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Nitrogen and phosphorus limitation effects on cell growth, biovolume, and toxin production in *Ostreopsis* cf. *ovata*

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ABSTRACT

Ostreopsis cf. *ovata* is an epiphytic/benthic dinoflagellate that produces palytoxin-like compounds (putative palytoxin, ovatoxin-a, -b, -c, d and -e). Here we report on effects of nitrogen (N) and phosphorous (P) limited conditions on cell growth, cell size, biovolume, and toxin production of an *O.* cf. *ovata* strain isolated from the Adriatic Sea (Italy). Experiments were carried out in batch cultures using nitrate (NO_3^-) and phosphate (PO_4^{3-}) as nutrient sources, and testing N:P ratios of 16, 5, 92 (control, N-limited and P-limited conditions, respectively). Residual N and P in the medium, cell yield, toxin concentrations, and toxin composition were analyzed throughout the growth.

Two distinct cell size classes were identified and named Class 1 (small cells) and Class 2 (large cells), whose relative contribution under control condition was about 30 and 70%, respectively. N-limitation affected cell size, with significantly higher abundance (16%) of small cells being recorded under N stress than under control and P stress conditions. Conversely, P-limitation induced an increase of cell volume all over the growth cycle. Nutrient limitations affected growth rates and reduced final cell yields of 2.2-fold and 1.8-fold for N- and P-limited treatments vs control, respectively. Under all tested conditions *O.* cf. *ovata* showed the same qualitative profile, leading to a slight different contribution of each toxin to the total toxin content. On overall, toxins showed increasing concentrations from early to late stationary growth phase; particularly under control condition total toxin content increased from 13 to 24 pg $cell^{-1}$. Nutrient limitations affected toxin production, which resulted significantly lower than control in late stationary phase, especially under N-deficiency: a 53% and 40% decrease in toxin cell content was observed under N- and P-limited conditions, respectively.

1. Introduction

Blooms of toxic dinoflagellates belonging to the *Ostreopsis* genus once confined to tropical and subtropical areas have recently spread to more temperate regions worldwide including the Mediterranean Sea (Rhodes, 2011 and references therein) where *Ostreopsis* cf. *ovata* outbreaks caused human intoxications (Tognetto et al., 1995; Tichadou et al., 2010 and references therein) and death of benthic invertebrates (Di Turi et al., 2003; Sansoni et al., 2003). Increasing monitoring surveys carried out in these last years have revealed the recurring presence of *O.* cf. *ovata* along several Mediterranean coasts developing in some cases seasonal blooms (e.g. Bianco et al., 2007; Monti et al., 2007; Mangialajo et al., 2008; Aligizaki et al., 2008; Spatharis et al., 2009; Totti et al., 2010; Mangialajo et al., 2011). *Ostreopsis* species are described as epiphytic-benthic dinoflagellates that occur in association with red and brown macroalgae and seagrasses, but they are also capable of growing on sand, hard substrata, such as rocks, and invertebrates by forming reddish-brown mats (Taylor, 1979; Faust and Morton, 1995; Totti et al., 2010). Moreover, *Ostreopsis* spp. are common components of the epiphytic/benthic dinoflagellate assemblages found in tropical ciguatera areas which includes, besides *Ostreopsis*, the genera *Gambierdiscus*, *Prorocentrum*, *Coolia*, and *Amphidinium* (Faust et al., 1996; Tindall and Morton, 1998; Tosteson et al., 1998). *Ostreopsis* spp. are known to produce palytoxin-like compounds which are complex nitrogen-containing molecules (Ukena et al., 2001; Lenoir et al., 2004; Ciminiello et al., 2006, 2008, 2010) that are listed among the most potent marine toxins so far known (reviewed by Deeds and Schwartz, 2010). High resolution liquid chromatography–mass spectrometry (HR LC–MS) studies on the Mediterranean *O.* cf. *ovata* (reviewed by Ciminiello et al., 2011a,b) highlighted a complex

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toxin profile of *O.* cf. *ovata* that contained, besides very minute amounts of putative palytoxin (pPLTX), five palytoxin-like compounds, named ovatoxin-a (OVTX-a), -b, -c, -d, and -e, OVTX-a being the major component.

Research on *Ostreopsis* spp. has been mainly focused on geographical distribution, chemical characterization of the toxin profile (Lenoir et al., 2004; Rhodes, 2011; Louzao et al., 2011; Ciminiello et al., 2011a,b), and risk to human health (Rhodes et al., 2002; Granéli et al., 2002; Taniyama et al., 2003; Deeds and Schwartz, 2010; Louzao et al., 2010; Ramos and Vasconcelos, 2010). Recently, a controversial triggering role of *Ostreopsis* blooms on trophic cascade effects has been also highlighted (Shears and Ross, 2009). Conversely, the role of environmental factors on both growth and toxicity of *Ostreopsis* spp. have been much less investigated. Up to now, worldwide in situ surveys suggest that temperature, wave action, and substrate typology play major roles for *Ostreopsis* growth (reviewed by Pistocchi et al., 2011). Nevertheless, despite the recurrent presence of *Ostreopsis* spp. in warm coastal waters the occurrence of the genus *Ostreopsis* has been recently reported in Russian waters, characterized by cold winter temperatures, from August to October at water temperatures of 7–25 °C (Selina and Orlova, 2010). Focusing on *O.* cf. *ovata* from the coastal areas of the Mediterranean Sea, its cell growth shows a seasonal trend with high cell numbers being recorded during warm periods and in regions characterized by low hydrodinamism (Brescianini et al., 2006; Totti et al., 2010; Pistocchi et al., 2011). Laboratory studies have shown that *O.* cf. *ovata* strains from different Italian coasts (Tyrrhenian and Adriatic) display different growth temperature optima which parallel with the in situ temperature typical of the blooming period of the single strain; conversely, the highest cell toxin levels were not associated to the best temperature growth conditions (Guerrini et al., 2010; Granéli et al., 2011; Pezzolesi et al., 2012). Additionally, in relation to an Adriatic strain, cell growth has been favored at high salinity values, while cell toxicity showed a less clear trend (Pezzolesi et al., 2012).

The role of nutrients and/or nutrient unbalanced conditions on growth and toxicity of *Ostreopsis* spp. is almost unknown. Generally, environmental surveys, carried out for monitoring epiphytic/epibenthic dinoflagellates, report low field nutrient concentrations in the nearby water column that can be limiting for growth, even if anthropogenic nutrient inputs can occur in some cases (e.g. Delgado et al., 2006; Parsons and Preskitt, 2007). Although nutrient concentrations appear to be related to dinoflagellate abundances in some of these studies, results based on correlation analyses are not univocal (Pistocchi et al., 2011; Accoroni et al., 2011).

As general, nitrogen to phosphorous ratio (N:P) supply is a topic of particular concern for the development of nuisance algal blooms with or without enhancement through eutrophication (Heisler et al., 2008). However, the extent to which changes in nutrient concentrations and ratios are linked to toxic/harmful phytoplankton blooms and their toxin production is poorly understood and highly controversial especially for benthic/epiphytic toxic dinoflagellates (Tindall and Morton, 1998; Parsons et al., 2010). Changes of the nutrient pool affect not only cell growth but also biochemical composition and rates of the different metabolic pathways (John and Flynn, 2000).

Generally, P-stress has been associated with the development of major toxicity in flagellates (e.g. *Prymnesium parvum*, Johansson and Granéli, 1999a; *Chrysochromulina polylepis*, Edvardsen et al., 1990; Johansson and Granéli, 1999b; Dahl et al., 2005), including planktonic dinoflagellates (e.g. *Protoceratium reticulatum*, Guerrini et al., 2007; Frangópulos et al., 2004) and benthic ones such as *Gambierdiscus toxicus* (Tindall and Morton, 1998) and *Prorocentrum lima* (Tomas and Baden, 1993; Varkitzi et al., 2010; Vanucci et al., 2010). However, depending on elemental composition of the toxin profile, different nutrient treatments may be expected to have different impacts. Levels of N-containing toxins such as paralytic shellfish poisoning (PSP) toxins enhance under P-limited or high N:P ratio conditions (Granéli and Flynn, 2006 and references therein; Lim et al., 2010); whereas, N stress has been found to depress PSP toxin synthesis (John and Flynn, 2000, 2002). In other studies the increase of cellular toxin amount has been found under simultaneous N and P limitations (Flynn et al., 1994; John and Flynn, 2000). Despite that, some Authors arise attention on drawing conclusions that nutrient limitation induces toxin production based only upon evidence of high cell toxin quota in growth-limiting nutrient-deficient cells (Cembella and John, 2006). An increase in toxin cell quota may results merely from a decrease in the rate of cell division relative to toxin synthesis (Cembella, 1998). Additionally, cell size varies in accordance to various physiological changes and toxin per cell will consequently vary to some extent (Garcés et al., 2002; Granéli and Flynn, 2006).

So far, information on laboratory experiments assessing effects of nutrient unbalanced conditions on *O.* cf. *ovata* growth and toxin production are almost lacking. Thus, the aim of the present study was to investigate on effects of nutrient limited conditions on *O.* cf. *ovata* cell growth and toxins' content, paying particular attention to the synoptic changes in cell size and volume which reflect biochemical and physiological status of the organism. To this aim, a *O.* cf. *ovata* strain isolated from the North-Western Adriatic Sea was grown under nitrogen and phosphorus limited conditions.

2. Materials and methods

2.1. Sampling site and laboratory cultures

O. cf. *ovata* strain OOAN0601 was isolated using capillary pipette method (Hoshaw and Rosowski, 1973) from water samples collected at Numana site (43°30′ N and 13°37′ E) located along the Ancona coast (North-Western Adriatic Sea; Italy) characterized by rocky bottom and shallow depth. Sampling was carried out in October 2006 in proximity of *Cystoseira* sp. and *Alcidium corallinum* seaweeds. After initial growth in microplates, cells were cultured in sterile Erlenmeyer flasks sealed with cotton plugs at 20 °C under a 16:8 h L:D (ca. 90 μmol m^{-2} s^{-1} by cool white lamp) in a thermostatic room. Cultures were established in natural seawater adjusted to salinity value of 36, with macronutrients added at a five-fold diluted f/2 concentration (Guillard, 1975) plus selenium.

In order to evaluate nutrient limitation effects on cell growth and toxin content the following three different nutrient conditions and relative N:P ratios were tested: control (nominally 105 μM nitrate, 6.56 μM phosphate; N:P ratio = 16), N-limited (30 μM NO_3^-, 6.56 μM PO_4^{3-}; N:P ratio = 5) and P-limited condition (105 μM NO_3^-, 1.14 μM PO_4^{3-}; N:P ratio = 92).

Experimental cultures were carried out in 2 L Erlenmeyer flasks containing 1500 mL of medium. All batch cultures were grown under the same temperature, light, and salinity values as reported above. The inocula (about 130–240 mL) were performed by using cells axenically collected at early stationary phase (day 9) from pre-adapted cultures at the three nutrient conditions described above.

For each nutrient condition 2 series of batch cultures were set up in parallel; one series (4 flasks) was used for determining cell growth and cell measurements, the second one (4 flasks) for toxin content. The experiment was carried out in duplicate.

Cell counts and measurements were performed at day 0, 2, 6, 9, 13, and 22. Since the evaluation of *O.* cf. *ovata* growth pattern in batch cultures is difficult due to the presence of mucous aggregates, cell sampling for cell counts was performed by using