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Modulation of ecdysal cyst and toxin dynamics of two *Alexandrium* (Dinophyceae) species under small-scale turbulence

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Abstract

In some dinoflagellate species, physiological processes appear to be altered by exposure to certain turbulent conditions. Here we investigated how two levels of turbulent kinetic energy dissipation rates (ε = 0.4 and 27 $cm^2\ s^{-3}$) affected the toxin and ecdysal cyst dynamics of two bloom forming species, *Alexandrium minutum* and *A. catenella*. The most striking responses were observed at the high ε generated by an orbital shaker. In *A. catenella*, lower cellular toxin content was measured in cultures shaken for more than 4 days. The same trend was observed in *A. minutum*, although variability masked statistical significance. For the two species, inhibition of ecdysal cyst production occurred immediately and during the period of exposure of the cultures to stirring (4 or more days) at any time during their growth curve. Recovery of cyst abundances was always observed when turbulence stopped. When turbulence persisted for more than 4 days the net growth rate significantly decreased and the final biomass yield was lower than in the unshaken cultures. This study suggests that high levels of small-scale turbulence would contribute to the modulation of the harmful bloom dynamics through the interaction at the level of toxin and encystment processes.

1 Introduction

Many dinoflagellate species have been reported to be sensitive to small-scale turbulence in both field and laboratory studies. Particular water circulation patterns, coinciding with relatively calm weather and water column stability, may favour the occurrence of dinoflagellate red tides (e.g., Wyatt and Horwood, 1973; Margalef et al., 1979; Pollingher and Zemel, 1981; Berman and Shteiman, 1998; Smayda and Reynolds, 2001). Laboratory data obtained using different species and experimental designs and setups show that dinoflagellate cells can be somehow directly affected by turbulence (e.g. Estrada et al., 1987; Peters and Marrasé, 2000; last review by Berdalet and Estrada, 2005). While some studies have noted positive or indifferent responses

(Berdalet and Estrada, 1993; Sullivan and Swift, 2003; Havskum et al., 2005; Havskum and Hansen, 2006), many others reported negative effects that, in general, point to the interference of small-scale turbulence with cell division and life cycle processes (including migration) (e.g., Berdalet, 1992; Havskum et al., 2005; Yeung and Wong, 2003, Yeung et al., 2006). Although direct comparison among studies is not possible (Peters and Marrasé, 2000), the available data suggest that the response to small-scale turbulence should be species-specific and dependent on turbulence intensity and quality (Berdalet and Estrada, 1993; Sullivan and Swift, 2003; Berdalet et al., 2007).

In the present study we investigate the effect of turbulence on two red-tide forming dinoflagellates, *Alexandrium minutum* Halim and *A. catenella* (Whedon and Kofoid) Balech, with special emphasis on the modulation of toxin and cyst production dynamics. The two organisms are reported to bloom in diverse coastal areas (e.g. Halim, 1960; Honsell et al., 1995; Hallegraef et al., 1998; Vila et al., 2001a, b). Dinoflagellates have complex life cycles that include alternation of resting stages (cysts) and vegetative cells, with benthic or planktonic phases, respectively (e.g. Wyatt and Jenkinson, 1997; Garcés et al., 2002). In turn, cysts can be formed sexually by fusion of haploid gametes (producing a diploid planozygote that subsequently undergoes encystment) or asexually from ecdysis of a vegetative cell (loss of flagella and cell wall). Different factors can trigger encystment and excystment after latency, but the mechanisms involved and the role of cysts in the dynamics of blooms in nature are not well understood. Both, *A. minutum* and *A. catenella* are heterothallic species (Yoshimadzu, 1984; Figueroa et al., 2007) and are reported to produce ecdysal cysts in clonal strains. A previous study revealed that high turbulence intensity decreased the growth of *A. minutum* and interfered somehow with cyst production, although a clear conclusion was not drawn (Berdalet et al., 2007). Further, exposure to small-scale turbulence has been reported to cause poor sexual encystment in *A. tamarense* (Anderson and Lindquist, 1985), inhibition of sexual cyst production in *Akashiwo sanguinea* (Tynan, 1993, as indicated in Thomas et al., 1997) and in *Scrippsiella lachrymosa* (Smith and Persson, 2005), and increase of cellular toxin content in *A. fundyense* (Juhl et al., 2001). This

study was done using the same experimental devices and designs of previous studies (Berdalet, 1992; Berdalet and Estrada, 1993; Havskum et al. 2005; Berdalet et al., 2007) to allow direct comparison between results.

2 Materials and methods

The clonal strain of *Alexandrium minutum* (strain IEO - AL1V, isolated by S. Fraga from the Ría de Vigo)) was provided by the Vigo Oceanographic Center (Spain) and that of *Alexandrium catenella* (isolated by M. Delgado from the NW Mediterranean coast, Tarragona) belongs to the ICM culture collection. Non-axenic stock and experimental unialgal cultures were maintained in a temperature controlled room under identical temperature (20°C±1°C), irradiance (120 μmol photon m^{-2} s^{-1}, 12:12 h LD cycle, light period starting at 08:00 a.m.) and culture media (f/2-enriched seawater without silicate addition, Guillard, 1975; seawater of salinity 38 obtained from Blanes Bay -NW Mediterranean-, 1 km offshore at a 5 m depth).

Turbulence was generated with either an orbital shaker or a vertically oscillating grid system. The orbital shaker was operated at 120 rpm and a displacement of 30 mm. We used 4-L spherical (Florence) flasks (containing 3 L culture medium). An average ε of 27 cm^2 s^{-3} was calculated from the equation $\log_{10} \varepsilon = -8.667 + 5.05 \cdot F$, where F is frequency in Hz. This equation was derived from data acquired with acoustic Doppler velocimetry technology. The oscillating grids device was designed by one of us (F. Peters) as described by Dolan et al. (2003). The grids were made of stainless steel coated with a plastic polyamide, had a diameter of 11.9 cm, a 0.38 cm bar thickness and a mesh size of 1.42 cm. We used 2-L cylindrical Plexiglas containers, an oscillating frequency of 9.1 rpm and a stroke of 10 to 11 cm. An average ε of 0.4 cm^2 s^{-3} was calculated following Peters and Gross (1994), considering a drag coefficient of 0.7 for the grid.

Experimental vessels were inoculated after several transfers of exponentially growing stock cultures to new media. The initial cell concentration was around 400 cells mL^{-1}

for *A. minutum* and 65 cells mL^{-1} for *A. catenella*. The cultures were allowed to reach exponential phase before turbulence was started. Turbulence was applied during the exponential phase (Exponential), the stationary phase (Stationary) or during both phases (Always) of the growth curve (Table 1). In each experiment, two flasks remained under still conditions throughout the entire experiment (Control). All treatments were done in duplicate. The response of *A. catenella* to the turbulence generated by the orbital shaker was studied in two replicate experiments (Table 1). In the first one, 4 treatments were applied and only samples for microscopic observations were obtained. In the second one, with only two treatments (Control and Always), we also sampled for toxin analyses. Samples for microscopic cell observations and toxin analyses were taken at noon (12:00 p.m.), after gently swirling the flasks.

Cell abundances were estimated using a Sedgewick-Rafter or a sedimentation chamber (depending on the cell density of the sample), after fixation with Lugol's iodine solution (Utermöhl, 1958). Net exponential growth rates, μ (day^{-1}), as defined by Guillard (1973), were calculated as the slope of the regression line of ln(N) versus time (t), where N is the estimated cell concentration. Cyst identification was based both on the external morphology and subsequent staining of a subsample with Calcofluor White M2R (Fritz and Triemer, 1985). For PSP-toxin analysis, algae in samples (100 to 300 ml) were concentrated by vacuum filtration (–25 Kpa) onto 25 mm GF/F filters (Whatman, Kent, UK). Filters were subsequently blotted on filtration paper until no humidity was observed (Latasa et al., 2001), wrapped in aluminium foil and stored frozen (–25°C) until extraction. The filters were extracted in 2.0 mL 0.05 M analytical-grade acetic acid, using an ice-cooled cell-homogenizer (Edmund-Bühler Vibrogen, Tübingen, Germany). Extracts were subsequently centrifuged (2355 g, 15 min at 5°C) to remove cell debris and filter fragments. Particle-free aliquots (1.0 mL) were transferred to amber injection vials and stored at 5°C in the HPLC auto-sampler until injection. Extracts of toxins were analyzed with the HPLC procedures described by Oshima (1995), based on post-column oxidation with periodic acid and fluorescence detection. Toxins were separated on an Agilent Technologies Zorbax-SB C8 (250×4.6 mm i.d.)

column fitted with a BetaBasic C8 Javelin precolumn. The HPLC equipment (Thermo Separation Products, San José, CA, USA) was tested and calibrated with toxin standards obtained from the National Research Council of Canada (Hallifax, NS), including GTX1&4-b, GTX2&3-b, STX-d, dcSTX, STXdiAc and Neo-b. Due to the lack of standards for N-sulfocarbamoyl-11-hydroxysulfate toxins (C1 to C4) these compounds were converted to their carbomate analogues with a hydrolization step (1 mL acetic acid extract plus 1 mL HCl 0.4 N and boiled at 100°C for 15 min), which were subsequently quantified during a second HPLC run.

Comparison of treatments over time for the different parameters was done using the non-parametric Kruskal-Wallis test (Motulsky, 2003). Growth rates were compared by testing for the heterogeneity of the slopes (analysis of covariance). Statistical analyses were conducted with Systat 5.1.2 for MacIntosh.

3 Results

Table 1 summarizes the results of net growth rate and final biomass yield estimated in the turbulence treatments for the 5 experiments. Note that the estimation of growth rates under still conditions include 4 replicates, corresponding to the 2 Control flasks and to the 2 vessels that were kept unshaken during the exponential period and subsequently stirred during the stationary one (i.e. Stationary treatment).

Exposure to the low turbulence intensity generated by the vertically oscillating grids favoured population development of the two species (Table 1) but had no significant effect on toxin or cyst dynamics (not shown).

Alexandrium minutum cultures exposed to the high ε intensities of the orbital shaker for more than 4 days (Always treatment), had a significantly ($p<0.0001$) lower exponential growth rate compared to that of the unshaken ones (Table 1, Fig. 1a). In contrast, the growth rate of *A. catenella* (Table 1, Fig. 2a) was not significantly affected by shaking for 4 days ($p=0.375$) or longer ($p=0.392$ and $p=0.105$, in the first and second experiment, respectively). Besides this differential response in the net growth rate, the two

species showed the lowest final biomass (in terms of cell abundance) when shaken for more than 4 days, namely, 55.4% of the abundances obtained in the Control cultures of *A. minutum* and 24.7% or 21.6% in the two experiments with *A. catenella* (Table 1).

Cyst abundances (expressed as % of total cell numbers) tended to progressively increase in the *A. minutum* Control cultures from the exponential to the stationary phase (Fig. 1b). A sudden decrease in the cyst number was observed when the cells were shaken for 4 days either during the exponential (Fig. 1c) or stationary (Fig. 1d) phases. Immediate restoration of cyst abundances occurred at the cessation of shaking in the two treatments. Minimum cyst proportions remained during the whole agitation period in the Always flasks (Fig. 1e). The same general trend was observed in *A. catenella* (not shown).

The toxin content of this *A. catenella* strain consisted mainly of the isomer pair GTX1 and GTX4, plus minor amounts of C1 and C2. As illustrated in Figs. 2b and 2c, the Always treatments of *A. catenella* had significantly lower C(1+2) (Mann-Whitney U test statistics 253.5, p=0.004) and GTX(1+4) (U=255.0, p=0.003) toxin content per cell. The same trend was observed in the Always treatments of the *A. minutum* experiment, while the toxin content in the cultures shaken during either the exponential or the stationary phase had no significant differences with that of the Control flasks (not shown).

4 Discusion

The experiments using the vertically oscillating grids were performed at ε intensities (ca. 0.4 $cm^2\,s^{-3}$) considered to naturally occur in the upper 10 m of the ocean under storm events (MacKenzie and Leggett, 1993; Kiørboe and Saiz, 1995; Petersen et al., 1998). The much higher ε generated in the orbital shaker (27 $cm^2\,s^{-3}$) would be even higher than those associated with intense wind conditions (>20 $m\,s^{-1}$, Granata and Dickey, 1991; MacKenzie and Leggett, 1993; Kiørboe and Saiz, 1995). Although our experimental values of ε are very high, both in intensity and persistence (Guadayol and Peters, 2006), these conditions may help to ascertain the underlying mechanisms

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of cell adaptations.

Interestingly, the highest net growth rates were estimated for the two species shaken at the lowest ε intensity with the vertically oscillating grids. Under similar experimental and turbulence intensity conditions, the net growth rates of *Oxyrrhis marina* (Havskum, 2003) and of *Ceratium tripos* decreased (Havskum et al., 2005). In contrast, *Fragilidium globosum* was not affected (Havskum et al., 2005) and the growth of *Heterocapsa triquetra* was favoured (Havskum and Hansen, 2006). This last observation suggested that turbulence facilitated gas exchange in the experimental vessels where high biomass developed. Note that in our experiments both *A. minutum* and *A. catenella* reached higher growth rates and biomass yields in the Control Plexiglass cylindric vessels than in the Control Pyrex sphaerical ones (Table 1). These differences in population development could be related to differences in shape or light wavelength transmission of the experimental vessels.

Considering the experiments in the orbital shakers, the trends observed in the biomass yields and growth rates are similar to those observed in previous studies with *A. minutum* under similar experimental conditions (Berdalet et al., 2007) and *A. catenella* conducted at ε of ca. 10^{-4} and ca. $1\,cm^2\,s^{-3}$ generated by horizontal rods oscillating in 20-L tanks (Sullivan and Swift, 2003). Regarding toxins, the results are opposite to those for *A. fundyense* that increased its cellular toxin content when exposed to $0.1\,cm^2\,s^{-3}$ in Couette devices (Juhl et al., 2001). Likely, differences in the physiological state of the cultures and/or the experimental setup used in each study have conditioned these opposite responses. In consequence, it is too soon to draw general conclusions and the question about the possible effect of turbulence on toxin production is an open one for future research.

In contrast, the immediate decay of ecdysal cyst abundances when the cultures were intensively shaken and the subsequent and fast recovery once turbulence stopped agree with previous studies (Anderson and Lindquist, 1985; Smith and Persson, 2004, 2005). Magnetic stirring for up to one month of *Scrippsiella lachrymosa* and *Alexandrium fundyense* cultures prevented their sexual encystment. Once stirring was

stopped, dinoflagellates resumed their regular mating behaviour. Turbulence would have denied them the stability for this process, without alteration of their physiological capacity to encyst. Smith and Persson (2004) suggested that sexual cyst formation would require a surface or boundary layer to facilitate the gamete meeting and initiation of mating. Subsequently, during the cyst formation, the cell wall gets stickier and zygotes sink to the bottom (of the container) or to the sediment. Although we are dealing here with asexual ecdysal cysts of *A. minutum* and *A. catenella*, a similar mechanism of interference by turbulence could also be acting. In nature, a certain degree of stability is usually associated with the outbreak, development and maintenance of dinoflagellate blooms (e.g. Margalef et al., 1979; Berman and Shteiman, 1998; Smayda and Reynolds, 2001) and dinoflagellates selectively accumulate in thin layers (e.g. *A. catenella* as observed by Sullivan et al., 2003). In our present study, the inhibition of the net population development observed in *A. catenella* and *A. minutum* during the long exposure to high turbulence intensities could be a combination of a direct alteration of the vegetative cell division and the interference with the ecdysal cyst formation. Our observation also indicates that the asexual encystment of these two species did not occur as a response to an environmental stress such as high turbulence. On the contrary, ecdysal cysts must be essential phases of the life cycles of these organisms playing a major role in population dynamics of certain dinoflagellates and requiring stability of the water column to proceed. Certainly, careful studies focussing on the link between small-scale turbulence and the different aspects of the life cycle of dinoflagellates will shed further light to understand the dynamics of this phytoplankton group in nature.

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Table 1. Net exponential growth rates (μ, day^{-1}) and final biomass yield (cells mL^{-1}) in each treatment of the 5 experiments performed in this study. "n" indicates the number of replicates considered for every calculation. For the estimations of μ we indicate the duration of the exponential growth phase (Expon. duration, days) considered for the calculations of the regression line and its associated standard error and adjusted multiple r^2; "p" indicates the degree of significance of the heterogeneity of the slopes tests (analysis of covariance) run to compare the growth rate during the shaking period with that of the Control (unshaken) ones. A comparison between the final yield obtained at the end of each experiment under turbulent conditions and the still ones is indicated as the %T/S percentage.

			Effect on growth rate						Effect on biomass yield			
Experiment	Treatment	Turbulent duration (days)	n	Expon. duration (days)	μ (day^{-1})	err	r^2	p	n	cells mL^{-1}	err	%T/S
A. minutum Orbital	Control	none	4	5–13	0.250	0.011	0.983		2	8458	825	
	Exponential	5–9	2	5–13	0.226	0.008	0.988	0.704	2	8142	258	96.3
	Stationary	11–15	2						2	7317	333	86.5
	Always	5–21	2	5–13	0.186	0.015	0.945	0.000	2	4690	130	55.4
A. minutum. Grids	Control	none	2	0–10	0.261	0.007	0.994		2	7725	158	
	Always	4–14	2	0–10	0.282	0.011	0.986	0.077	2	9813	263	127.0
A. catenella. Orbital shaker I	Control	none	4	5–13	0.213	0.011	0.934		2	2228	597	
	Exponential	4–8	2	5–9	0.214	0.008	0.899	0.375	2	2066	161	92.7
	Stationary	12–16	2						2	2047	522	91.9
	Always	4–21	2	5–9	0.228	0.065	0.620	0.392	2	549	49	24.7
A. catenella. Orbital shaker II	Control	none	2	0–10	0.227	0.015	0.954		2	3425	700	
	Always	4–21	2	0–10	0.204	0.013	0.959	0.105	2	739	61	21.6
A. catenella. Grids	Control	none	4	0–11	0.312	0.011	0.970		2	4820	330	
	Exponential	3–7	2	0–11	0.330	0.013	0.983	0.096	2	5016	4	104.1
	Stationary	11–15	2						2	5625	1350	116.7
	Always	3–20	2	0-11	0.348	0.010	0.991	0.001	2	7233	133	150.1

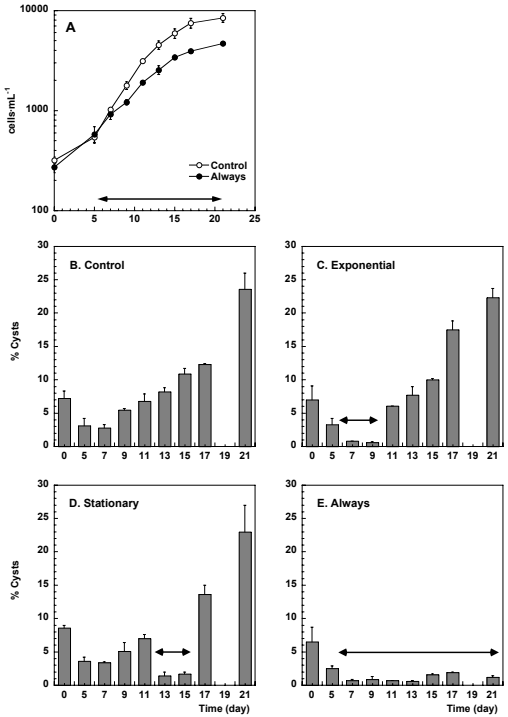

Fig. 1. (A): Temporal changes in *A. minutum* cell abundance in the unshaken Control (white symbols) and the turbulence Always treatment (black symbols). The turbulence treatment was done with an orbital shaker between days 5 to 21 (Table 1). The temporal changes of the other two shaken treatments (Exponential and Stationary, Table 1) were not significantly different from those of the Control ones and are not shown for clarity. **(B)** to **(E)**: Temporal changes in ecdysal cysts abundances (expressed as percentage of the total cell numbers) in each treatment of the experiment. Vertical bars indicate the standard error of the mean, and the shaken period of each treatment is marked by the double arrow horizontal line.

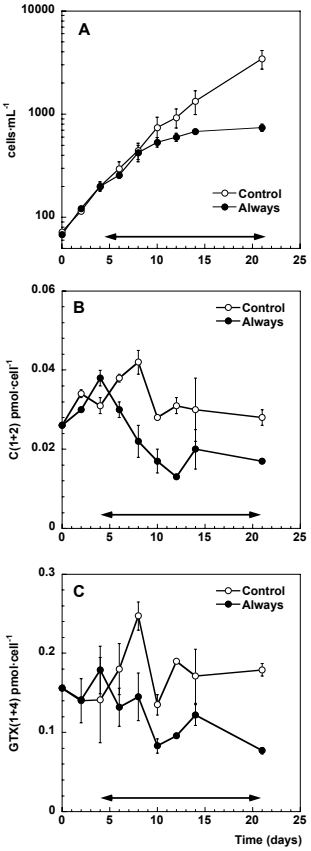

Fig. 2. Temporal changes in the cell abundances **(A)** and the toxin content (**B** and **C**) of the second experiment performed with *A. catenella* in the orbital shaker (Table 1), with only two treatments (Control and Always). Vertical bars indicate the standard error of the mean, and the shaken period of each treatment is marked by the double arrow horizontal line.