other related dinoflagellate consensus LSU rRNA
gene sequences available from GenBank and [20] using OLGIGO
6.0 software. The sequence alignment was constructed using
CLUSTALX2 [23]. The species-specific primers for amplification
of 204 bp (Tm = 81.8°C) of *O. cf. ovata* were Ovata rt forward (5’-
TTGTATCATTCTGGCAATCT-3’) and Ovata rt reverse (5’-
TGAACTTTACCATGCCATTAG-3’). The primers were syn-
thetised by Eurofins MWG operon (Ebersberg, Germany).

**Species-specificity of the qrt-PCR assay**

The species-specificity of the primers was examined in *silico*
using BLAST and tested in qrt-PCR with purified genomic DNA
of *O. cf. ovata* and *O. cf. siamensis* from cultures. Species-specificity
was also assayed with purified genomic DNA from 10 macrophyte
samples collected in a coastal area (Pesaro, central Adriatic Sea)
where *Ostreopsis* spp. had not been detected by microscopy analysis.
Moreover, the potential presence of target extracellular DNA
fragments of *O. cf. ovata* in the environmental samples (macrophytes and surface seawater) was also checked. A total amount of 40 ml of 10 environmental samples of *U. rigida* collected during an *O. cf. ovata* bloom was filtered onto filter type TSTP with 3 μm size pores (Millipore, Billericia, MA, USA) to separate cells from the seawater matrix. Aliquot of 2 μl of the flow through was analysed by the qrt-PCR assay before (first qrt-PCR), and after (second qrt-PCR) by centrifugation at 4000 xg for 10 min at room temperature.

**Construction of plasmid and standard curves**

The 638 bp partial LSU rDNA region was amplified with LSU D1R and LSU D2C primers [24] from purified *O. cf. ovata* CBA163 genomic DNA. The fragment was cloned into the pCR 2.1 vector (Invitrogen, Carlsbad, CA, USA) and the derived pLSUO plasmid DNA was purified using Qiaprep Miniprep kit (QIAGEN, Valencia, CA, USA). Plasmid concentration was measured with a Qubit fluorometer following the manufacturer’s instructions. Plasmid copy number was calculated using the following formula: molecules μl⁻¹ = (A×6.022×10²³)/(660×B)⁻¹, where A is the plasmid concentration (g μl⁻¹), B is the plasmid length containing the cloned sequence, 6.022×10²³ is the Avogadro’s number and 660 is the average molecular weight of one base pair. The plasmid standard curve for *O. cf. ovata* was obtained amplifying a specific internal fragment of 204 bp from 10-fold scalar dilutions with copy number ranging from 10⁶ to 10² (three replicates), and from 10, 5 and 2 molecules (4 replicates) (pLSUO standard curve). A second standard curve (gold standard) was generated by amplifying the 204 bp specific fragment from a mixed *O. cf. ovata* crude extract of 2000 cell pool from *U. rigida* samples (n = 8) collected during the bloom event. This calibration curve was generated using selected cell dilutions as illustrated below (see Assay reproducibility in Results section). In all experiments, negative controls (NTC) containing MilliQ water were tested in triplicate.

**Quantitative real-time PCR assays of cultured and field samples**

Qrt-PCR of *O. cf. ovata* was performed in a final volume of 25 μl containing Hot-Rescue Real Time PCR Kit SG (Diathera, Fano, Italy) based on double-stranded DNA binding dye SYBR Green I, primers at a final concentration of 300 nM, 0.5 U of Hot-Rescue Taq DNA polymerase, and 2 μl undiluted, 1:10 and 1:100 diluted sub-samples of crude extracts. All amplification reactions were carried out using a Step-one Real-time PCR System (Applied Biosystems, 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 60 °C for 1 min. A gold standard curve was included in each PCR reaction.

**Data analysis**

Acquisition of qrt-PCR data and subsequent analyses were carried out using StepOne Software v. 2.1. Because of our assay used SYBR Green I based amplicon detection, a dissociation curve was constructed after the real-time PCR to check for primer dimers, contaminating DNA, and PCR products from misannealed primers.

Standard curves were created automatically and accepted when the slopes were between −3.44 and −3.32 (95–100% efficiency) and the minimum value of the correlation coefficient (R²) was 0.96. The amplification percentage efficiency was calculated as (10⁻¹⁻slope−1)×100. The rDNA copy number per cell and number of *O. cf. ovata* cells in the environmental samples (macrophyte and seawater) were calculated by interpolation of the Ct (threshold cycle) experimentally determined on pLSUO and gold standard curves, respectively, taking into account the lysis buffer volume and dilution factor of the crude extracts. The LSU rDNA copy and cell numbers of *O. cf. ovata* were determined in the environmental samples.

In addition, where there was non-amplification, the samples were further analysed and classified as follows: a) if a qrt-PCR amplification yielded a C<sub>t</sub> value < C<sub>t</sub> value from 2-copy plasmid and 0.0008 cell of gold standard, the sample was quantifiable and the LSU rDNA and cell numbers were determined (sample n = 20); b) if the C<sub>t</sub> value > C<sub>t</sub> value derived from 0.0008 cell, the sample was defined as positive for the presence of *O. cf. ovata* but below this quantification limit (sample n = 21); c) if a negative amplification was reproduced, samples were checked in the spike-qrtPCR for the presence of PCR inhibitors by adding 2 plasmid copies to 1:1, 1:10 and 1:100 crude extract dilutions. If the C<sub>t</sub> values corresponded to those obtained from 2 pLSUO copy number amplification, the samples were not inhibited and these data were reported as not detected (n.d.). *O. cf. ovata* abundance on *U. rigida* was normalised to cells g⁻¹ fw, while *O. cf. ovata* abundance in surface seawater was normalised to cells l⁻¹.

Statistical analyses were performed with non-parametric Mann-Whitney, Kruskal-Wallis and Spearman correlation tests with the MedCalc program (MedCalc Software, Mariakerke, Belgium), with a p<0.05 determining significance.

## Results

### Optimisation of lysis procedure

In order to check the efficiency of the DNA extraction procedure, 4 samples of cultured *O. cf. ovata* CBA163 harvested at 6<sup>th</sup> day of growth and containing 30000 (a), 20000 (b), 10000 (c) and 5000 (d) cells respectively were lysed with 500 μl of buffer and tested by qrt-PCR assay. The rRNA gene copy numbers per cell (± SD) calculated from these samples (9875±275; 7604±96; 8902±1269; 9517±1621 for samples a, b, c and d, respectively) were not significantly different (p>0.05). The results showed that the DNA extraction procedure was not affected by different cell concentrations within the tested range.

### Assay specificity

The species-specificity of the primers designed to target *O. cf. ovata* was examined *in-silico* using BLAST and the results indicated that they were highly specific. Species-specificity was also tested by qrt-PCR on crude extracts from macrophyte samples where *Ostrea* spp. had not been detected by microscopy. Negative amplifications were obtained. The species-specificity of the primers was also assayed in cultures of *O. cf. ovata* and other species of *Ostrea*, such as *O. cf. siamensis*. Positive amplification only with *O. cf. ovata* cultures demonstrated the species-specificity of primers. Furthermore, other several environmental samples containing high background of different microbial taxa assemblages collected in different coastal localities of the Mediterranean Sea in 2008–2009 summers were analysed. The results showed the taxon – specificity signal of the amplification of the mixed microphyto-benthic assemblage DNA only in those samples resulted positive for the presence of *O. cf. ovata* without any other aspecific amplified product (data not shown). The potential presence of *O. cf. ovata* extracellular DNA fragments in two different types of environmental samples, macrophytes and surface seawater (n = 6), was tested by first and second qrt-PCR assay as reported in the Materials and Methods section. The qrt-PCR assays gave C<sub>t</sub> value means of 34.56±0.8 (n = 3 macrophyte samples) and 33.4±0.5...
(n = 3 seawater samples)> the Ct value obtained with 2-copy plasmid dilutions (32.09±0.71), demonstrating a negligible presence of free O. cf. ovata DNA.

**PCR inhibitors and spiking experiments**

To check for the presence of potential inhibitors, ten-fold serial dilutions of crude extract (1:1, 1:10, 1:100) were tested by qrt-PCR and the amplification efficiency evaluated. To obtain optimal quantification, only the PCR products of the same sample with a Ct difference between 3.3 and 3.4 (ΔCt of 3.3 corresponds to an optimal efficiency of 100%) were accepted. None of the dilutions was eliminated for the quantification assay, as they all fell within this range (data not shown). In addition, the putative presence of PCR inhibitors in O. cf. ovata samples with negative amplification was verified by adding 2 copies of pLSUO plasmid to the 1:1, 1:10 and 1:100 dilutions of crude extracts of environmental samples. The spiked qrt-PCR amplifications showed that the Ct values of the 2 molecules (31.09±0.45, n = 26) were not significantly different to the 2 molecules of the plasmid standard curve (32.09±0.71, n = 8) (p>0.05), indicating that the negative amplification of the environmental samples was in fact due to the absence of LSU rDNA target sequences and not to the presence of inhibitors.

**Standard curves, dynamic range and sensitivity of the assay**

The adoption of pLSUO plasmid as standard was validated. The conditions of the environmental samples were simulated by adding 2 μl of crude extract sample, which had been analysed by qrt-PCR to ensure absence of O. cf. ovata DNA, to all pLSUO scalar dilution samples. The results showed that this standard had the same efficiency as that obtained only with pLSUO scalar dilutions (slope = 3.30 and 3.44, respectively, ΔCt<0.01).

A total of 40 qrt-PCR cycles of 10-fold serial (from 10^6 to 10) and 5- and 2- molecule dilutions yielded a pLSUO standard curve with a dynamic range of 6 orders of magnitude and a strong linear correlation (R² mean: 0.99) between the Ct values for each input amount (from 10^5 to 2 molecules) and the log_{10} of the starting pLSUO copy number. The qrt-PCR efficiency was 95%, and the mean standard curve (γ = -3.44±34.04, n = 8 experiments) with a sensitivity of 2 copies/reaction was used to calculate the number of LSU gene copies per PCR sample. The gold standard curve generated by amplifying the species-specific O. cf. ovata fragment from a lysed pool of macroalgae samples (n = 4) had a linear correlation range of 5 log_{10} (R² mean: 0.96), a quantification limit of 0.0008 cell per PCR reaction (Ct mean = 34.33±1.06) and an efficiency of 98%. This standard curve mean of γ = -3.36±23.72 (n = 6 experiments) derived from plotting the Ct values of each input amount (from 8 to 0.0008 cells) against log_{10} of the starting cell number, enabled us to calculate the initial amount of cells for each PCR sample. As the two standard curves (Fig. 1) had the same efficiency (ΔCt = 0.00), the data from the environmental samples were normalized and expressed as LSU gene copy number per cell.

**Assay reproducibility**

The method’s reproducibility (inter-assay variation) was assayed by calculating the CV_{Ct} (coefficient of variation of cycle threshold) of both pLSUO and gold standards, and for the estimated pLSUO plasmid copy number (CV_{Ct}) extrapolated from the mean equation of the standard curve in 8 independent experiments run on different days using 8 different sets of dilutions for the two standard curves (Tables 1 and 2). The CV_{Ct} mean values of the pLSUO and gold standard curves were 1.7% (range 10^6 – 2) and 3.0% (range 8 – 0.0008 cells) respectively, and remained at 1.5% in the low copy number range (10^2 – 2) and at 3.0% in the low cell number range (0.008 – 0.0008). Moreover, the CV_{Ct} mean values of the pLSUO standard curve was 25% in the 10^6 – 2 copy number range. The precision (intra-assay variation) of each of the two curves was measured 10 times within one PCR run. The mean intra-assay variation based on the Ct was 1.4% and 3.0% for the pLSUO and gold standard curves respectively.

**Estimation of rDNA in O. cf. ovata culture system and environmental samples**

Five O. cf. ovata isolates collected from different regions of the Mediterranean Sea were grown in a culture system. Aliquots of 5.0×10^7 cells were harvested at 6, 13 and 28 days and lysated in 500 μl buffer. The qrt-PCR experiment was carried out using the crude extract derived from 20, 8, 2, and 0.2 cells per sampling day. The mean of LSU gene copy number per cell (n =3) was calculated by plotting the Ct values on the pLSUO standard curve based on the dilution factor. Within the same isolate a significant variation in rDNA copy number per cell between the 6th and 13th days was found (p<0.05). On the other hand, no significant differences in copy number were detected between the 6th and 28th days and the 13th and 28th days (p>0.05). Meanwhile, a significant variation in copy number per cell was found among the different O. cf. ovata isolates (p<0.05) except for O. cf. ovata CBA1377 vs CBA13577 at 13th day, and CBA166 vs CBA1273, and CBA1377 vs CBA1346 at 28th day (Table 3).

The LSU gene copy number per O. cf. ovata cell from environmental samples was also evaluated in qrt-PCR by extrapolating it from pLSUO and gold standard comparison curves (Tables 4 and 5). The mean value of 1030±49 derived from all both macroalga and surface seawater sample measurements, which no significant variability displayed (p>0.05).

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

All samples were analysed by qrt-PCR to check for the presence of O. cf. samens. These amplifications yielded negative results, excluding the presence of this species (data not shown), then all Ostreopsis spp. cells observed by microscopy were considered belonging to O. cf. ovata.

Environmental samples of U. rigida microepiphytic assemblages and surface water were analysed with both microscopy and molecular (qrt-PCR assay) methods (Fig. 2). The temporal trend in O. cf. ovata mean abundances during the study period is shown in Tables 4 and 5, for epiphytic cells and water column respectively. The first occurrence of O. cf. ovata cells on macroalgal samples was detected on August 6th by microscopy, and on August 21st by qrt-PCR. Cell abundances increased during September and reached the peak between September 23rd and October 6th. Maximum cell densities in the U. rigida samples were observed on September 29th (6.2×10^4 cells g^-1 fw by qrt-PCR and microscopy, respectively). The bloom declined at the end of October. Ostreopsis cells were detected until October 21st by both qrt-PCR and microscopy, while on October 28th a positive result was obtained only with the qrt-PCR method.

In the water column, the first cells were observed on August 27th with both microscopy and qrt-PCR methods. Maximum values were observed on September 3rd (1.3×10^7 and 9.2×10^6 cells l^-1 detected by qrt-PCR and microscopy, respectively). As already observed with respect to the benthic substrata, cells disappeared at the end of October (last detection 20 cells l^-1 on October 21st, only with microscopy). There was a significant positive correlation
Figure 1. Dynamic range, sensitivity and specificity of the qrt-PCR assay. A typical amplification plot (a, d) and the corresponding standard curve (b, e) for the pLSUO and gold standards are shown, respectively. A 204 bp fragment of the LSU gene were amplified in a 10^6, 10^5, 10^4, 10^3, 10^2, 10, 5 and 2 copy dilutions of the pLSUO plasmid (a) and in a 8, 4, 0.8, 0.16, 0.08, 0.016, 0.008, 0.004 and 0.0008 O. cf. ovata cell dilutions (d) of the lysed pool from macroalgae samples. The fluorescence intensity (ΔRn) expressed as logarithmic scale is plotted vs cycle number. Only one replicate is shown. The mean standard curve is obtained by the correlation of Ct values and log_{10} input plasmid copy (b) or environmental O. cf. ovata cell number (e) measured in 8 independent experiments ± SD, respectively. Melting curve of the the 204 bp amplified fragments of the pLSUO plasmid (c) and environmental O. cf. ovata cells (f) generated by qrt-PCR. To better display the melting curves, only the low input pLSUO plasmid copy number (10, 2 and NTC) and cell number (0.008 and 0.0008) are shown. As few as two copies of pLSUO plasmid and 0.0008 cell dilutions are clearly detected. The specificity of the 204 bp amplicon was also confirmed by gel electrophoresis and sequencing analysis (data not shown).

doi:10.1371/journal.pone.0017699
settled for the microscopy, resulted in a positive detection (no. 21) 
(Spearman r = 0.98 and 0.97 respectively, samples from no. 14 to no. 19 and from no. 36 to no. 41 correlation was particularly evident during the bloom event in microscopy and those found with molecular methods. This high in which no 
of low cell numbers, the PCR reaction of two macrophyte samples 
and in the water column. 

Discussion

In order to understand the dynamics of toxic microalgae blooms and to mitigate their impact on human health, the environment and the economy, it is important to improve monitoring of them in terms of frequency, sensitivity and rapidity. To achieve this goal, a reliable and fast molecular method with high sample throughput and a low quantification limit is desirable. Identification of Ostreopsis spp. in natural samples is usually based on light or epifluorescence microscopy, but because of the high morphological and morphometrical variation within each species this is

| Table 1. Reproducibility of the qrt-PCR assay based on pLSUO plasmid standard curve. |
|---|
| Copy number | Mean Ct ± SD| CVc%| CVt% |
| 10⁰ | 12.88±0.06 | 0.4 | 4.0 |
| 10¹ | 16.89±0.61 | 3.6 | 33 |
| 10² | 20.21±0.41 | 2.0 | 26 |
| 10³ | 23.68±0.38 | 1.6 | 23 |
| 10⁴ | 27.15±0.38 | 1.4 | 23 |
| 10⁵ | 30.89±0.49 | 1.6 | 33 |
| 5 | 31.36±0.23 | 0.7 | 15 |
| 2 | 32.09±0.71 | 2.2 | 46 |

*Mean Ct values measured in 8 independent experiments ± standard deviation (SD). 
*Coefficient of variation of cycle threshold. 
*Coefficient of variation of copy number. 
doi:10.1371/journal.pone.0017699.t001

(n = 18, Spearman r = 0.97, p<0.05) between cell densities on Ulva rigida and in the water column. 
A correspondence was found between the results obtained with microscopy and those found with molecular methods. This high correlation was particularly evident during the bloom event in samples from no. 14 to no. 19 and from no. 36 to no. 41 (Spearman r = 0.98 and 0.97 respectively, p<0.05). In the range of low cell numbers, the PCR reaction of two macrophyte samples in which no Ostreopsis cells were found using the volume sample settled for the microscopy, resulted in a positive detection (no. 21) and quantification of 7 cells g⁻¹ fw (no. 12). Whereas, microscopy analyses revealed the presence of 6 cells g⁻¹fw in sample no. 10, and 20 cells l⁻¹ in sample no. 42, while the same samples analysed by qrt-PCR produced negative results.

| Table 2. Reproducibility of the qrt-PCR assay based on gold standard curve. |
|---|
| Dilution factor | Cell number | Mean Ct ± SD | CVc%| CVt% |
| 1:1 | 8 | 20.58±0.70 | 0.034 | 3.4 |
| 1:2 | 4 | 21.57±0.78 | 0.036 | 3.6 |
| 1:10 | 0.8 | 24.13±0.82 | 0.034 | 3.4 |
| 1:50 | 0.16 | 26.50±0.91 | 0.034 | 3.4 |
| 1:100 | 0.08 | 27.52±1.12 | 0.041 | 4.1 |
| 1:500 | 0.016 | 29.99±1.02 | 0.034 | 3.4 |
| 1:1000 | 0.008 | 30.40±0.96 | 0.030 | 3.2 |
| 1:2000 | 0.004 | 31.36±0.65 | 0.021 | 2.1 |
| 1:10000 | 0.0008 | 34.35±1.06 | 0.031 | 3.1 |

*Mean Ct values measured in 8 independent experiments ± standard deviation (SD). 
*Coefficient of variation of cycle threshold. 
*Coefficient of variation of copy number. 
doi:10.1371/journal.pone.0017699.t002

| Table 3. LSU gene copy number per cell in Ostreopsis cf. ovata isolates during growth phase. |
|---|
| Ostreopsis cf. ovata isolates | LSU copy number ± SD |
| 6th day | 13th day | 28th day |
| CBA 166 | 3908±188 | 1812±384 | 848±175 |
| CBA 1273 | 8488±64 | 1372±294 | 3687±366 |
| CBA 1298 | 1577±168 | 881±236 | 1908±300 |
| CBA 1377 | 1520±155 | 410±54 | 849±210 |
| CBA 1346 | 1225±217 | 536±121 | 81±13 |

*Mean LSU copy number measured in triplicates ± standard deviation (SD). 
doi:10.1371/journal.pone.0017699.t003

| Table 4. Qrt-PCR assay and microscopy analysis of Ostreopsis cf. ovata cell number from macroalgae Ulva rigida samples collected in 2009 at Conero Riviera (northern Adriatic Sea) and calculation of LSU gene copy number per cell by qrt-PCR. |
|---|
| Sample no. | Sampling period | Abundance a (cells g⁻¹ fw ± SD) | LSU copy no.c (cell⁻¹ ± SD) |
| | | qrt-PCR | Microscopy |
| 1 | March 25 th | n.d. b | n.d. |
| 2 | April 7 th | n.d. | n.d. |
| 3 | May 7 th | n.d. | n.d. |
| 4 | May 18 th | n.d. | n.d. |
| 5 | June 3 rd | n.d. | n.d. |
| 6 | June 15 th | n.d. | n.d. |
| 7 | July 3 rd | n.d. | n.d. |
| 8 | July 17 th | n.d. | n.d. |
| 9 | July 31 st | n.d. | n.d. |
| 10 | August 6 th | n.d. | n.d. |
| 11 | August 11 st | n.d. | n.d. |
| 12 | August 21 st | 7±3 | n.d. |
| 13 | August 27 th | 11±4 | 91±42 |
| 14 | September 3 rd | 11551±840 | 13838±692 |
| 15 | September 9 th | 955±203 | 703±84 |
| 16 | September 15 th | 757±123 | 892±196 |
| 17 | September 23 rd | 56456±3740 | 55611±2224 |
| 18 | September 29 th | 62120±6798 | 73573±2943 |
| 19 | October 6 th | 28784±4056 | 43058±1292 |
| 20 | October 21 st | 4±2 | 48±55 |
| 21 | October 28 th | n.d. | n.d. |
| 22 | November 19 th | n.d. | n.d. |

*Mean abundance measured in triplicates ± standard deviation (SD). 
*Not detected. 
*Mean LSU copy number measured in triplicates ± standard deviation (SD). 
doi:10.1371/journal.pone.0017699.t004
Table 5. qrt-PCR assay and microscopy analysis of Ostreopsis cf. ovata cell number from surface seawater samples collected in 2009 at Conero Riviera (northern Adriatic Sea) and calculation of LSU gene copy number per cell by qrt-PCR.

| Sample no. | Sampling period | Abundance* (cells l⁻¹ ± SD) | LSU copy no.* (cell⁻¹ ± SD) |
|------------|-----------------|-----------------------------|-----------------------------|
|            |                 | qrt-PCR                     | Microscopy                  |
| 23         | March 25th      | n.d.                        | n.d.                        |
| 24         | April 7th       | n.d.                        | n.d.                        |
| 25         | May 7th         | n.d.                        | n.d.                        |
| 26         | May 18th        | n.d.                        | n.d.                        |
| 27         | June 3rd        | n.d.                        | n.d.                        |
| 28         | June 15th       | n.d.                        | n.d.                        |
| 29         | July 3rd        | n.d.                        | n.d.                        |
| 30         | July 17th       | n.d.                        | n.d.                        |
| 31         | July 31st       | n.d.                        | n.d.                        |
| 32         | August 6th      | n.d.                        | n.d.                        |
| 33         | August 11th     | n.d.                        | n.d.                        |
| 34         | August 21st     | n.d.                        | n.d.                        |
| 35         | August 27th     | 11 ± 3                      | 160 ± 131                   | 1142 ± 74 |
| 36         | September 3rd   | 125924 ± 5100               | 92480 ± 3699                | 971 ± 39  |
| 37         | September 9th   | 100 ± 50                    | 360 ± 241                   | 1080 ± 48 |
| 38         | September 15th  | 250 ± 75                    | 840 ± 370                   | 1058 ± 65 |
| 39         | September 23rd  | 16625 ± 775                 | 15200 ± 1216                | 1029 ± 58 |
| 40         | September 29th  | 15675 ± 2700                | 13750 ± 2062                | 1013 ± 52 |
| 41         | October 6th     | 2475 ± 75                   | 680 ± 313                   | 1030 ± 40 |
| 42         | October 21st    | n.d.                        | 20 ± 40                     | n.d.      |
| 43         | October 28th    | n.d.                        | n.d.                        | n.d.      |

*Mean abundance measured in triplicates ± standard deviation (SD).
Not detected.

The mean LSU copy number measured in triplicates ± standard deviation (SD).
doi:10.1371/journal.pone.0017699.0005

difficult, time consuming, labour-intensive, and requires a high level of taxonomic expertise, especially when different Ostreopsis species co-occur in natural samples.

Figure 2. Micrograph of Ostreopsis cf. ovata assemblage in Scanning Electron Microscopy (SEM). Scale bar 50 μm (photo by Romagnoli T.).
doi:10.1371/journal.pone.0017699.g002

In this study, we report the development of a qrt-PCR assay for specific O. cf. ovata quantification. The species-specific primers were designed on a partial (D1/D2 domains) LSU rDNA sequence. The qrt-PCR assay was based on binding the SYBR Green I dye into double-stranded PCR products. The SYBR Green methodology seems to be a better option for quantification than compared to sequence-specific fluorescent probes in the TaqMan or TaqMan MGB based assay, since it requires only one set of specific primers, hence providing additional experimental flexibility, and it reduces assay set-up and running costs while providing similar levels of accuracy in optimised assays [25]. Moreover, the fluorescent-probe-based assay, such as TaqMan, can fail to detect target DNA that contains even only a single mismatched base at the 5’ end of the probe nucleotide sequence. This would prevent hybridization of the 5’-3’ exonuclease activity of the Tag polymerase and therefore no liberation of the fluorescent reporter, making quantification impossible [26,27].

We demonstrated that the significant variation in rDNA copy number per cell of O. cf. ovata in culture systems made it impossible to develop a reliable and accurate quantification method based only on a plasmid standard curve or on a pool of DNA target from cultured samples. The main differences between our assay and other qrt-PCR methods were the elimination of the DNA purification step and the introduction of a second calibration curve, such as “gold standard”, which was created with pooled crude extracts of O. cf. ovata from environmental samples collected during the bloom event. This allowed us to recover total DNA of the target microbial species, eliminate the different amplification efficiencies among standards and unknown samples, and normalise the rDNA copy number per cell of O. cf. ovata in environmental samples and thus obtain a specific absolute quantification.

A series of parameters for obtaining this goal were examined. Lysis buffer performance in recovering total DNA from cells was validated by testing different samples of O. cf. ovata CBA165 collected at the same day of growth and lysed. Thus, this procedure was effective and reproducible in the range of 5000 to 50000 cells. The significant differences in LSU copy number per cell among different O. cf. ovata isolates found during growth phases is therefore due to high variability in the LSU gene content which could be due to the age of culture, as already postulated for other marine planktonic dinoflagellates [28], or to the potential presence of extra-chromosomal rDNA molecules. Indeed, in some alveolate species rRNA genes are organised in linear and circular rDNA copy number for this species in the environmental samples is not precisely known, the fact that the quantification limit is largely below 1 cell indicates that the rDNA operon is tandemly repeated up to thousands copies, as in other protists [31]. The mean efficiency of the pLSUO standard curve was 95% with a dynamic range of 6 orders of magnitude (from 10⁶ to 2 copies) and a strong linear correlation. The similar efficiencies allowed us to precisely calculate the rDNA copy number per O. cf. ovata cell. Quantification with standards requires evaluation of
specification of the assay was demonstrated: (i) in silico using BLAST; (ii) by qrt-PCR carried out with purified DNA from *O. cf. ovata* and *O. cf. siamensis* cultured cells; (iii) by qrt-PCR test performed with macrophyte samples containing mixed microphytobenthos assemblages to ensure the absence of non-specific amplification products. Furthermore, the results revealed that the sets of primers exclusively identified the *O. cf. ovata* based on the amplification of rDNA from whole cells, refuting the hypothesis that extracellular target rDNA molecules could interfere with target cell quantification. Thus, the abundance of *O. cf. ovata* calculated with our optimized assay was species-specific and accurate.

The qrt-PCR assay was validated in 43 environmental samples collected from the Conero Riviera during both non-bloom and bloom conditions. Cell lysates were used as templates in the PCR reactions without further purification and, to rule out the possibility that inhibitory substances affect amplification reactions, (i) the cellular pellet was washed with artificial sea water before the lysis procedure to remove traces of formalin and other inhibitors; (ii) after lysis, cellular components were eliminated by centrifugation; (iii) the efficiencies of crude extract amplifications were assayed by qrt-PCR. In addition, the spiking experiments demonstrated the absence of inhibitors. Therefore, all these devices were able to remove all PCR inhibitors from the procedure, from sample collection to PCR reaction.

Abundances determined in natural samples by qrt-PCR using the gold standard, correlated significantly with counts obtained by light microscopy during the bloom event. As far as the reliability of the microscopy method is concerned, it should be noted that in order to obtain a statistically acceptable estimate of cell numbers using the Utermohl method, it is recommended that at least 50 units be counted for each taxon [32]. This limit is a problem for samples containing low cell abundances, such as those taken in non-bloom or pre-bloom conditions. Bearing this in mind, where cell numbers are low, data obtained through microscopic counts often overestimate cell abundances and are therefore considered highly unreliable, with an error of 200% [33]. The lack of correspondence observed between microscopic and qrt-PCR methods in environmental samples containing low cell numbers, may be explained if we take into account the threshold limits in microscopy counts. In particular, the *Ostreopsis* abundance of 20 cells l\(^{-1}\) in sample no. 42 was obtained from counting only one cell in 40 ml of settled sample, and in sample no. 10 (6 cells g\(^{-1}\) lw) only 2 cells were counted in 10 ml of settled sample. On the other hand, the sensitivity of the qrt-PCR assay made a positive detection in two macrophyte samples in which no *Ostreopsis* cells were found by microscopy. Furthermore, when using microscopy, sample volumes must be adjusted according to target species abundances. Concentrated samples need to be diluted, while samples with low *O. cf. ovata* abundances need to be counted in higher volumes or even in multiple sub-samples. These complications mean that it could be take as long as two weeks to fully process the analyses of the 43 samples. In contrast, with our qrt-PCR method it takes few hours to analyse a set of standards and unknown samples, reducing working time drastically compared with microscopy-based methods when a large number of samples need to be analysed. The qrt-PCR we developed had a high specificity, sensitivity, reproducibility and efficiency in a broad dynamic range over which cell abundance could be quantified, and did not require morphological taxonomic expertise. This approach involved the analysis of 40-ml field samples and resulted in a quantification limit based on both sample and lysis buffer volumes. This quantification limit could be reduced by filtering a larger sample volume, which would increase the sensitivity of the qrt-PCR assay.

The similar efficiencies of the pLSUO and gold standard curves that we found allowed us to correctly quantify the mean copy number of rDNA per cell (1030±49) in the *O. cf. ovata* bloom event. This is very important because for the first time a molecular assay that was validated on *O. cf. ovata* quantification by pLSUO and gold standards has allowed us to quantify the toxic benthic dinoflagellate in a survey activity using only the pLSUO standard curve. To demonstrate the robustness of the qrt-PCR method we applied it to new samples collected during the *O. cf. ovata* bloom occurred at the Conero Riviera in 2010 summer. The comparison of results obtained from these preliminary experiments with those obtained in the present study, showed high reproducibility and efficiency of the method at time scale. Therefore, the assay may be considered versatile to any environmental bloom in different Mediterranean coastal localities.

Furthermore, the timely and specific detection of harmful algal species prior to bloom development is a crucial component of most HAB management programmes and is also a necessary tool for researchers studying population dynamics and developing models to forecast HAB events.

Acknowledgments

We thank Dr. Galluzzi L. for comments on the manuscript. We would like to thank Environmental Agencies ARPAT and ARPAL, Prof. Tumbolo A., Drs. Giacobbe M.G. and Chiantore M.C. for providing us field samples of *Ostreopsis* spp.

Author Contributions

Conceived and designed the experiments: AC FP AP. Performed the experiments: FB CB. Analyzed the data: FP AC CB CT SA AP. Contributed reagents/materials/analysis tools: AP. Wrote the paper: FP AC AP.

References

1. Galluzzi L, Penna A, Bertozzini E, Vila M, Garecs E, et al. (2004) Development of a real-time PCR assay for rapid detection and quantification of *Alexandrium minutum* (a dinoflagellate). Applied and Environmental Microbiology 70: 1199–1206.
2. Godlie A, Asplund ME, Harnstrom K, Saravanam V, Tyagi A, et al. (2008) Quantification of diatom and dinoflagellate biomasses in coastal marine seawater samples by real-time PCR. Applied and Environmental Microbiology 74: 7174–7182.
3. Erdour DL, Perry L, Keeler B, Lewis J, Anderson DM (2010) A quantitative real-time PCR assay for the identification and enumeration of *Alexandrium* cysts in marine sediments. Deep Sea Research Part II: Topical Studies in Oceanography 57: 279–297.
4. Thornhill DJ, Leprieuse TC, Santos SR (2007) Measuring rDNA diversity in eukaryotic microbial systems: how intragenomic variation, pseudogenes, and PCR artifacts confound biodiversity estimates. Molecular Ecology 16: 5326–5340.
5. Torres-Machorro AL, Hernández R, Cevallos AM, López-Villaseor I (2010) Ribosomal RNA genes in eukaryotic microorganisms: witnesses of phylogeny? FEBS Microbiology Review 34: 59–86.
13. Totti C, Accoroni S, Cerino F, Cucchiari E, Romagnoli T (2010) 
12. Mangialajo L, Bertolotto R, Cattaneo-Vietti R, Chiantore MC, Grillo C, et al. 
11. Vila M, Garce’s E, Maso´ M (2001) Potentially toxic epiphytic dinoflagellates 
10. Yasumoto T, Seino N, Murakami Y, Murata M (1987) Toxins produced by 
14. Riobo` P, Paz B, Franco JM (2006) Analysis of palytoxin-like in 
19. Battocchi C, Totti C, Vila M, Maso` M, Capellacci S, et al. (2010) Monitoring 
17. Penna A, Vila M, Fraga S, Battocchi C, Casabianca S, Robiò P, et al. (2010) A 
20. Penna A, Fraga S, Battocchi C, Casabianca S, Robiò P, et al. (2010) A 
21. Smayda TJ (1997) Harmful algal blooms: their ecophysiology and general 
relevance to phytoplankton blooms in the sea. Limnology and Oceanography 42: 1137–1153. 
22. Totti C, De Stefano M, Facca C, Ghirardelli LA (2004) Microphytobenthos. In: 
Gambi MC, Dappiano M, eds. Mediterranean marine benthos: a manual of 
Methods for its sampling and study. Genoa: SMB. pp 247–266. 
23. Larkin MA, Blackshields G, Brown NP, Chernia R, McGregor PA, McWilliam H, et al. (2007) ClustalW2 and ClustalX version 2. Bioinformatics 23: 2947–2948. 
24. Scholin CA, Herzog M, Sogin M, Anderson JM (1994) Identification of group- 
and strain-specific genetic markers from globally distributed Alexandrium 
(Dinophyceae). II. Sequence analysis of fragments of the LSU rRNA gene. 
Journal of Phycolgy 30: 999–1011. 
25. Karsai A, Müller S, Platz S, Hauser MT (2002) Evaluation of a home-made 
SYBR green I reaction mixture for real-time PCR quantification of gene 
expression. Biotechniques 32: 790–796. 
26. Désiré N, Dehie A, Schneider V, Jacomet C, Guillon C, et al. (2001) 
Quantification of human immunodeficiency Virus type 1 proviral load by a 
TaqMan real time PCR assay. Journal of Clinical Microbiology 39: 1303–1310. 
27. Casabianca A, Gori C, Orlanetti C, Forbici F, Perino CF, et al. (2007) Fast and 
sensitive quantitative detection of HIV DNA in whole blood leucocytes by SYBR 
green I real–time PCR assay. Molecular and Cellular Probes 21: 368–378. 
28. Galluzzi L, Bertozzini E, Penna A, Perini F, Garce’s E, Magagn M (2010) 
Analysis of rRNA gene content in the Mediterranean dinoflagellate 
Alexandrium catenella and Alexandrium tamathi: Implications for the quantitative real-time PCR- 
based monitoring methods. Journal of Applied Phycology 22: 1–9. 
29. Manuyma S, Nozaki H (2007) Sequence and Infracommunal Location of the 
Extrachromosomal rDNA Plasmid of the Amoebo-Flagellate Nassulea guidei. 
Journal of Eukaryotic Microbiology 54: 333–337. 
30. Greenwood SJ, Schooner MR, Cook JR, Gray MW (2001) Analysis of intragenic 
space transcripts suggests “read around” transcription of the extrachromosomal 
circular rDNA in Euglena guttata. Nucleic Acid Research 29: 2191–2198. 
31. Saito K, Drogue T, Robledo JA, Keupkina DN, Vasta GR (2002) Characterization of the rRNA locus of Plostrera pisicola and development of 
standard and quantitative PCR-based detection assays targeted to the non 
transcribed spacer. Applied and Environmental Microbiology 68: 5394–5407. 
32. Rott E, Salmaso N, Hoehn E (2007) Quality control of Utermöhl-based 
phytoplankton counting and biovolume estimates - an easy task or a Gordian 
knot? Hydrobiologia 578: 141–146. 
33. Eder L, Elbracht E (2010) The Utermöhl method for quantitative 
phytoplankton analysis. In: Karlson B, Cunac C, Bremo E, eds. Microscopic 
and molecular methods for quantitative phytoplankton analysis. Paris: UNESCO. pp 13–20.