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Ecophysiological responses of the toxic dinoflagellate *Ostreopsis* cf. *ovata* under different water motion conditions

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Abstract

Hydrodynamic conditions affect marine microalgae. In the case of some harmful epiphytic species, field observations suggest that water motion and wave action play a selective role in determining their spatial distribution and ecology and regulating cell physiology. In order to obtain new insights on this topic, laboratory experiments were performed with Mediterranean strains of *Ostreopsis* cf. *ovata*. Monospecific cultures were exposed during 3 weeks to the turbulent motion generated by an orbital shaker at 50 rpm in order to simulate the wave movements in their natural habitat. The growth curve and toxin concentrations in the shaken cultures were compared to those maintained under still, control conditions. Shaken *O.* cf. *ovata* populations entered stationary phase earlier, reached lower cell yield and had 30% lower ovatoxin-a intracellular content compared to control ones. In the two treatments, the cell toxin content in the exponential phase was lower than in the stationary phase. These results contribute to understand the dynamics of benthic HABs and their impacts to the ecosystem and human health.

Keywords: Ostreopsis, BHAB, Mediterranean, toxicity, small-scale turbulence

Introduction

Since the late 1990s, the toxic benthic dinoflagellate *Ostreopsis* has caused recurrent blooms in temperate areas, including the Mediterranean coasts. Some of these bloom have been associated with human respiratory disorders (Fig. 1 in Ciminiello *et al.* 2014 and references therein). Interestingly, these adverse effects only occurred during certain phases of the bloom (Vila *et al.* 2016). Some of the blooms have also been associated with massive macrofauna mortalities (e.g. Shears and Ross 2009). These negative impacts on the marine habitat and human health have motivated research on *Ostreopsis* bloom dynamics. Field observations suggest that water motion and wave action, among other environmental factors, could play a selective role on the spatial distribution and ecology of this harmful epiphytic species although no clear relatrionship has been defined so far. For instance in the Mediterranean, *O.* cf. *ovata* was reported from shaken and slightly shaken habitats by Vila *et al.* (2001) while it was found in sheltered areas by Accoroni *et al.* (2012). These apparent contradictory observations arise in part from the lack of quantification of water turbulence in the field, and from the difficulty to discriminate the specific role of this factor from its interaction with other drivers (e.g. depth, light intensity, macroalgal substrates). However, small-scale turbulence has been described to exert negative species-specific effects in some planktonic dinoflagellates (e.g. Berdalet et al., 2011 and references there in). In this study, we present the preliminary results of our investigation of physiological responses of an *Ostreopsis* cf. *ovata* strain exposed to still and turbulent conditions.

Material and Methods

Ostreopsis cf. *ovata* strain ”Ostreo BCN1_2014” was isolated from Llavaneres beach, a hot spot where the species bloomed annually since, at least, 2004 but probably since 1998. Aliquots of an exponential monospecific culture grown in f/2 medium were used as inoculum for the 12 experimental (250 ml sterile plastic flasks) cultures with 200 $cells \cdot ml^{-1}$ as initial concentration. The 12 flasks were incubated at

23ºC under a 12-12 hours light-dark cycle, and an irradiance of 150 µmol photon·m^{-2}·s^{-1}. After 24h, the initial cell concentration in each flask was determined on 1-ml Sedgewick Rafter chamber (in duplicate). Six experimental flasks, so called "Control", were maintained under still conditions. The other six flasks, referred to as "Turbulence" were continuously agitated on an orbital shaker at 50 rpm with a 0 – 10º angle inclination variability range in order to simulate the wave movement in their natural habitat. Shaking started on day 1, i.e. 24 hours following inoculation. The experiment lasted for three weeks.

Population growth was characterized by sampling each flask every 2 days. Cell counts on Lugol fixed samples were performed in duplicate as described above. Growth rate was calculated following Guillard (1973) where the growth rate is the slope of the Ln of the cell counts over time during the exponential phase.

At the beginning of the stationary phase (day 11), when the differences between the growth curves in Control and Turbulence treatments were evident, cell size analyses were conducted on around 100 randomly chosen cells from each treatment. Four cell measures were done: dorsoventral diameter including the theca (DVt), dorso-ventral diameter of the inner cytoplasm (DVc), trans-diameter including the theca (Wt) and trans-diameter of the inner cell (Wc). Cell size parameters at each treatment were compared by one-way analyses of variance (one-way ANOVA; STATISTICA). The shape of each measured cell was noted and several microphotographs were also taken a using Leica-Leitz DMIRB inverted microscope (Leica Microsystems, Wetzlar, Germany) and ProgRes CapturePro image analysis software (JENOPTIK Laser, Optik Systeme).

Samples for toxin determination were collected twice, during the exponential phase (day 7) and at the end of the experiment (day 23). Each sampling day, the total remaining content of three Control and Turbulence flasks (from 100 to 230 ml depending on the day) were filtered through GF/F fiber filter (Whatman) and kept frozen at -80ºC until analysis. Thus, after day 7, the number of flask replicates of each treatment was reduced from 6 to 3. Filters were extracted with 100% methanol and palytoxin and ovatoxins were determined by UHPLC-HRMS using a Hypersil Gold C18 column (100 x 2.1 mm, 1.9 µm, Thermofisher Scientific) and a mobile phase gradient elution of acetonitrile : water (0.1% formic acid) for the chromatographic separation and coupled to a Q-Exactive quadrupole-Orbitrap mass spectrometer (Thermofisher Scientific) with electrospray as ionization source in positive ion mode.

In summary, two treatments were done, six 250-ml plastic culture flasks containing *O.* cf. *ovata* were maintained under still conditions, used as Control and six 250-ml culture flasks were permanently shaken after day 1 (Turbulence). Parameters measured were cell number, cell size and shape, toxin content and growth rate. The effect of turbulence on these parameters is discussed.

Results and Discussion

Ostreopsis cf. *ovata* showed the typical sigmoid growth curve and grew similarly under Control (still) and Turbulence (shaken) conditions (Fig. 1). However, the final cell numbers reached in the Controls (5300 cells·ml^{-1}) almost doubled the final yield reached by the Turbulence experiments (3000 cells·ml^{-1}). Furthermore, whereas *O.* cf. *ovata* growth rate was similar in both treatments (0.32 d^{-1} and 0.39 d^{-1} in Control and Turbulence, respectively), the exponential phase lasted for 11 days in the still flasks and only 5 days in the shaken ones. This result suggests some kind of disturbance on their reproduction or life history processes as has been observed previously in dinoflagellates (e.g. Berdalet *et al.* (2011) and references there in). In the natural environment, indeed, notably high *Ostreopsis* cell concentrations have been recorded during long lasting calm sea conditions (e.g. Giussani 2016, Accoroni & Totti 2016 and references there in).

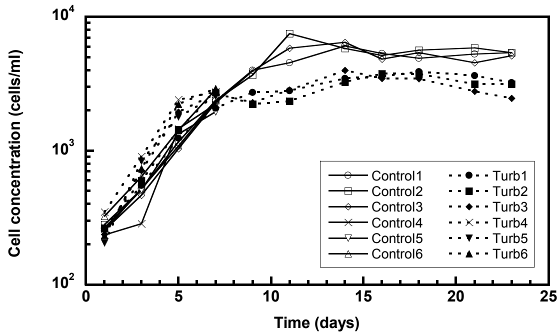

Fig. 1. Growth curves of *O.* cf. *ovata* in the Control (still) and Turbulence (shaken) treatments. Note that, for each treatment, there were 6 replicates until day 7, and 3 replicates until the end of the experiment. Each data point

corresponds to the average of two replicate cell counts per flask.

The mean cell size measured in the Turbulence treatment (see Table 1) was statisticaly significantly larger than cells measured in the Control ($p<0.01$) considering the four measured parameters.

Table 1. Cell sizes of Control and Turbulence treatments considering all the cells together (without taking account morphotypes).

	Control				Turbulence			
	n	Mean	Min-Max	SD	n	Mean	Min-Max	SD
DVt	97	32,7	22,1-51,0	5,3	101	34,0	20,2-49,9	5,0
DVc	101	26,4	14,7-45,1	5,5	103	28,2	17,6-41,7	4,4
Wt	97	24,4	16,0-43,0	6,1	101	24,9	13,6-41,8	4,5
Wc	101	20,4	11,8-39,1	6,0	103	20,9	10,7-37,5	4,3

Such increase in cell size under turbulence conditions has been described in other experiments and is related to interference of turbulence with cell division. However, as has already been reported by several authors, cultures of *Ostreopsis* show a large variability in shape and size (e.g. Accoroni *et al.* 2014). Bravo *et al.* (2012) classified cultured cells into three size-categories (25–35 µm, 35–50 µm and >50 µm in DV diameter). In this experiment, five morphotypes were identified at the beginning of the stationary phase based on their shape (drop-shaped to round-shaped cells), content (clear or dark cytoplasm), life history stage (vegetative or pellicle cysts), and cell size range (from 20 to 51 µm). The five morphotypes were designated as DropClear (DC), Dark (D), Round (R), Without theca (WT) and others (O). The three dominant morphotypes are illustrated Fig 2.

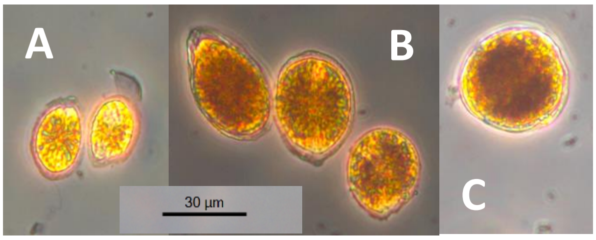

Fig. 2. Microphotographs of the three dominant morphotypes at the beginning of the stationary phase (day 11). A) Drop-shaped clear cells (”DC”), B) Dark cells (”D”) and C) Round cells (”R”).

DC comprised small cells (Table 2) that clearly dominated both treatments (61% in Control and 63% in Turbulence), followed by large dark cells (D) (16% in Control vs. 22% in Turbulence conditions); and finally, by rounded cells (R) (13% vs. 11%). The two last categories (WT and O) represented less than 10% of cell counts.

Table 2. Mean cell sizes (DVt, Wt) of the three dominant morphotypes in Control and Turbulence treatments. Standard deviation is indicated.

		Control	Turbulence
DropClear (DC)	**DVt**	31,0 ± 2,8	33,6 ± 3,7
	Wt	21,6 ± 3,2	23,0 ± 3,3
	n	62	65
Dark (D)	**DVt**	41,5 ± 5,7	38,2 ± 5,0
	Wt	34,4 ± 5,9	30,2 ± 4,0
	n	16	23
Round (R)	**DVt**	30,9 ± 4,3	27,3 ± 3,5
	Wt	26,8 ± 3,9	23,8 ± 2,6
	n	13	11

In this study we observed that whereas the most abundant DC cells were larger in the Turbulence treatments than in the Control ones (for the 4 parameters measured), D and R cells were larger in the Control than in the Turbulence flasks (Fig. 3). More studies are required to understand the specific role that each *Ostreopsis* morphotype plays in the life history of this organism.

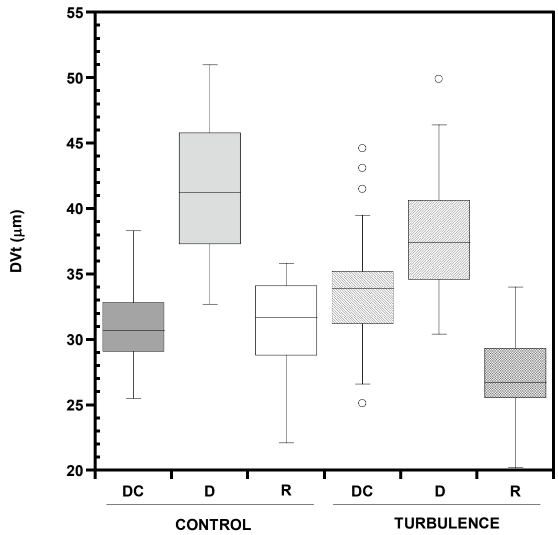

Fig. 3. Median (horitzontal line), 25 and 75 quartiles (box), minimum and maximum (whiskers) and outlier (point) of the dorsoventral diameter including the theca (DVt) measured for

the three dominant morphotypes (see Fig. 2) in Control and Turbulence treatments.

Regarding toxins, OVTXa was the dominant one with small amounts of palytoxin analogues such as OVTXb-g and putative palytoxin (not shown). *O.* cf. *ovata* toxin production was four times higher in the stationary phase than in the exponential one (Fig. 4); shaken cells had 30% lower toxin content (23 pg OVTXa·cell^{-1}) than the still cultures (32 pg OVTXa·cell^{-1}). In addition, intracellular toxin concentration was also lower in the Turbulence than in the Control flasks. Such trends have also been observed in *Alexandrium minutum* and *A. catenella* (Bolli *et al*. 2007) exposed to laboratory generated turbulence. These results reinforce the possible link of toxin production with reproduction processes.

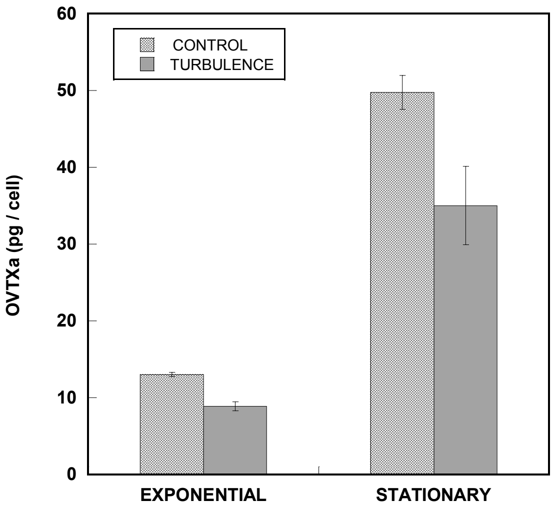

Fig. 4. Toxin content as OVTXa in Control and Turbulence conditions during the exponential and the stationary phase.

Respiratory outbreaks caused by suspected toxic aerosols seem to occur under low wind episodes (below 4 m·s^{-1}, Vila *et al*. 2016). In this study, the unshaken control treatments resulted in higher cell densities than the corresponding turbulence treatment. Control stationary phase cells also had higher toxin content per cell. Though it is not possible to extrapolate the results of this laboratory study to the natural events with certainty, the data do indicate that calm conditions may have allowed higher densities of more toxic stationary phase cells to accumulate. Release of these toxins from this higher biomass population, either by excretion or by lysis of senescent cells, accompanied by onshore wind direction, may account for observed intermitant respiratory illness. More work is needed, but results of this study do provides a new piece to the puzzle with respect to understand the *Ostreopsis* bloom and its negative effects on human health.

Acknowledgements

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