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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.** Some dinoflagellate species have shown different physiological responses to certain turbulent conditions. Here we investigate how two levels of turbulent kinetic energy dissipation rates ($\varepsilon$ = 0.4 and 27 $cm^2 s^{-3}$) affect the PSP toxins and ecdysal cyst dynamics of two bloom forming species, *Alexandrium minutum* and *A. catenella*. The most striking responses were observed at the high $\varepsilon$ generated by an orbital shaker. In the cultures of the two species shaken for more than 4 days, the cellular GTX(1+4) toxin contents were significantly lower than in the still control cultures. In *A. minutum* this trend was also observed in the C(1+2) toxin content. For the two species, inhibition of ecdysal cyst production occurred 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 shaking persisted for more than 4 days, the net growth rate significantly decreased in *A. minutum* (from 0.25±0.01 $day^{-1}$ to 0.19±0.02 $day^{-1}$) and the final cell numbers were lower (ca. 55.4%) than in the still control cultures. In *A. catenella*, the net growth rate was not markedly modified by turbulence although under long exposure to shaking, the cultures entered earlier in the stationary phase and the final cell numbers were significantly lower (ca. 23%) than in the control flasks.

The described responses were not observed in the experiments performed at the low turbulence intensities with an orbital grid system, where the population development was favoured. In those conditions, cells appeared to escape from the zone of the influence of the grids and concentrated in calmer thin layers either at the top or at the bottom of the containers.

This ecophysiological study provides new evidences about the sensitivity to high levels of small-scale turbulence by two life cycle related processes, toxin production and encystment, in dinoflagellates. This can contribute to the understanding of the dynamics of those organisms in nature.

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## 1 Introduction

Many laboratory studies have investigated the sensitivity to small-scale turbulence by phytoplankton (reviews by Thomas et al., 1997; Peters and Marrasé, 2000; Berdalet and Estrada, 2005). Dinoflagellates appear as the group showing a diversity of particular responses to this, not well understood yet, environmental factor. The available data indicate that the direct effect of turbulence is species-specific and dependent on the experimental conditions (Berdalet and Estrada, 1993; Sullivan and Swift, 2003; Berdalet et al., 2007), although direct comparison between studies is not straightforward (Peters and Marrasé, 2000). Some studies have found positive (or indifferent) biological 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; Pollingher and Zemel, 1981; Yeung and Wong, 2003).

This kind of experimental studies have been inspired on the observation that the occurrence of dinoflagellate red tides is favoured by relatively calm weather and water column stability coinciding with particular water circulation patterns (e.g., Wyatt and Horwood, 1973; Margalef et al., 1979; Pollingher and Zemel, 1981; Berman and Shteiman, 1998) although they also develop in frontal zones (Estrada and Blasco, 1979; Smayda and Reynolds, 2001). Given the ecological and socioeconomic importance of dinoflagellate blooms, it is important to study the different factors that can modulate their dynamics. Although the laboratory approaches will never mimic nature, they can help to ascertain the underlying mechanisms of cell responses to particular factors that cannot be separated from the rest of forcings in the field.

The aim of the present study was to increase the information on the sensitivity to small-scale turbulence of two red-tide forming dinoflagellates, *Alexandrium catenella* (Whedon and Kofoid) Balech and *Alexandrium minutum* Halim,

with special emphasis on the modulation of toxin and cyst production dynamics. The two organisms are reported to bloom in coastal areas (e.g. Halim, 1960; Delgado, 1990; Honsell et al., 1995; Hallegraef et al., 1998; Vila et al., 2001a, b). A previous study combining both field and laboratory work (Sullivan et al., 2003), hypothesized that *A. catenella* actively concentrated at depths with low turbulence and shear. In another experimental study (Berdalet et al., 2007), high turbulence intensity decreased the growth of *A. minutum* and interfered somehow with cyst production, although a clear conclusion was not drawn and the authors mentioned the need of further tests. Dinoflagellates have complex life cycles that include alternation of resting stages (cysts *senso lato*) and vegetative cells, with benthic or planktonic phases, respectively (e.g. Wyatt and Jenkinson, 1997; Garcés et al., 2002). In turn, cysts can be sexually formed from the 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). In general, sexually and asexually formed cysts are considered, respectively, resting and temporary cysts. 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. 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).

In this study we chose two experimental setups that were used previously in other studies performed in our laboratory with *A. minutum* and other species (Berdalet, 1992; Berdalet and Estrada, 1993; Havskum et al., 2005; Berdalet et al., 2007) with the aim of allowing direct comparison between data. High turbulence was produced in 4-l sphaerical flasks containing 3 l of medium agitated by an orbital shaker. This setup and shaking intensities guaranteed turbulent conditions in the whole container and provided the large volumes needed for the appropriate sampling of biological parameters (in particular, toxin concentration) while at the same time allowed direct comparison to previous experiments. A lower level of turbulence intensity was achieved with a vertically-oscillating grid system and 2-l cylindric vessels.

## 2 Materials and methods

The clonal strain of *Alexandrium minutum* (strain IEO – AL1V) was provided by the Vigo Oceanographic Center (Spain) and that of *Alexandrium catenella* (isolated by M. Delgado from the Tarragona Harbor, NW Mediterranean) 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 $\mu$mol 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 $\varepsilon$ of 27 $cm^2\,s^{-3}$ ($2.7\times10^{-3}$ Watt $kg^{-1}$) was calculated from the equation $\log_{10}\varepsilon$ –8.667 + 5.05 F, where F is frequency in Hz. This equation was derived from data acquired with a side-looking acoustic Doppler velocimeter (NDVlab, Nortek AS, Rud, Norway) for a range of oscillating frequencies and positions within the flask (Guadayol et al., 2007[1]). The power spectra from the velocity time series were analysed to calculate $\varepsilon$ using the linear regression method developed by Stiansen and Sundby (2001). The oscillating grids device, as described by Dolan et al. (2003), was designed by one of us (F. Peters). 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 $\varepsilon$ of 0.4 $cm^2\,s^{-3}$ ($4\times10^{-5}$ Watt $kg^{-1}$) 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 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.). Because cells showed

[1]Guadayol, Ò., Peters, F., Stiansen, J. E., Marrasé, C., and Lohrmann, A: Evaluation of oscillating grids and orbital shakers as means to generate isotropic and homogeneous small scale turbulence in laboratory enclosures commonly used in plankton studies, in review, 2007.

**Table 1.** Net exponential growth rates ($\mu$, 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 $\mu$ 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 final cell numbers | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 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* | *Control* | none | 4 | 5-13 | 0.25 | 0.01 | 0.983 | | 2 | 8458 | 825 | |
| Orbital | *Exponential* | 5-9 | 2 | 5-13 | 0.23 | 0.01 | 0.988 | 0.704 | 2 | 8142 | 258 | 96.3 |
| 27 cm$^2$s$^{-3}$ | *Stationary* | 11-15 | | | | | | | 2 | 7317 | 333 | 86.5 |
| | *Always* | 5-21 | 2 | 5-13 | 0.19 | 0.02 | 0.945 | 0.000 | 2 | 4690 | 130 | 55.4 |
| *A. minutum.* | *Control* | none | 2 | 0-10 | 0.26 | 0.01 | 0.994 | | 2 | 7725 | 158 | |
| Grids | *Always* | 4-14 | 2 | 0-10 | 0.28 | 0.01 | 0.986 | 0.077 | 2 | 9813 | 263 | 127.0 |
| 0.4 cm$^2$s$^{-3}$ | | | | | | | | | | | | |
| *A. catenella.* | *Control* | none | 4 | 5-13 | 0.21 | 0.01 | 0.934 | | 2 | 2228 | 597 | |
| Orbital shaker I | *Exponential* | 4-8 | 2 | 5-9 | 0.21 | 0.01 | 0.899 | 0.375 | 2 | 2066 | 161 | 92.7 |
| 27 cm$^2$s$^{-3}$ | *Stationary* | 12-16 | | | | | | | 2 | 2047 | 522 | 91.9 |
| | *Always* | 4-21 | 2 | 5-9 | 0.23 | 0.06 | 0.620 | 0.392 | 2 | 549 | 49 | 24.7 |
| *A. catenella.* | *Control* | none | 2 | 0-10 | 0.23 | 0.02 | 0.954 | | 2 | 3425 | 700 | |
| Orbital shaker II | *Always* | 4-21 | 2 | 0-10 | 0.20 | 0.01 | 0.959 | 0.105 | 2 | 739 | 61 | 21.6 |
| 27 cm$^2$s$^{-3}$ | | | | | | | | | | | | |
| *A. catenella.* | *Control* | none | 4 | 0-11 | 0.31 | 0.01 | 0.970 | | 2 | 4820 | 330 | |
| Grids | *Exponential* | 3-7 | 2 | 0-11 | 0.33 | 0.01 | 0.983 | 0.096 | 2 | 5016 | 4 | 104.1 |
| 0.4 cm$^2$s$^{-3}$ | *Stationary* | 11-15 | | | | | | | 2 | 5625 | 1350 | 116.7 |
| | *Always* | 3-20 | 2 | 0-11 | 0.35 | 0.01 | 0.991 | 0.001 | 2 | 7233 | 133 | 150.1 |

heterogeneous distributions in the flasks it was necessary to carefully mix them in order to obtain representative samples. The sphaerical still flasks were gently swirled and all the 2-l cylinders (shaken or not) were turned upside down 10 times.

Cell abundances were estimated using a Sedgewick-Rafter or a sedimentation chamber (Utermöhl, 1958), depending on the cell density of the sample, after fixation with Lugol's iodine solution. Net exponential growth rates, $\mu$ (day$^{-1}$), as defined by Guillard (1973), were calculated as the slope of the regression line of ln(N) versus time (t), where N was 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 paralytic shellfish toxin (PSP) analysis, 100 to 300 ml of culture were required for detection. Due to this required sample volume, sampling could only be done at the beginning (from the mother culture) and at the end in the 2-l vessels of the vertically oscillating grids setup. In those experiments sampling could only be done at the beginning (from the mother culture) and at the end in the 2-l vessels of the vertically oscillating grids setup. The samples 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 in the latter (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 cold (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 NRC (Hallifax, Canada), 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 cell numbers estimated in the different 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

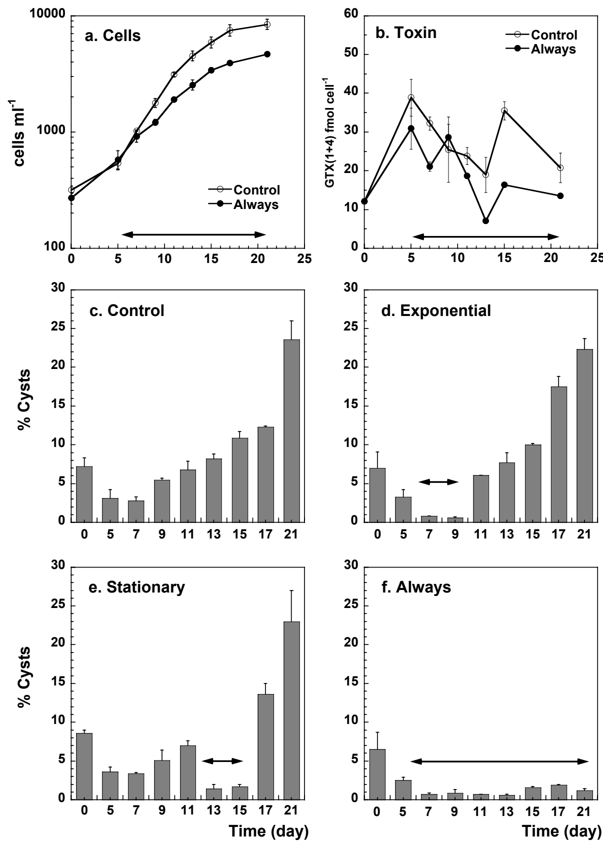

**Fig. 1.** **(a)**: Temporal changes in *A. minutum* cell numbers in the unshaken *Control* (white symbols) and the turbulence *Always* treatment (black symbols). The turbulence treatment was done with an orbital shaker between days 5 to 21 (Table 1). The temporal changes of the other two shaken treatments (*Exponential* and *Stationary*, Table 1) were not significantly different from those of the *Control* ones and are not shown for clarity. **(b)**: Temporal changes in the GTX(1+4) toxin content in the *Control* and the *Always* treatments. **(c)** to **(f)**: 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.

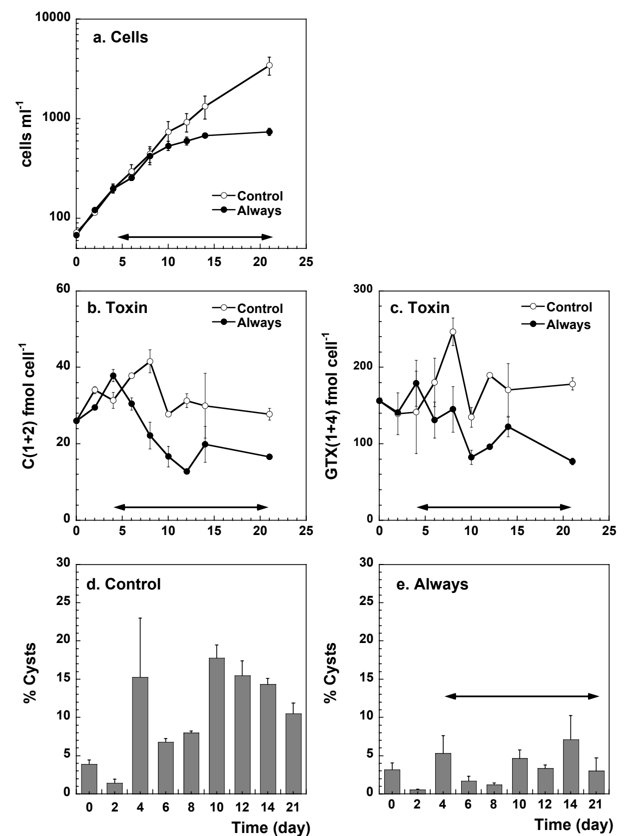

**Fig. 2.** Temporal changes in the cell numbers **(a)**, the toxin content (**b** and **c**) and the ecdysal cyst abundances in the second experiment performed with *A. catenella* in the orbital shaker with only two treatments (*Control* and *Always*, Table 1). Vertical bars indicate the standard error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

during the exponential period and subsequently stirred during the stationary one (i.e. *Stationary* treatment). Table 2 contains the statistics of the comparison of the toxin content and the percentage of cyst abundances between the different shaken treatments in each experiment with their corresponding *Control*.

Experiments with the orbital shaker

In general, exposure to the high $\varepsilon$ intensities generated by the orbital shaker did not significantly modify the exponential growth rate of the shaken treatments compared to that of the unshaken ones (Table 1, Figs. 1a and 2a). Only the *A. minutum* cultures exposed to turbulence for more than 4 days (*Always* treatment, Table 1, Fig. 1a) grew significantly slower ($0.19\pm0.02$ day$^{-1}$) than their Control ($0.25\pm0.01$ day$^{-1}$). The two species showed slightly lower final cell numbers when shaken for 4 days either during the exponential or the stationary phase, namely between 86.5% and 96.3% of the abundances obtained in the *Control* cultures (Table 1). The lowest final cell concentrations were estimated in the *Always* treatments, 55.4% in the *A. minutum* experiment (Fig. 1a) and 24.7% or 21.6% in the ones with *A. catenella* (Fig. 2a).

The toxin content of both, the *A. minutum* and the *A. catenella* strains, consisted mainly of the isomer pair GTX1 and GTX4, plus minor amounts of C1 and C2. At the beginning of the experiment with *A. minutum* its C(1+2) and GTX(1+4) contents were, respectively, 0.46 fmol cell$^{-1}$ and 12 fmol cell$^{-1}$. Those values peaked on day 5 and decreased afterwards with some oscillation until the end of the experiment in all treatments. There were not significant differences (Table 2) between the contents of the two toxin estimated in

**Table 2.** Statistics values of the comparison between the toxin content and the cyst abundances of each turbulent treatment with its corresponding still *Control*, for each of the 5 experiments performed in this study. U: Mann-Whitney non-parametric analysis of variance; p: degree of significance; n: number of data included in the statistical test. The tests compared the data during the shaking period of each treatment and the corresponding days of the *Control*. However, in the experiments with the oscillating grids, the comparison of the toxin contents was done only on the last day of each experiment.

| | | | Effect on Cx toxin | | | Effect on GTX toxin | | | Effect on % cysts | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Experiment | Treatment | Turbulent duration (days) | U | p | n | U | p | n | U | p | n |
| *A. minutum* | *Control* | none | | | | | | | | | |
| Orbital | *Exponential* | 5-9 | 10.0 | 0.564 | 8 | 13.0 | 0.149 | 8 | 32.0 | 0.006 | 12 |
| 27 $cm^2s^{-3}$ | *Stationary* | 11-15 | 10.0 | 0.564 | 8 | 7.0 | 0.165 | 6 | 32.0 | 0.006 | 12 |
| | *Always* | 5-21 | 83.5 | 0.505 | 28 | 151.5 | 0.014 | 28 | 196.0 | 0.000 | 28 |
| *A. minutum*. | *Control* | none | | | | | | | | | |
| Grids | *Always* | 4-14 | 1.000 | 0.439 | 4 | 4.0 | 0.121 | 4 | 30.0 | 0.131 | 20 |
| 0.4 $cm^2s^{-3}$ | | | | | | | | | | | |
| *A. catenella* | *Control* | none | | | | | | | | | |
| Orbital shaker I | *Exponential* | 4-8 | no data | | | no data | | | 64.0 | 0.001 | 16 |
| 27 $cm^2s^{-3}$ | *Stationary* | 12-16 | | | | | | | 64.0 | 0.001 | 16 |
| | *Always* | 4-21 | | | | | | | 467.0 | 0.000 | 44 |
| *A. catenella*. | *Control* | none | | | | | | | | | |
| Orbital shaker II | *Always* | 4-21 | 130.5 | 0.001 | 24 | 134.0 | 0.000 | 24 | 215.0 | 0.000 | 30 |
| 27 $cm^2s^{-3}$ | | | | | | | | | | | |
| *A. catenella*. | *Control* | none | | | | | | | | | |
| Grids | *Exponential* | 3-7 | 2.00 | 1.00 | 4 | 2.00 | 1.00 | 4 | 16.0 | 0.021 | 8 |
| 0.4 $cm^2s^{-3}$ | *Stationary* | 11-15 | 2.00 | 1.00 | 4 | 2.00 | 1.00 | 4 | 12.0 | 0.248 | 8 |
| | *Always* | 3-20 | 3.00 | 0.431 | 4 | 3.00 | 0.431 | 4 | 142.0 | 0.598 | 32 |

the *Control* and in the flasks shaken during the exponential or the stationary phase, nor in the C(1+2) levels estimated in the *Always* treatments. Only the GTX content in the *Always* flasks was significantly higher (19.5±2.2 fmolcell$^{-1}$; n=14; mean ± SEM) than in the *Control* (26.9±2.4 fmol cell$^{-1}$, n=14; Fig. 1b, Table 2). Overall, the range of the two toxins measured in *A. minutum*, considering the whole data set was 0.9±0.05 fmol cell$^{-1}$ and 21.3±1.3 fmol cell$^{-1}$ (n=57).

The toxin levels of *A. catenella* were several times higher than that of *A. minutum* (Figs. 2b and c). At the beginning of the experiment the C(1+2) and GTX(1+4) contents were, respectively, 26 fmol cell$^{-1}$ and 156 fmol cell$^{-1}$. As illustrated in Figs. 2b and c, the levels of the two toxins in this species were significantly lower (Table 2) in the *Always* treatments (109.2±9.2 fmol cell$^{-1}$ GTX(1+4) and 19.8±1.9 fmol cell$^{-1}$ C(1+2), n=12) than in the *Control* (169.7±9.2 fmol cell$^{-1}$ GTX(1+4) and 32.6±1.2 fmol cell$^{-1}$ C(1+2), n=21).

Cyst abundances (expressed as % of the total cell numbers) tended to progressively increase in the *A. minutum Control* cultures from the exponential to the stationary phase (Fig. 1c). A sudden decrease in the cyst numbers were observed when the cells were shaken for 4 days either during the exponential (Fig. 1d) or stationary (Fig. 1e) phases. In those shaking periods, the percentage of ecdysal cysts were significantly lower compared to the corresponding days (days 5–9 and 11–15, respectively) of the experiment in the *Control* (Table 2). Immediate restoration of cyst abundances occurred at the cessation of shaking in the two treatments. In the *Always* flasks, significantly lower cyst proportions remained during the whole agitation period (Fig. 1f, Table 2, U=196.0, p=0.0001, compared to the % of cysts from days 6 to 21 in the *Control*).

The same general trend was observed in the two experiments with *A. catenella*. In the first experiment with this species, the cyst numbers in the *Control* flasks contributed 15.3%±3.1 (n=32) of the total cell numbers along the whole experiment. During the shaking periods, the percentage of cysts dropped significantly (Table 2) with values around 1.1%±0.3 (n=8) in the *Exponential*, 7.8%±1.6 (n=8) in the *Stationary* and 1.6%±0.3 (n=26) in the *Always* treatments. A similar figure was found in the second experiment (Figs. 2d and e, Table 2), concerning the *Always* and the *Control* cultures.

Experiments with the vertically oscillating grids

Exposure to the low turbulence intensity generated by the vertically oscillating grids favoured the population development of the two species (Figs. 3a and b, Table 1). In the *A. catenella* experiment both, the net growth rate and the final cell numbers increased with the duration of the shaking period (Table 2). After 2 days of shaking, visual inspection of the containers seemed to point towards an accumulation of cells at the top and the bottom of the containers, suggesting that dinoflagellates could have possibly escaped turbulence since the grid did not reach the ends of the cylinders. At the bottom, the aggregation areas conformed to the geometry of the grids, i.e. with cells concentrated forming empty squares. Aggregates homogeneously distributed at the bottom of the cylinders were also observed in the still treatments, when they entered the stationary phase.

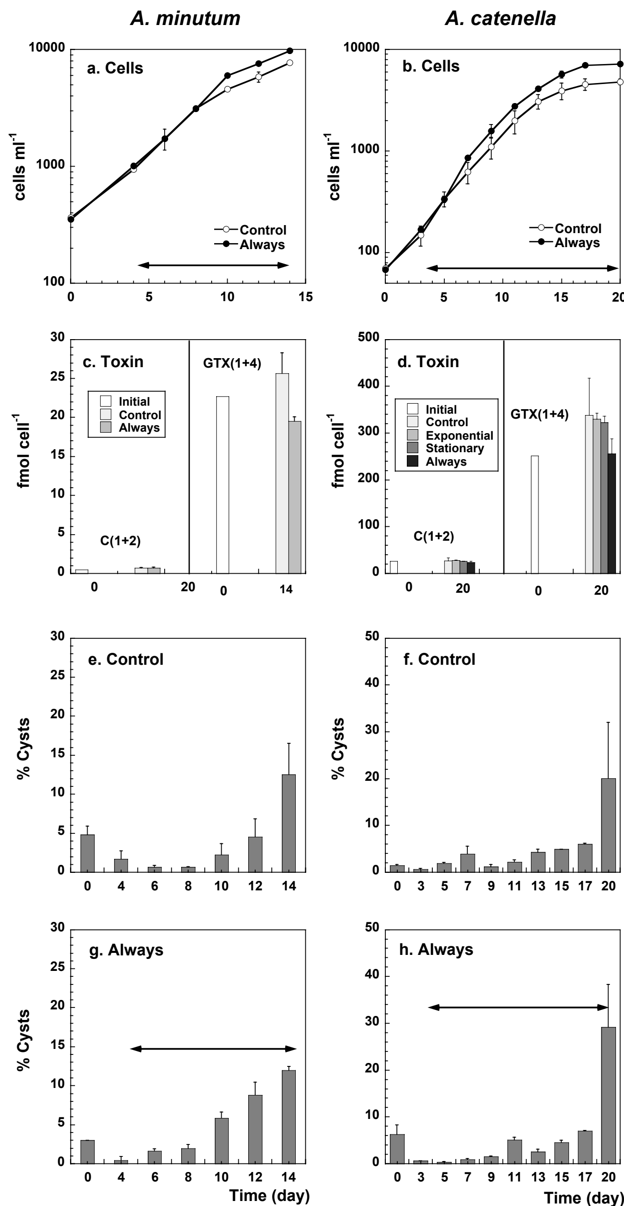


**Fig. 3.** Temporal changes in the different parameters estimated in the experiments performed with the vertically oscillating grids in *A. minutum* (**a**, **c**, **e** and **g**) and *A. catenella* (**b**, **d**, **f** and **h**). For simplicity, only the cell numbers and % cyst abundances of the *Control* and the *Always* treatments of the *A. catenella* experiment are shown; the description of the *Exponential* and *Stationary* cultures can be found in the text and in Table 2. (a) and (b) correspond to the temporal changes in cell numbers. (c) and (d) show the cell concentration of the C(1+2) and the GTX(1+4) on the first day and at the end of each experiment. (e) and (f) display the changes in the cyst abundances of the still *Control* and (g) and (h) correspond to the *Always* treatments. Vertical bars indicate the standard error of the mean, and the shaken period of each treatment is marked by the double arrow horizontal line.

The experiments with grids showed inconclusive results concerning the cellular content of both GTX(1+4) and C(1+2), since samples could only be taken on the first and the last day (Figs. 3c and d). There were no significant differences between each shaken treatment and the unshaken ones on the last day of each experiment (Table 2).

The dynamics of the cyst abundances was similar between the different treatments (Figs. 3e to h). In the unshaken *Control* (Figs. 3e and f) they remained below the 10% or the 5% in *A. catenella* and *A. minutum*, respectively, and tended to increase (up to the 30% or the 12%, respectively) when the cultures entered the stationary phase. In general, turbulence did not significantly modify this trend (Table 2), except in the *Exponential* treatment of the *A. catenella* experiment, where the cyst abundances were significantly lower than in the *Control* treatment during the shaking period (days 4–7, Table 2). When turbulence stopped, cyst abundances increased and reached similar levels than in the *Control*.

## 4 Discussion

The experiments using the vertically oscillating grids were performed at $\varepsilon$ 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 $\varepsilon$ 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), and would only be experienced by cells in the surf zone or rocky intertidal. Our experimental values of $\varepsilon$ were large, both in intensity and persistence, compared to Mediterranean turbulence events (Guadayol and Peters, 2006), although 4-day sustained events of 0.4 $cm^2 s^{-3}$ do occur sometimes. Thus we chose this duration for some experimental conditions during either the exponential or the stationary phase of the growth curve. The physiological responses at this time scale also served to compare with the trends under still control conditions and under longer periods of exposure to shaking (16–17 days). The turbulence generated is also qualitatively different for each laboratory setup as it is different from field turbulence (Peters and Redondo, 1997). With their limitations, but also with their particular advantages, these experimental conditions may help to ascertain the underlying mechanisms of cell adaptations. The following discussion is developed within a physiological context with no aim to directly extrapolate to nature.

Both the net growth rates and the final cell numbers of the two species increased when mixed at the lowest $\varepsilon$ intensity with the vertically oscillating grids for 4 or more days. With the same setup and similar experimental design and turbulence conditions, different species-specific responses have been observed: while the net growth rates of *Oxyrrhis marina* (Havskum, 2003) and of *Ceratium tripos* (Havskum et

al., 2005) decreased, the one of *Fragilidium subglobosum* was not affected (Havskum et al., 2005) and that of *Heterocapsa triquetra* was favoured (Havskum and Hansen, 2006). In this last study, those authors suggested that turbulence facilitated gas exchange and avoided excessive pH increase in the experimental vessels allowing higher cell numbers than in the still flasks. This mechanism could have operated also in our experiments, where the two species concentrated either at the top or the bottom of the cylinders where the grids did not pass. There, cells could proceed with their life cycle relatively undisturbed, including cyst formation (see below). Given that dinoflagellates managed to escape from the mixed zone, we cannot conclude that low turbulence stimulated dinoflagellate growth nor suggest any hypothesis for natural conditions. The noticeable result here is that the two species were likely sensitive to the tested turbulence intensity, as they swam away from it and chose preferentially calm zones.

In contrast, it is not possible to escape from the turbulence generated by the orbital shaker. However, the decrease in the net growth rate in *A. minutum* and the lowest final numbers in the two species were only observed when shaking lasted for more than 4 days. Those trends were similar to those described in previous studies with *A. minutum* under similar experimental conditions (Berdalet et al., 2007) and with *A. catenella* conducted at $\varepsilon$ of ca. $10^{-4}$ and ca. $1\,\mathrm{cm^2\,s^{-3}}$ generated by horizontal rods oscillating in 20-l tanks (Sullivan and Swift, 2003). In the two studies, shaking lasted for 1–2 weeks. Overall the data indicate that even the highest intensities and persistence levels of turbulent conditions that can be generated naturally, would have no lethal effects on our tested dinoflagellates.

The most immediate response was the decay of ecdysal cyst abundances when the cultures were intensively shaken for only 4 days, and the subsequent and fast recovery once turbulence stopped. The persistence of significantly lower cyst numbers in the cultures shaken for more than 4 days confirmed this trend. Those results 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 high turbulence was not a factor that induced the asexual encystment of these two species. On the contrary, ecdysal cysts must be an essential phase 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.

Regarding toxins, the results in the two experiments with the orbital shaker are opposite to those for *A. fundyense* that increased its cellular toxin content when exposed to $0.1\,\mathrm{cm^2\,s^{-3}}$ in Couette devices (Juhl et al., 2001). Likely, differences in the physiological state of the cultures and/or the experimental setup and design 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 continues to be an open one.

Certainly, detailed 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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