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Genetic diversity of the genus *Ostreopsis* Schmidt: phylogeographical considerations and molecular methodology applications for field detection in the Mediterranean Sea

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Abstract – This study reports some recent phylogeographical considerations on the genus *Ostreopsis* distribution worldwide, with particular attention to the Mediterranean Sea, and new recent advances on the quali-quantitiative detection of *Ostreopsis* species along coastal areas of the Mediterranean Sea based on the PCR and quantitative real time PCR (qrt-PCR) assays. It was found that *O.* cf. *ovata* is widely dispersed throughout tropical and warm

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temperate coastal areas. In the Atlantic/Mediterranean region it represents a panmictic population that is highly divergent from Indo-Pacific populations. Furthermore, we demonstrated that the developed qrt-PCR assay is specific, robust and high sample throughput for the quantification of the toxic *O.* cf. *ovata* in the environmental samples. This molecular approach may be considered alternative to traditional methods of microscopy and applied for the survey of benthic toxic microalgal species in marine ecosystems.

Benthic dinoflagellate / distribution / Mediterranean Sea / *Ostreopsis* / ribosomal genes / qrt-PCR

INTRODUCTION

Ostreopsis is a benthic and epiphytic dinoflagellate known to produce palytoxin-like compounds. The genus *Ostreopsis* has recently been receiving greater attention from researchers and public authorities, since its proliferations have been associated with human intoxications by toxic aerosols along the western Mediterranean coasts. Two species based on morphology and genetic characters, as *O.* cf. *ovata* Fukuyo and *O.* cf. *siamensis* Schmidt, are present in the Mediterranean Sea (Penna *et al.,* 2005). In particular, *O.* cf. *ovata* genotype is being found with increasing frequency and abundance in the temperate area of the Mediterranean Sea (Fig. 1) (Mangialajo *et al.,* 2011; Parson *et al.,* 2012), causing negative impact on economical activities and environmental health, and above all, its blooms are considered potential causative agent of human intoxication cases due to inhalation of marine aerosols along coastal areas (Durando *et al.,* 2007; Barroso Garcia *et al.,* 2008). *Ostreopsis* species can produce different palytoxin analogues with different toxicological and ecological implications to human health and environmental communities (Ciminiello *et al.,* 2011). Therefore, species-specific taxonomic attribution to strains and assemblages in field samples is an essential criterion.

Fig. 1. Geographical distribution of the *Ostreopsis* cf. *ovata* (Atlantic/Mediterranean clade) in the Mediterranean Sea.

Ostreopsis is distributed worldwide, from tropical to temperate coastal waters, with nine described different morphotype species. But only few identified genetic species, reported as *O.* cf. *ovata*, *O.* cf. *siamensis*, *O. lenticularis* and *O. labens*, were analyzed based on the phylogeny and nucleotide diversity at inter- and intra- species especially at the Mediterranean area. Regarding the phylogenetic position of genus *Ostreopsis,* it clearly grouped within the Gonyaulacales together with the other Ostreopsidaceae genus *Coolia* based on the SSU and LSU gene sequence data (Saldarriaga *et al.,* 2004; Guerrini *et al.,* 2010). Phylogenetic and phylogeographic analyses were carried out based on the ribosomal gene sequences from partial nuclear LSU (D1/D2 domains) and 5.8S genes and non-coding internal transcribed spacer (ITS) of several isolates of different *Ostreopsis* species collected in numerous localities throughout the world (Penna *et al.,* 2010). The rDNA phylogeny revealed different clades within genus *Ostreopsis* as mentioned above. Different genetic lineages of *O.* cf. *ovata* were correlated with macrogeographical distribution. Further, at the Atlantic/ Mediterranean regions, *O.* cf. *ovata* seemed to constitute a panmictic population highly differentiated from the Indo-Pacific populations. The other *Ostreopsis* genetic lineages turned out restricted to just one of the two main warm-water oceanic basins of the Atlantic/Mediterranean and Indo-Pacific.

The species-specific identification by optical methods of microscopy is difficult due to the high morphological variability, and thus different species having similar morphotypes can be easily misinterpreted. Molecular primers for the species-specific identification and quantification were designed and validated using PCR based technologies. In the monitoring activities of the toxic blooms, the PCR based methods proved to be effective tools, complementary or alternative to microscopy for rapid and species-specific estimation of *Ostreopsis* spp. In this report, new preliminary findings will be introduced with regard to population genetic of Mediterranean *O.* cf. *ovata* analysis, as well as new rapid and sensitive detection of *Ostreopsis* spp. in marine environment.

METHODS USED IN LITERATURE

Phylogenetic and phylogeographical analyses of numerous isolates of *Ostreopsis* spp. based on ITS, 5.8S and LSU genes were reported by Pin *et al.,* (2001), Penna *et al.,* (2005; 2010), Guerrini *et al.,* (2010) and Laza Martinez *et al.,* (2011). Molecular PCR based assay for the detection and monitoring of toxic *Ostreopsis* species in coastal waters of the Mediterranean Sea, combined with light microscopy was illustrated in Battocchi *et al.,* (2010) and Accoroni *et al.,* (2011). Finally, new and innovative molecular quantitative real time PCR method for the enumeration of toxic benthic *O.* cf. *ovata* in the coastal system of the Mediterranean Sea was developed by Perini *et al.,* (2011).

PHYLOGENETIC CHARACTERIZATION OF THE GENUS *OSTREOPSIS*

In the Mediterranean Sea, molecular phylogenetic coupled with morphological investigations showed that all *Ostreopsis* spp. isolates grouped into two distinct species, *Ostreopsis* cf. *ovata* and *O.* cf. *siamensis.* The phylogenetic

analyses, which included also isolates from SW Atlantic and Indo-Pacific areas, confirmed the clustering of the *Ostreopsis* isolates in two distinct species of *O.* cf. *ovata* and *O.* cf. *siamensis* (Penna *et al.,* 2005) based on the ITS regions and 5.8S gene. Based on the NJ, ML and Bayesian Inference analyses, the Mediterranean and Atlantic *O.* cf. *ovata* clade resulted well separated from the Asian clade. This means that probably the European and Asian isolates evolved independently over time as also shown by the high percentage of net nucleotide differences. Further, based on the higher divergence values of single or concatenated ITS, 5.8S and LSU sequences, as shown in Penna *et al.,* (2010), each *O.* cf. *ovata* grouping can constitute a genetically distinct clade, or species, if in the future, the taxonomical analysis of cultured type material of both Mediterranean and Indo-Pacific areas will prove this assumption. In this latter study, different *Ostreopsis* species were analyzed based on the molecular diversity index among isolates. The ribosomal sequences showed that the species *O.* cf. *siamensis*, *O.* cf. *ovata*, *O. lenticularis* and *O. labens* were highly differentiated, with greatest divergence between Atlantic/Mediterranean and Malaysian/Indonesian *O.* cf. *ovata* isolates. Moreover, in the phylogenetic study of Pin *et al.,* (2001) it was shown that *O.* cf. *ovata* was phylogenetically distinct from *O. lenticularis* based on the ITS-5.8S rDNA sequence data, and further, within the *O.* cf. *ovata* isolates two different groups merged in the Malaysian waters: the Port Dickinson, and Kota Kinabalu and Pulau Redang clusters that belonged to the Indian and Pacific areas, respectively. The isolates of *O.* cf. *siamensis* from different western Mediterranean localities made a clade and shared identical nucleotide sequences. This species was detected and identified in the Mediterranean Sea, and recently in the eastern Atlantic coasts (Laza-Martinez *et al.,* 2011), whereas *O. siamensis* morphotype was originally described by Schmidt (1901) in the Indo-Pacific region.

PHYLOGEOGRAPHICAL IMPLICATIONS FOR THE GENUS *OSTREOPSIS*

Ostreopsis is a benthic dinoflagellate that can be expected showing a geographical pattern at macrogeographical scales, since it is distributed worldwide. To test if genus *Ostreopsis* has geographical distribution and genetic differentiation, a phylogeographic structure within and among different species of *Ostreopsis* isolates from different geographical locations based on ribosomal genes was demonstrated and analysed. The phylogenetic analyses based on single and concatenated ribosomal genes of 5.8S, LSU (D1/D2) and ITS regions evidenced that different clades corresponded to different species within the *Ostreopsis* spp. (Figs 2-3). In particular, a clade represented by isolates of *Ostreopsis* sp. VGO881, KC84 and KC86; a clade constituted by *O. lenticularis* and *O. labens* or *O. labens* and *Ostreopsis* sp. from Hawaii; a clade constituted by *O.* cf. *siamensis* and a clade comprising *O.* cf. *ovata*. The clade that includes new different genotypes of VGO881, KC84 and KC86 probably corresponding to a new species of *Ostreopsis* based on morphology and genetic features, was found both on the Atlantic coast of Canary Islands and Greece and Cyprus in the Mediterranean Sea (Penna pers. comm.). The clade of *O.* cf. *siamensis* included isolates from the Mediterranean Sea and E Atlantic without isolates from the Indo-Pacific region, where *O. siamensis* was originally described by Schmidt (1901). This evidence can

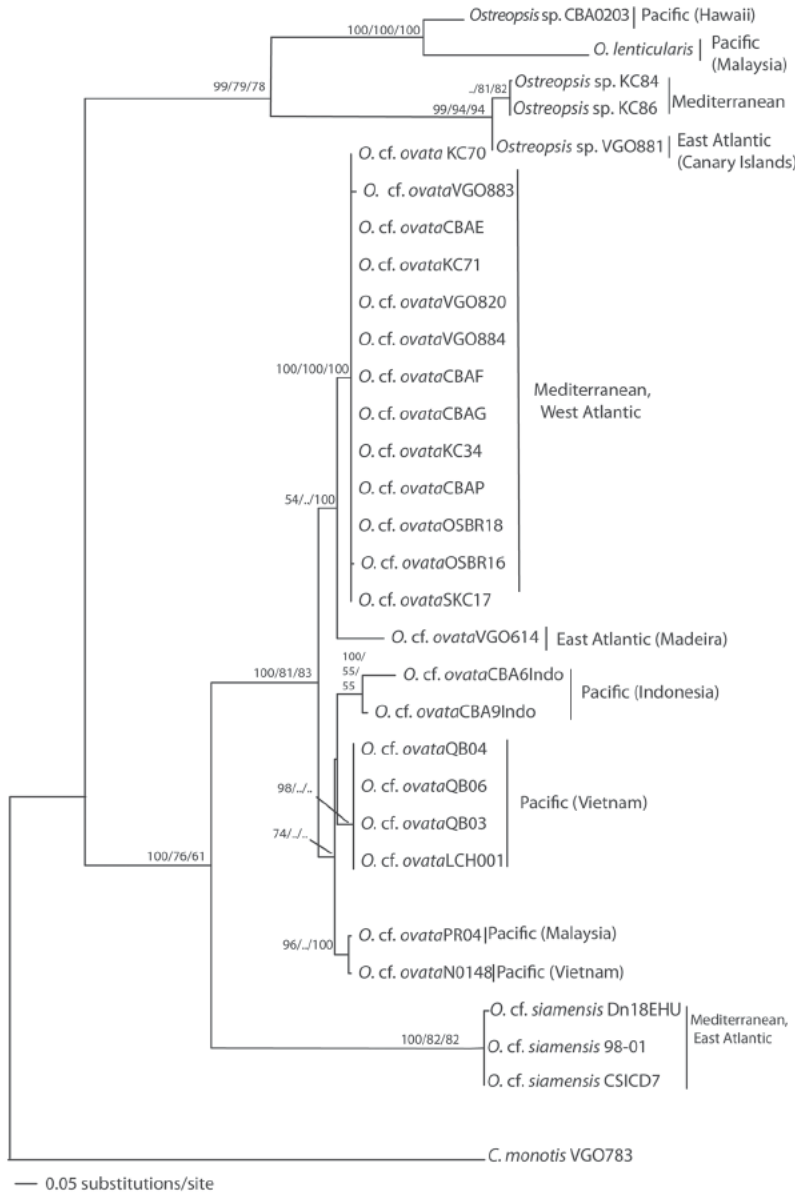

Fig. 2. Maximum likelihood (ML) phylogenetic trees of genus *Ostreopsis* based on the LSU rDNA sequences; numbers on the major nodes represent, from left to right, neighbour-joining (NJ) (1,000 pseudoreplicates), maximum parsimony (MP) (1,000 pseudoreplicates) and ML (1,000 pseudoreplicates) bootstrap values. The tree was rooted using *Coolia monotis* as outgroup.

demonstrate that these European isolates may represent a different species from *O. siamensis*. The *O.* cf. *ovata* isolates were widely dispersed from the western to eastern Atlantic coasts, all over the Mediterranean Sea, in the Indian and Pacific

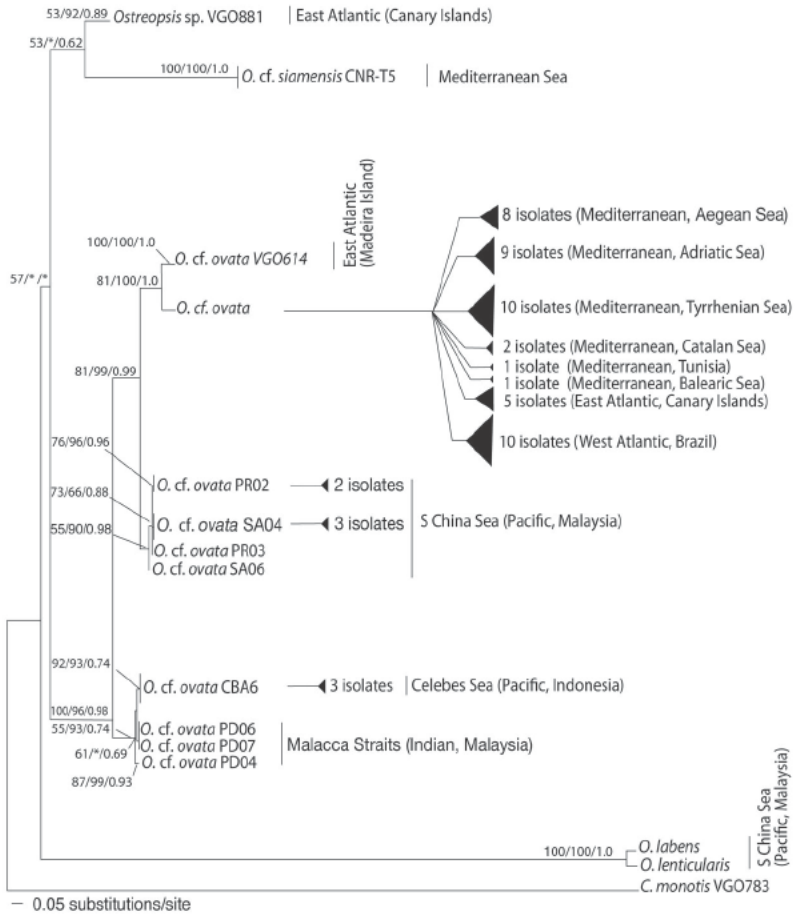

Fig. 3. Maximum likelihood (ML) phylogenetic trees of genus *Ostreopsis* based on the ITS-5.8S rDNA sequences; numbers on the major nodes represent, from left to right, maximum parsimony (MP) (1,000 pseudoreplicates), ML (1,000 pseudoreplicates) bootstrap values and Bayesian posterior probability values from Penna *et al*., (2010). Only bootstrap values > 50% are shown. Asterisks at the major nodes mark discrepancies between the different methods. The tree was rooted using *Coolia monotis* as outgroup.

Ocean. Within the *O*. cf. *ovata* different genetic lineages were present; these resulted to be correlated with geographical distribution at macrogeographical scale. *O*. cf. *ovata* grouped into Mediterranean/Atlantic clade, Malacca Strait clade, and the South China Sea and Celebes clades. This peculiar geographic distribution of the *O*. cf. *ovata* clades has been hypothesized in relation to the geographical scenario of the different oceanic basins and barrier land of continents (see Penna *et al*., 2010). The Atlantic/Mediterranean *O*. cf. *ovata* clade analysed by the statistical parsimony method based on the concatenated ITS-5.8S-LSU sequences have shown that these two areas might host a single panmictic population, since no clear correspondence was evident among geographical origin of isolates and different groupings into the network of haplotypes. No genetic

isolation and therefore, an existing gene flow were supposed to be consistent between the Atlantic and Mediterranean Sea thorough the Gibraltar Strait. The complex current systems that favoured dispersal events between the two seas for a range of pelagic organisms and therefore, gene flow would support the clear panmixia between Atlantic and Mediterranean isolates of *O.* cf. *ovata.* Furthermore, several new and worldwide isolates of *O.* cf. *ovata* were analysed based on new sequences of mitochondrial genes (Cox I and Cob). The network statistical analysis of both single and concatenated genes identified an unique homogeneous haplotype with no genetic differentiation among isolates from the Mediterranean and Atlantic; therefore, these molecular markers seemed not resolving any geographical cluster differentiation of the Atlantic/Mediterranean *O.* cf. *ovata* clade (Penna pers comm.).

MONITORING AND QUANTIFICATION OF *OSTREOPSIS* SPP. BY MOLECULAR METHODS

Molecular techniques utilize genetic differences within regions or distinct genes between species. Molecular analysis requires a previous knowledge of the genetic diversity of the phytoplankton taxa in a specific geographic area. To date, rDNA genes are one of the favoured multi-copy targets in the nuclear genome. The PCR amplification technique is widely used for the genetic characterization and enumeration of numerous species of harmful microalgae in marine environment (Galluzzi *et al.,* 2008). Further, quantitative PCR (qPCR) is an extremely sensitive method, which has been applied in recent years to detect and quantify different phytoplankton species in field samples (Godhe *et al.,* 2008). Its application could revolutionise the study of HAB microalgal population dynamics in marine systems as it allows the concurrent identification, enumeration and determination of viability of target species.

It was experienced that two *Ostreopsis* species, as *O.* cf. *ovata* and *O.* cf *siamensis*, can co-occur in natural samples of the Mediterranean Sea (Vila *et al.,* 2001; Aligizaki & Nikolaidis 2006; Battocchi *et al.,* 2010). The proper identification of the two species in bloom events is actually determinant since in relation to the specific production of toxins that can affect the human health and other organisms in a different way (Ciminiello *et al.,* 2008; Riobò *et al.,* 2008). On the other hand, the optical basic methods of microscopy have limited detection power of target species when the cells are present at low concentration.

First, an efficient PCR-based assay was applied to natural samples in order to monitor the presence of *Ostreopsis* species in several Mediterranean coastal areas. These first studies represented development, validation and application of the qualitative PCR-based assay (Battocchi *et al.,* 2010), where the molecular data were statistically correlated with conventional microscopy determinations. Samples were collected at several sites along the coasts of Italy and Spain, in the northern Adriatic and Catalan Sea in the summer 2007, where most of the localities sampled were commonly affected by blooms of *Ostreopsis* spp. (Totti *et al.,* 2010). The oligonucleotide primers used in the PCR were designed in the high variable ribosomal region of ITS and more conserved 5.8S sequence of *Ostreopsis* to satisfy the genus and species-specificity PCR reactions. Molecular taxonomical amplification signal for both genus and species derived by

the amplification of 92 bp for the genus *Ostreopsis*, and 210 and 223 bp for *O.* cf. *ovata* and *O.* cf. *siamensis*, respectively. All field samples contained mixed microphytobenthic assemblages including target species. The abundance of *Ostreopsis* ranged from undetectable to 10^6 cells g^{-1} fw or 10^4-10^2 cells L^{-1} in macrophytes and seawater samples, respectively. Due to the similar morphology and overlapping of sizes and morphotypes of the co-occurring of the two species the microscopy determinations didn't look at the identification of *O.* cf. *ovata* or *O.* cf. *siamensis* in the examined samples. Whereas, the PCR-based assay confirmed the identification, as well as the absence, of the two *Osteopsis* species in the natural samples. Both species were detected co-occurring only in the Spanish samples, whereas only *O.* cf. *ovata* was detected in the northern Adriatic coast. The positive detection by PCR was higher than microscopy determinations by 19% for the macrophyte samples and 32% for net concentrated and surface water samples. Further, both methods, molecular and microscopy, revealed also the absence of the genus *Ostreopsis* in the macroalgal and seawater samples examined in this study (Battocchi *et al*., 2010).

Furthermore, a new and more sensitive molecular approach using the SYBR I Green-based real-time PCR method for estimation of the toxic *Ostreopsis* cf. *ovata* in marine environment was developed (Perini *et al.,* 2011). The rDNA target was the D1/D2 domains of LSU gene and the quantification was obtained combining the use of a plasmid standard curve with a ''gold standard'' created with pooled crude extracts from field samples collected during a bloom event of *O.* cf. *ovata* in the NW Adriatic Sea (Mediterranean Sea). This strategy allowed to normalize the rDNA copy number per cell, and because of their similar PCR efficiencies, the exact *O.* cf. *ovata* rDNA copy number per cell in field samples was obtained. 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 and the sensitivity of the assay was of cells g^{-1} fw or L^{-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 range of quantification over time. The LSU gene copy number per *O.* cf. *ovata* cell was calculated at 1030 ± 49 extrapolating it from pLSUO and gold standard comparison curves ($p < 0.05$). Environmental samples of microepiphytic assemblages and surface water were analyzed with both microscopy and molecular methods to validate the qrt-PCR assay (Figs 4 and 5). A correspondence was found between the two methods with a high correlation particularly evident during the bloom event ($p < 0.05$). Moreover, the greater sensitivity of qrt-PCR *O.* cf. *ovata* detection was shown in those samples where no *Ostreopsis* were cells found or containing low abundance determined by microscopy. Further, the robustness of the qrt-PCR method based on performed lysis procedure of samples, on the similar efficiencies of the pLSUO and gold standard curves, on the significant correlation obtained between light microscopy and qrt-PCR during the bloom event, and on the specificity and high sample throughput for the absolute quantification of the *O.* cf. *ovata* in the environmental samples makes it to be considered very effective for problematic species, as these benthic toxic microalgae, in the monitoring activity applied to marine coastal systems.

In conclusions, due to the recent recognized current information on the *Ostreopsis* taxonomic ambiguities, molecular phylogenetic, morphological and physiological parameters should be strongly suggested to be applied together to reconstruct the taxonomy of this genus and contribute to update the biogeographical scenario of this taxon. As *Ostreopsis* blooms are causing serious

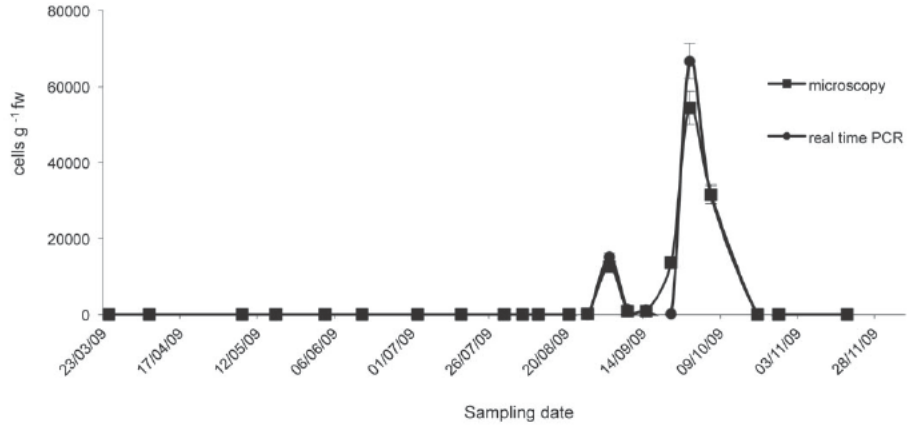

Fig. 4. *Ostreopsis* cf. *ovata* abundance (± SE) determined by both qrt-PCR and microscopy methods on epiphytic samples collected in 2009 at Conero Riviera (northern Adriatic Sea).

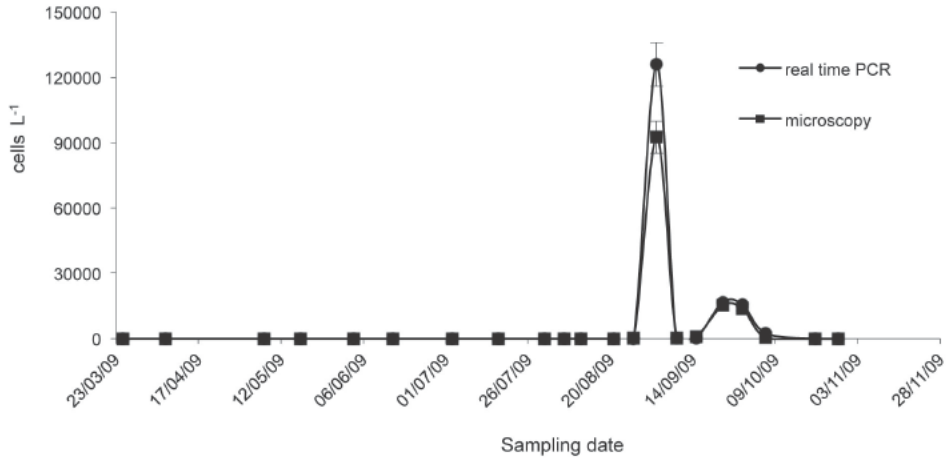

Fig. 5. *Ostreopsis* cf. *ovata* abundance (± SE) determined by both qrt-PCR and microscopy methods on seawater samples collected in 2009 at Conero Riviera (northern Adriatic Sea).

human and environmental health problems, undoubtedly research and monitoring plan activities should increase in the near future for adopting mitigation strategies of such toxic blooms. The coupling of traditional, as optical and epi-fluorescence microscopy, methods with more innovative technologies such as the molecular PCR assays, will permit in the monitoring plans the identification and estimation of specific genotype/s responsible of toxic blooms at temporal and spatial scales.

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