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New insights on the life cycle stages of the toxic benthic dinoflagellate *Ostreopsis* cf. *ovata*

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ABSTRACT

New observations on the life cycle stages of *Ostreopsis* cf. *ovata* are reported in the field and in cultures samples from the northern Adriatic Sea (Mediterranean Sea). Cultures of *O.* cf. *ovata* were performed using both replete and N-free growth media and analyzed for 5 months. *Ostreopsis* cells displayed a high morphological variability. Some cells were characterized by the presence of orange accumulation bodies, which represented a signal of stress conditions. Two mechanisms of gamete mating seem to occur. In the first (already reported) vegetative cells conjugated through the epitheca without plasmogamy; in this regard we observed a new 'process' on the top of the epitheca which might represent a structure involved in this mating mechanism. In the second, small cells acting as gametes were aligned laterally with the two cingula perpendicular to each other. Meiosis possibly occurred as suggested by tetrad formation, originating four vegetative cells. At least two types of cyst were formed, a non-dormant (pellicle) cyst germinating within 2 days and a resting cyst which is able to germinate after a 5-month dormancy only at temperatures over 25 °C.

1. Introduction

Considerable blooms of the toxic benthic dinoflagellates *Ostreopsis* spp. are reported worldwide in many tropical and temperate regions (Vila et al., 2001; Aligizaki and Nikolaidis, 2006; Mangialajo et al., 2011; Rhodes, 2011; Parsons et al., 2012). Along the Mediterranean coasts, massive blooms of *Ostreopsis*, mostly generated by the genotype *Ostreopsis* cf. *ovata* (Battocchi et al., 2010; Perini et al., 2011), typically proliferate in shallow and sheltered waters forming a rusty-brown colored mucilaginous film, which covers reefs, rocks, soft sediments, seaweeds, marine angiosperms and invertebrates (Vila et al., 2001; Turki, 2005; Aligizaki and Nikolaidis, 2006; Totti et al., 2010; Accoroni et al., 2011). *O.* cf. *ovata* toxicity is associated with the presence of palytoxins (Ciminiello et al., 2011), such as putative palytoxin, ovatoxin-a, b, c, d, e, f and mascarenotoxin-a and c in both field and cultured samples (Rossi et al., 2010; Accoroni et al., 2011; Ciminiello et al., 2012; Scalco et al., 2012). *O.* cf. *ovata* blooms have an impact on human health, causing intoxications through both inhalation and contact (Gallitelli et al., 2005; Kermarec et al., 2008; Tichadou et al., 2010). Such toxicity also affects benthic communities including bivalves, gastropods, cirripeds, echinoderms and fishes, causing suffering or mass mortalities in various parts of the world where massive *Ostreopsis* blooms occur (Shears and Ross, 2009; Accoroni et al., 2011; Faimali et al., 2012; Gorbi et al., 2012, 2013; Pagliara and Caroppo, 2012; Pezzolesi et al., 2012).

The life cycles of benthic ciguatera related dinoflagellates, such as those included in the genera *Ostreopsis*, *Coolia*, *Gambierdiscus* and *Prorocentrum*, are poorly understood. In the Ostreopsidaceae family, the only described life cycles are those of *Coolia monotis* (Faust, 1992) and recently *Ostreopsis* cf. *ovata* (Bravo et al., 2012). In *C. monotis*, vegetative cells and gametes have very similar morphologies; gamete fusion in this dinoflagellate gives rise to large biflagellated planozygotes that develop into short-term thin-walled cysts after several maturation stages, where a lightly pigmented cell becomes intensely dark brown in color and loses motility (Faust, 1992). Recently, Bravo et al. (2012) have shed light on the unknown *O.* cf. *ovata* life cycle, demonstrating sexuality in its life cycle and describing cysts in both culture and natural samples. These authors reported that mating gamete pairs were the only sexual stage clearly distinguishable from dividing cells and were observed either in the intracrosses or the intercrosses of different strains. The authors also suggested that gamete conjugation in *O.* cf. *ovata* could be carried out through karyogamy, without requiring plasmogamy, as only the transfer of a nucleus from one gamete to the other was seen.

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In this study, we investigated the *Ostreopsis* cf. *ovata* population in culture samples under two different nutritional conditions and in natural sediment samples, analyzing different morphotypes (vegetative cells, short-term and resting cysts, and some morphotypes which can be ascribed to the sexual cycle), and reporting new morphological features which may be involved in sexual reproduction. The temperature conditions affecting cyst germination were also tested.

2. Materials and methods

2.1. Cultures of Ostreopsis cf. ovata

Two strains of *Ostreopsis* cf. *ovata*, OoAPn0807/D and OoAPn0808/C1 were isolated using the capillary pipette method (Hoshaw and Rosowski, 1973) from water samples during the 2007 and 2008 blooms, respectively. After an initial growth in microplates, cells were cultured at 21 ± 0.1 °C under a 12:12 h L:D photoperiod and an irradiance of 90–100 μmol m^{-2} s^{-1}, in modified f/4 medium (Si free, Se added, see below). Two types of growth media were used, N-replete and N-free growth medium, the former for culture maintenance and control and the latter for the experiment (see below). The N-replete growth medium was prepared by adding macronutrients at a f/4 medium concentration (Guillard and Ryther, 1962) and selenium to filtered and autoclaved natural seawater (salinity 35). Trace metals, iron, vitamins (H, B1 and B12) and HEPES pH 7.1 were added at levels corresponding to f/2 medium. The N-free medium was prepared in the same way but without the addition of nitrogen in order to enhance sexual reproduction.

The experiment was conducted in three replicates: 300 ml flasks containing N-free growth medium were inoculated with *Ostreopsis* cf. *ovata* cells to a final concentration of 500 cells ml^{-1}, using two strains (250 cells ml^{-1} from each strain). The control was performed in the same conditions, but with replete growth medium. The cultures were monitored over a period of 2 months to observe if cysts were produced: samples (2 ml) were collected every 2 days from day 2 to day 28, and then the frequency was reduced from weekly (until day 34) to fortnightly (until the end of the second month). Samples were collected after homogenization from each flask and preserved with 0.8% neutralized formaldehyde in the dark until the analyses.

All the flasks were maintained for 5 months. At the end of this time, 1 ml of each flask was put in 6-well culture plates filled with 4 ml of growth medium containing antibiotic cocktail (250 μg/ml penicillin G, 250 μg/ml kanamycin and 500 μg/ml streptomycin) and monitored every 2 days for a week.

2.2. Sampling and treatment of natural sediments

Ostreopsis cysts were also searched for in natural sediments at Passetto station (Ancona, northern Adriatic Sea, Italy), a coastal site affected by intense *Ostreopsis* blooms during the late summer (Accoroni et al., 2012a). Sediment samples (three replicates) were collected during May–June 2012 (before the first appearance of *Ostreopsis* vegetative cells), using a cut-off plastic syringe (2.1 cm diameter). The upper 2 cm of the sediment surface were cut with a blade, collected in a Falcon tube and stored in darkness at 4 °C until analysis. Cysts were separated from the sediment by differential centrifugation, using an aqueous solution of sodium polytungstate (SPT), following the Bolch (1997) method. Purified sediment subsamples (1 ml) were then observed under a light microscope.

2.3. Light microscope analysis

Both culture and sediment samples were settled in counting chambers, according to the Utermöhl method (Hasle, 1978). In order to obtain a homogeneous settling in the bottom of the counting chamber, after gently stirring, samples or subsamples were poured with a pipette into a cylinder/chamber complex previously filled with filtered seawater. Cells were then stained with the DNA-specific dye DAPI to visualize the nucleus and the cellulose specific dye Calcofluor-White M2R to distinguish thecal morphology.

Samples were analyzed at 400× magnification, for the identification and counting of different cell morphotypes, using an inverted microscope (Zeiss Axiovert 135) equipped with phase contrast and epifluorescence apparatus using the following filter sets: (i) exciter filter BP 450–490, dichroic beam splitting mirror FT 510, barrier filter LP 520 (blue excitation) to detect chloroplast autofluorescence; (ii) exciter filter BP 395–440, dichroic beam splitting mirror FT 460, barrier filter LP 425 to detect Calcofluor White M2R staining; (iii) exciter filter BP 365/12, dichroic beam splitting mirror FT 395, barrier filter LP 397 for 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI) staining.

Ostreopsis cf. *ovata* cells were measured at 400× magnification using a micrometric ocular. For each sample, at least 10 cells were measured along the dorsoventral diameter (DV) and the transdiameter (W), with 5582 total cells measured.

2.4. Scanning electron microscope (SEM) analysis

Selected samples were treated for scanning electron microscopy (SEM) observations: samples (2 ml) were dehydrated by immersion in ethanol at increasing concentrations, filtered through a Nucleopore polycarbonate filter and treated in a Critical Point Dryer (Polaron CPD7501). Filters were placed on stubs and sputtered with gold–palladium in a Sputter Coater (Polaron SC7640) for observation under SEM (Philips EM 515, 25 kV).

2.5. Germination tests

Cysts were isolated from culture flasks and from sediment samples and used to perform germination tests. Seventy single cysts belonging to different morphotypes were placed separately in 24-well culture plates filled with growth medium. Plates were placed in the culture chamber in the conditions previously described and were observed every day for several weeks. Two different temperatures were tested for cyst germination: 21 ± 0.1 °C and 25 ± 0.5 °C.

2.6. Statistical analysis

Differences in total cell abundances and percent abundances of observed morphotypes between the experiment and the control were assessed through a one-way analysis of variance (ANOVA). When significant differences for the main effect were observed ($p < 0.05$), a Tukey's pairwise comparison test was also performed. Statistical analyses were performed using Statistica (Statsoft) software.

3. Results and discussion

From observations of cultured material, we observed that *Ostreopsis* cells displayed a high morphological variability (DV ranged from 20 to 88.75 μm and W from 13.75 to 80 μm, Table 1), as already reported in natural samples (Accoroni et al., 2012b). Gamete mating was also observed (Fig. 2A and B): vegetative cells, especially when placed in diluted or N-free culture medium, conjugated through the epitheca acting as gametes, as already described by Bravo et al. (2012), who reported that conjugation in *Ostreopsis* cf. *ovata* occurs without plasmogamy, with nuclear