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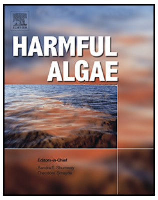

Ostreopsis cf. *ovata* from western Mediterranean Sea: Physiological responses under different temperature and salinity conditions

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ABSTRACT

The dinoflagellate *Ostreopsis* cf. *ovata* proliferates seasonally in the Mediterranean Sea, producing palytoxin-like compounds (ovatoxins) which are considered among the most potent marine toxins. Blooms have been related to several toxic events in which respiratory problems in humans and mortality of benthic marine organisms have been observed. In the coming decades, an increase in temperature and salinity is predicted in the Mediterranean Sea as a consequence of global warming that may provoke alterations in the dynamics of marine microorganisms. In this study, the physiological effects of changes in water temperature and salinity were analyzed, and their interaction through a multi-factorial experiment using two strains of *O.* cf. *ovata* in culture that had been isolated from the western Mediterranean Sea. In order to perform an accurate and reliable estimation of cell abundance, hydrochloric acid and sodium-ethylenediaminetetraacetic acid treatments were evaluated for the purpose of disaggregating cell clumps, with the former providing lower counting errors, especially after the stationary phase. Results of the physiological study showed that growth was inhibited at 19 °C for all salinities. The highest growth rates were registered at 24 °C for both strains (0.48 ± 0.05 div day^{-1}), and a significant variability in growth rate was found among salinities at 24 °C and 28 °C. Two groups were distinguished by cell size in all high temperature conditions and a positive correlation was found between the amount of small cells and growth rate. The concentration of palytoxin-like compounds in the cultures increased with time and significantly higher amounts of toxin were found at 28 °C in comparison to 24 °C. The results suggest that climate change may not affect intensity of blooms, but their toxicity may be enhanced.

1. Introduction

The genus *Ostreopsis*, Schmidt (1901) is a harmful benthic marine dinoflagellate, members of which are producers of palytoxin (PLTX)-like analogs. The nine species of the genus have very similar morphological features, resulting in recent ongoing revision of taxonomic identifications using molecular methods to clarify controversial issues (Penna et al., 2010, 2012; Sato et al., 2011). The genus *Ostreopsis* is characterized by massive proliferations that reach very high concentrations in benthic substrates such as macroalgae, rocks, or invertebrates. These blooms are associated with abundant amounts of mucilage, in which cells are embedded. This mucilaginous net is composed by acidic polysaccharides and a high number of trichocysts (Honsell et al., 2013). Cultures maintained in the laboratory produce these aggregates as well which constitute an impediment to accurate estimation of cell abundance. Previous studies have used different strategies to dissolve mucilage to better homogenize the samples and facilitate cell counting. Two chemicals have been tested for this purpose, sodium-ethylenediaminetetraacetic acid (Na-EDTA) (Scalco et al., 2012; Pezzolesi et al., 2012) and hydrochloric acid (HCl) (Guerrini et al., 2010; Vanucci et al., 2012b; Monti and Cecchin, 2012).

The dinoflagellate *Ostreopsis* cf. *ovata* has a widespread distribution, in both tropical and temperate climates. While

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O. cf. *ovata* in Pacific tropical waters has been reported to persist all year long (Pin et al., 2001; Parsons and Preskitt, 2007; Parsons et al., 2012), in the Mediterranean Sea, a seasonal pattern in bloom dynamics has been observed, coinciding with high temperatures in most cases. The strains of *O.* cf. *ovata* isolated from the Mediterranean Sea and the Atlantic Ocean belong to a genetic clade that presents a high degree of sequence homology (Penna et al., 2010). The threshold temperature for bloom formation varies, even within the Mediterranean, and correlation between temperature and *O.* cf. *ovata* outbreaks is not easy to determine, as other factors may also affect bloom dynamics (reviewed in Pistocchi et al., 2011). Considering projections for seawater warming for the 21st century, which are expected to be more intense in the Mediterranean Sea than in other areas (IPCC, 2014), it is necessary to intensify the efforts to understand how the growth and toxin production of *O.* cf. *ovata* is affected by higher temperature regimes. Several laboratory studies have been performed to characterize the optimal growth temperatures for *O.* cf. *ovata* strains from the Mediterranean Sea. Optimal growth rates were dissimilar; the optimum for Tyrrhenian strains was above 22 °C (Granéli et al., 2011; Scalco et al., 2012) and at 20 °C from the Adriatic Sea (Pezzolesi et al., 2012). From these results, differential adaptation to temperature may be expected among *O.* cf. *ovata* populations.

The response of marine microalgae to warming could differ depending on other environmental factors. Coastal waters often present pronounced fluctuations of physicochemical factors. The interaction of these factors with temperature may have consequences in *Ostreopsis* cf. *ovata* distributions (Fraga et al., 2012). Taking into account that changes in salinity are common in coastal waters due to evaporation, rainfall and/or riverine flow fluctuations (Tanimoto et al., 2013), it is very important to study the interactions of salinity with temperature to clarify the possible repercussions to microalgae physiology. The dinoflagellate *O.* cf. *ovata* from Japanese coastal waters has been studied under a wide range of conditions, suggesting that high salinities and temperatures would favor its growth (Yamaguchi et al., 2012). A recent physiological study performed in two *O.* cf. *ovata* strains from Thailand, highlighted the importance of this interaction and the environmental conditions of the place of origin of the strains (Tawong et al., 2015).

Environmental conditions also affect other physiological patterns apart from growth, such as morphology and toxicity. Morphometric changes in *Ostreopsis* cf. *ovata* have been described during different growth phases. A high abundance of small cells can be attributed to an increase in cell divisions (Silva and Faust, 1995). Strains of *O.* cf. *ovata* from the Mediterranean clade produce PLTX-like compounds, identified as putative palytoxin (pPLTX) (Ciminiello et al., 2006), and re-named as isobaric PLTX (isoPLTX) (García-Altares et al., 2015); and 8 PLTX analogs named ovatoxins (OVTX), OVTX-a, being the major component in most cases (Ciminiello et al., 2008), -b, -c, -d, -e (Ciminiello et al., 2010), -f (Ciminiello et al., 2012), -g (García-Altares et al., 2015) and -h (Brissard et al., 2014). There is evidence of accumulation of PLTX-like compounds in seafood from the Mediterranean Sea (Aligizaki et al., 2008, 2011; Amzil et al., 2012; Biré et al., 2013, 2015) but there is no record of human poisoning caused by consumption of contaminated seafood in this area. There have been many cases of human illness attributed to inhalation or direct contact with cells or aerosols, as well as mass mortalities of invertebrates (reviewed in Mangialajo et al., 2011). It has been observed that *O.* cf. *ovata* toxin content is inversely correlated with growth rate (Pezzolesi et al., 2012; Granéli et al., 2011), suggesting that under unfavorable conditions, a higher amount of toxin is produced.

Studying the physiological responses of *Ostreopsis* cf. *ovata* is important to understand the potential hazard this species may represent due to the production of potent toxins. The aim of this work was to determine: (1) growth performances and effects on toxin content with the interaction of temperature and salinity and (2) characterization of the presence of small cells associated with growth phases and environmental conditions.

2. Materials and methods

2.1. *Ostreopsis* cf. *ovata* cultures maintenance

Strains IRTA-SMM-11-09 and IRTA-SMM-11-10 of *Ostreopsis* cf. *ovata* were isolated from macroalgae samples, *Jania rubens*, collected in two sites of the southern coast of Catalonia in August 2011 (40°33′15.7176″ N; 0°31′58.242″ E and 40°50′47.8242″ N; 0°45′44.9532″ E). Both sites are rocky shore areas in the south and north of the Ebro Delta respectively (Fig. 1). Cells were isolated with a glass pipette by the capillary method (Hoshaw and Rosowski, 1973) under an inverted microscope (Leica DM-IL). After initial growth in a 24 well microplate, stock cultures were maintained in 25 cm^2 non-treated polystyrene sterile flasks (IWAKI) filled with autoclaved sterile filtered natural seawater containing a nutrient supplement of a five-fold dilution of f/2 medium (Guillard, 1975) (Sigma). Salinity was adjusted to 36 by adding autoclaved MilliQ water and re-inoculations were performed every 3 weeks. Cultures were maintained at a constant temperature of 24 °C and illumination was provided by fluorescent tubes with a photon irradiance of 100 μmol photons m^{-2} s^{-1} under 12:12-h light:dark photoperiod, which remained constant during all experiments.

2.2. DNA extraction, polymerase chain reaction amplification and sequencing

Extraction of DNA from culture samples of *Ostreopsis* cf. *ovata* strains IRTA-SMM-11-09 and IRTA-SMM-11-10 was achieved following Andree et al. (2011). The ITS and 5.8S ribosomal RNA (rRNA) gene regions were obtained by polymerase chain reaction (PCR), performed in an Eppendorf^{TM} MasterCycler Personal Thermal Cycler using oligonucleotide primers ITSA (5′-GTAACACGGTHTCCGTAGGT-3′) and ITSB (5′-AKATGCTTATRTTCAGCRGG-3′) (Sato et al., 2011). Resulting fragments of rRNA were evaluated by agarose gel electrophoresis and purified using spin column chromatography (Qiagen PCR Purification Kit, Valencia, CA). Purified fragments were sent to be sequenced bidirectionally (Sistemas Genómicos, LLC; Valencia, Spain) using the same primers as those used in the initial amplification. Forward and reverse sequence reactions were aligned and manually edited using BioEdit, version 7.0.0 (Hall, 1999). Genetic distances were obtained by Kimura's two-parameter model (Kimura, 1980) with MEGA 5.1.

2.3. Treatment experiments to improve cell quantification

The addition of HCl and Na-EDTA to samples was performed in order to disaggregate the mucilaginous net. Hydrochloric acid liberates H^+ protons in aqueous medium that may neutralize negative electric charges present in cell walls and polysaccharides. The mechanism of action of the Na-EDTA treatment causes the chelation of Ca^{2+} and Mg^{2+} that bind polysaccharides together, thereby disrupting the aggregation (Alldredge et al., 1993).

Two experiments with IRTA-SMM-11-10 strain were carried out in cultures grown in 600 mL, in 150 cm^2 non-treated polystyrene sterile flat bottom flasks (Nunc). Experiment 1 was performed using a late decaying phase culture (7 weeks, 4.0×10^3 cells mL^{-1}) in May 2012 and Experiment 2 using a late stationary phase culture (4 weeks, 2.5×10^3 cells mL^{-1}) in December 2012. Six different sample volumes (0.5, 1, 3, 5,