**Interactive effects of salinity and temperature on planozygote and cyst formation of
Alexandrium minutum (Dinophyceae) in culture**

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Running head: Factors inducing sexuality in *A. minutum*

ABSTRACT

The factors regulating dinoflagellate life-cycle transitions are poorly understood. However, their identification is essential to unravel the causes promoting the outbreaks of harmful algal blooms (HABs) because these blooms are often associated with the formation and germination of sexual cysts. Nevertheless, there is an absolute lack of knowledge on the factors regulating planozygote-cyst transitions in dinoflagellates due to the difficulties of differentiating planozygotes from vegetative stages. In the present study, two different approaches were used to clarify the relevance of environmental factors on planozygote and cyst formation of the toxic dinoflagellate *Alexandrium minutum*. In the first, the effects of changes in initial phosphate (P) and nitrate (N) concentrations in the medium on the percentage of planozygotes formed were examined using flow cytometry. In the second, two-factorial designs were used to determine how salinity (S), temperature (T), and the density of the initial cell inoculum (I) affect planozygote and resting-cyst formation. These experiments led to the following conclusions:

- 1) Low P/N ratios seem to induce gamete expression because the percentage of planozygotes recorded in the absence of added phosphate (-P) was significantly higher than that obtained in the absence of added nitrogen (-N), or when the concentrations of both nitrogen and phosphate were 20 times lower (N/20+P/20).
- 2) Salinity (S) and temperature (T) strongly affected both planozygote and cyst formation, as sexuality in the population increased significantly as salinity decreased and temperatures increased. (S, T) combinations that resulted in no significant cyst formation were, however, favorable for vegetative growth, ruling out the possibility of negative effects on cell physiology.
- 3) The initial cell density is thought to be important for sexual cyst formation by determining the chances of gamete contact. However, the inoculum concentrations tested did not explain either planozygote formation or the appearance of resting cysts.

Key words: *Alexandrium minutum*, encystment, response surface methodology, salinity, sexuality, temperature, planozygotes.

Abbreviations: P: phosphate, N: nitrate, N/20+P/20: medium with both nitrogen and phosphate 20 times lower in concentration than in regular L1 medium, S: salinity, T: temperature, I: cell inoculum.

INTRODUCTION

Alexandrium minutum (Halim) is a toxic dinoflagellate species that causes paralytic shellfish poisoning (PSP) in many parts of the world. Because dinoflagellate blooms- also known as red tides- have important negative impacts on fisheries, aquaculture, water quality, and public health (Smayda 1990), it is essential to unravel the factors controlling these algae proliferation.

Generally, the important oceanographic factors for red tide formation are known to be water temperature (Erard-Le Denn 1997), salinity (Anderson et al. 1983, Anderson et al. 2005) and nutrients (Anderson et al. 2002, Glibert et al. 2001). In the case of *Alexandrium* species, several studies have shown that salinity is a key exogenous factor influencing the bloom dynamics and throughout the world, blooms of *A. minutum* are associated with salinity stratification and local nutrient-rich freshwater inputs (Blanco et al. 1985, Cannon 1990, Chang et al. 1996, Giacobbe et al. 1996, Erard-Le Denn 1997, Bravo et al. 2008). *A. minutum* blooms have been also reported in coastal environments with salinity gradients (Bravo et al. 2009). Because of this ability not only to survive but also to grow asexually in various salinities (10-40psu in culture with toxins (PST) mainly produced at salinities of ≤ 25 psu (Grzebyk et al. 2003)), *A. minutum* is characterized as a “euryhaline” alga, indicating that this species must have physiological properties supporting this organism’s salinity tolerance. Regarding temperature, the vegetative growth in cultures of *A. minutum* increases with increasing temperature (from 15 to 25°C) (Lim et al. 2006), and growth rates of up to 0.5 divisions day⁻¹ can be reached at 12°C after a period of adaptation (Cannon 1996). Nutrient inputs have been shown to sustain *A. minutum* blooms in different ways; cell densities showed a positive significant correlation with NO₃ but a negative correlation with NH₄ in the Mediterranean sea (Bravo et al. 2008) and Brittany coast (Maguer et al. 2004), whereas reduced N, mainly as NH₄ sustained a bloom in the Cape Town harbour (Pitcher et al. 2007).

Although salinity, temperature and nutrients are the most commonly studied variables to explain bloom development, sexuality is another factor that in theory should be important to

promote and sustain blooms (Garcés et al. 1999). The traditional role of sexuality is explained as follows: A bloom begins as a small population of dinoflagellate cells, but under appropriate conditions an exponential growth phase (mitotic, i.e., asexual division) causes a tremendous increase in their population. In time, the depletion of nutrients and carbon dioxide in the water, and degraded environmental conditions caused by the bloom, decrease population growth. At this phase of the bloom, many dinoflagellate species are thought to undergo sexual fusion and form resting cysts (hypnozygotes) that sink to the bottom of the sea. Resting cyst formation is important due to several reasons. Because resting cysts are sexual, they indicate the existence of sexual fusion and recombination in the blooming population, but additionally, the formation of cysts deposits in the sediments may be used as inoculum for future blooms, a theory supported by evidence in both *A. minutum* (Garcés et al. 2004, Bravo et al. 2009) and *A. tamarense* (Anderson et al. 2005). Some studies also indicate that massive encystments may contribute to bloom collapse (Matsuoka and Takeuchi 1995, Kremp and Heiskanen 1999, Wang et al. 2007). Therefore, to explain bloom development and recurrence is important to understand how the asexual (vegetative growth) and sexual (planozygote and resting cyst formation) routes are linked and which factors from the already discussed above promote one or the other. However, contrasting conclusions have been reached by studies aimed at clarifying the factors inducing cyst formation, linking it either to stressful (for eg. Anderson et al. 1984) or to optimal conditions (for e.g. Kremp et al. (2009)).

In the case of *A. minutum* (but this example may be extended to other studied species), even when nutrient stress seems to be essential to trigger encystment in culture (Probert et al. 2002, Figueroa et al. 2007), encystment in the field may be better explained as a naturally occurring phase within the life history during optimum growth conditions (Garcés et al. 2004). For example, natural resting cyst formation in *A. minutum* has been observed to start in periods in which high vegetative cell densities are present in the water column, the cyst flux covering the periods of maintenance and decline of the bloom. The low but continuous percentage of

encysting population suggests that encystment is not triggered by any particular stressful environmental condition (Garcés et al. 2004, Pitcher et al. 2007). This apparent discrepancy indicates the need to assess nutrient limitation per cell in the field, as proposed by Olli and Anderson (2002), but this factor is difficult to measure in natural samples.

Additionally, it has also been pointed out that temperature and irradiance may not be essential but instead only influence the rate of encystment to a limited extent (Sgrosso et al. 2001, Nagai 2004). Nevertheless, the importance of combined factors, such as nitrogen and temperature, has been shown in *Peridinium cinctum* Stein (Grigorszky et al. 2006). Furthermore, factors that influence cell density may also affect cyst production, since cell contact seems to provide important signals for planozygote encystment (Uchida 2001). Regarding salinity, it is difficult to find studies relating salinity and sexual induction. However, this relation may be happening because cellular C and N concentrations, factors that did have being related with sexuality, vary in response to salinity changes (Nielsen 1996).

If our objective is to study whether some factor is affecting the number of cells undergoing sexuality, we need to find a reliable checkpoint in the sexual route. Resting cyst formation depends on the successful completion of two steps, formation and fusion of compatible gametes, and planozygote encystment. However, resting cyst formation has been traditionally the sole criterion used to estimate the amount of sexuality in a given dinoflagellate population. Indeed, almost all studies (in culture) on the effect of nutrients on sexuality have uniquely counted resting cysts formation, and therefore, it may be possible that their results only showed that planozygote encystment, but not necessarily planozygote formation, is affected by nutrient deficiency. It is well known that attempts to quantify the different life cycle stages most often results in highly inaccurate estimates of gamete and planozygote numbers. For unknown reasons, many planozygotes may remain in culture without encysting (e. g. Anderson et al. 1985, Figueroa et al. 2005), but also planozygotes may divide (e. g. Uchida 2001, Figueroa and Bravo 2005a, b, Figueroa et al. 2007). To our knowledge, only Anderson and Lindquist (1985), in their

study of *A. tamarense*, used the criterion of cell size to count planozygotes. Those authors noted the limitations also posed by this approach and thus the need for a cytological marker that recognizes early planozygote stages. Indeed, size was subsequently shown to be an inadequate basis for characterizing planozygotes in *A. minutum* (Figuerola et al. 2007). The main problem in measuring the true extent of sexuality in cyst-forming dinoflagellates is to differentiate gametes and planozygotes from vegetative stages (see, for example, Anderson and Lindquist 1985; von Stosch 1973, Pfiester 1975, 1976, 1977; Walker and Steidinger 1979). This problem can be resolved using flow-cytometry, such as the method recently used to count planozygotes in *A. minutum* (Figuerola et al. 2007). As this method is based on DNA content, it is also able to identify newly formed (small) planozygotes.

The objective of the present work was to answer concrete questions not yet known about the dinoflagellate sexuality: i) Is planozygote formation, and not only planozygote encystment, affected by the amount of nutrients in culture? ii) Are other environmental factors apart from nutrients, such as salinity, temperature and initial inoculum (cell contact) affecting planozygote and cyst formation in culture?, and iii) Are conditions favourable for growth unfavourable for sex and vice versa?.

MATERIAL AND METHODS

Culture conditions. *A. minutum* strains VGO 650 and VGO 651 belong to the culture collection of the Centro Oceanográfico de Vigo. Cultures were grown at 20°C with an irradiance of approx. $90 \mu\text{mol photons. m}^{-2} \text{s}^{-1}$ and a photoperiod of 12:12 h L:D (light:dark). Culture stocks were maintained in Erlenmeyer flasks filled with 50 mL of L1 medium (Guillard and Hargraves 1993) without added silica. The medium was prepared using Atlantic seawater adjusted to a salinity of 27 psu by the addition of sterile distilled water.

Effects of nutrients on planozygote formation using flow cytometry.

Sexual crosses. Duplicate crosses (VGO 650 × VGO 651) were conducted in sterile polystyrene Petri dishes (Iwaki, Japan, 16 mm diam.) containing either 10 mL of L1 medium with a 1:20 dilution of nitrates and phosphates (L/20), or L1 medium without added phosphate (L-P), or L1 medium without added nitrate (L-N). The dishes were inoculated with exponentially growing cells (2000–4000 cells mL⁻¹) to a final concentration of 300 cells mL⁻¹ (150 cells mL⁻¹ from each compatible strain). Cultures were sampled for planozygote formation at days 3, 4, and 5 after crossing.

Flow cytometric analyses. Planozygote formation was calculated following the method described by Figueroa et al. (2007), which takes advantage of the dark-induced synchronization of cell division in *Alexandrium* in order to calculate planozygote formation by flow cytometry. Since *A. minutum* cells arrest in the G1 phase of the cell cycle after 48 h of incubation in the dark, the number of diploids (2n) formed in a synchronized sexual cross sampled immediately after the end of the dark period are planozygotes and not dividing cells containing double the amount of DNA. Therefore, cultures sampled for planozygote formation (3, 4 and 5 days after crossing) entered the 48h dark period at days 1, 2 and 3 after crossing. The entire contents of the Petri dish (10mL) were recovered, fixed with 1% paraformaldehyde for 10 min, and washed in PBS (pH 7, Sigma-Aldrich, St. Louis, USA) (1200 g × 10 min). The pellet was resuspended in 2 mL of cold methanol and stored for at least 12 h at 4°C to allow chlorophyll extraction. The cells were then washed twice in PBS (pH 7) and the pellet was resuspended in a staining solution (PBS, 3 µg propidium iodide mL⁻¹ and 1.1 µg RNaseA mL⁻¹) for at least 2 h before analysis. A Becton and Dickinson FACScalibur bench machine with a laser emitting at 488 nm was used. Samples were run at low speed (approx. 18 µL min⁻¹) and data were acquired in linear and log modes until around 10000 events had been recorded. As an internal standard, 10 µl of a solution containing yellow-green 0.92-µm polysciences latex beads (10⁶ mL⁻¹) was added to each sample.

Fluorescence emission of propidium iodide was detected at 617 nm. ModFit LT (Verity software

House, Topsham, ME, USA) was used to compute peak numbers, coefficients of variation (CVs), and peak ratios for the DNA fluorescence distributions in a population.

Statistical experimental designs (factorial or Box, Hunter & Hunter designs)

Due to volume/surface related factors, cyst formation decreases drastically when the flask volume is increased (Smith and Persson 2004). For this reason, a first factorial experiment was designed to optimize cyst formation using small volumes. However, flow cytometry analysis requires a considerable number of cells. On top of this, dinoflagellate sexuality is induced by nutrient-limiting conditions and with an initial cell inoculum containing very few cells. Therefore, a second factorial experiment using bigger flasks was designed to estimate planozygote formation, despite the fact that cyst production would thus be dramatically reduced.

In both experiments, first-order factorial designs were used (Box et al. 2005). These designs were always orthogonal, that is, variance-covariance matrix is diagonal. Therefore, the estimators of effects are uncorrelated and the variances are minimised. For the calculation of experimental error, a quadruple replication of experiments in the center of the experimental domains was carried out. A factorial design also known as standard design or Box, Hunter & Hunter design is a powerful statistical tool, included in the response surface methodologies (RSM), which by means of a small number of experiments and empirical equations fittings is used to predict and to optimize the joint effect of several independent variables on a studied response (dependent variable). The independent variables in the first experimental design were salinity (S), temperature (T), and density of the initial inoculum (I). In the second factorial experiment, the variables were salinity (S) and temperature (T).

Experimental design and statistics

The results of the factorial designs were used to obtain, by means of orthogonal least-squares calculation, polynomial equations describing the following responses (*R*), or dependent variables: 1) cells. mL⁻¹, 2) ratio of cysts per cell and 3) percent diploids, each one as a function of T, S and

I concentrations (first experimental design) or T and S levels (second experimental design). The general form of the polynomial equations is:

$$R = b_0 + \sum_{i=1}^n b_i x_i + \sum_{i=1}^n \sum_{j>i}^n b_{ij} x_i x_j + \sum_{i=1}^n \sum_{j>i}^n \sum_{k>j}^n b_{ijk} x_i x_j x_k$$

where, R represents the dependent variable to be modelled; b_0 , b_i , b_{ij} and b_{ijk} are the coefficients of the model; x_i , x_j and x_k represent the independent variables.

Statistical significance of the coefficients was verified by Student's t -test ($\alpha=0.05$). Mathematical models consistency was quantified using analysis of variance (Fisher's F -test, $\alpha=0.05$) applied to the following mean squares ratios:

$$F_1 = \frac{\text{Model}}{\text{Total error}}$$

the model is acceptable if $F_1 \geq F_{den}^{num}$

$$F_2 = \frac{\text{Model} + \text{Lack of fitting}}{\text{Model}}$$

the model is acceptable if $F_2 \leq F_{den}^{num}$

$$F_3 = \frac{\text{Total error}}{\text{Experimental error}}$$

the model is acceptable if $F_3 \leq F_{den}^{num}$

$$F_4 = \frac{\text{Lack of fitting}}{\text{Experimental error}}$$

the model is acceptable if $F_4 \leq F_{den}^{num}$

where the corresponding sum of squares, degrees of freedom and mean squares were obtained as it is explained in Table 1.

The goodness of fit was established by means of the determination coefficient in its conventional expression:

$$r^2 = \frac{SSM}{SST}$$

In addition, this equation was corrected to penalize the increase of independent variables significant, in accordance with the function:

$$r_{adjusted}^2 = 1 - \left(\frac{SSE \times total \ degrees \ of \ freedom}{SST \times error \ degrees \ of \ freedom} \right)$$

Experiment 1: Effect of salinity, temperature, and density of the initial inoculum on cyst production.

The sexual cross of *A. minutum* strains VGO 650 and VGO 651 was performed in 60 flasks of 50 mL (Iwaki, Tokio, Japan) filled with 25 mL of medium containing a 1:20 dilution of nitrates and phosphates (L/20). The flasks were inoculated with exponentially growing cells (2000-4000 cells.mL⁻¹) to three different final cell concentrations (300, 500, and 700 cells.mL⁻¹), half from each compatible strain. The codified values for the variables are shown in Table 2. Based on the combinations shown in Table 3, the inoculation medium (L/20) was prepared at three different salinities (18, 27, and 36 psu), and the cultures were incubated at three different temperatures (15, 19, and 24°C). Within 20 days after the crossing, five samplings were prepared and the following variables measured: salinity (S), photosynthetic efficiency (Fv/Fm), cell concentration (cells mL⁻¹), and cyst concentration (resting cysts mL⁻¹). Subsamples were fixed with Lugol for cell and cyst enumeration and placed in Sedwick-Rafter chambers (SPI, West Chester, PA, USA); at least 200 cells were counted. Resting cysts (Fig. 3) were identified following the methods of Bolch et al. (1991) and Figueroa et al. (2007). As described by Guillard (Guillard, 1973), net duplication growth rates (day⁻¹) were calculated from the slopes of the regression lines of ln (N) versus time, with N as the mean number of cells during the time period of maximum growth; $K = \ln(N_1/N_0)/(t_1 - t_0)$, and the duplication time for each treatment as $K_d = \ln 2/k$.

Salinity was measured with a standard-conductivity cell (TetraCon 325 WTW, Munich, Germany). Fv/Fm was determined, after 20–30 min of dark adaptation (Schreiber et al. 1995), with a Phyto-PAM with Phyto ED measuring head (Heinz Walz GmbH, Pfullingen, Germany) that included a measuring chamber with a 15-mm-diameter quartz cuvette. Fv/Fm is interpreted as the quantum yield of photochemical energy conversion in PSII (photosynthetic efficiency).

Experiment 2: Effect of salinity and temperature on planozygote formation.

The second experiment was carried out under the same conditions as the first but using IWAKI flasks (200 mL) filled with 75 mL of culture and prepared according to the conditions shown in Table 4, which also lists the codified values for the variables. Within 18 days after crossing, data describing cyst formation and the percent of diploids were recorded for the different salinity and temperature combinations. Each culture sampled for planozygote formation (2-20 days after crossing) entered the 48h darkness period two days before sampling (0-18 days after crossing). As example, Table 5 summarized the full-statistical analysis for the diploids concentration production. Resting-cyst formation was also quantified by extracting a 3-mL aliquot, which was then processed according to the protocol described above.

RESULTS

Effects of nutrients on planozygote formation.

The percentage of planozygotes formed under the three different nutritional levels was measured (Table 6). The results indicated that significantly more planozygotes (ANOVA, $p < 0.05$) were formed in medium without added phosphates (L-P) than in medium without added nitrates (L-N) or medium containing 20-fold lower amounts of nitrates and phosphates (L/20). In L-P medium, and depending on the time elapsed after crossing of the parental strains, 70–78% of the population consisted of planozygotes (Fig. 1); in L-N medium, 61–65% of the population consisted of planozygotes whereas in L/20 medium planozygotes accounted for only 32–46% of the population.

Effects of salinity (S), temperature (T), and density of the initial inoculum (I) on cyst formation (experiment 1) and planozygote formation (experiment 2).

Experiment 1.

During the 18 days following sexual induction, data pertinent to the division rates and cyst formation under the different combinations of salinity (S), temperature (T), and initial inoculums (I) were recorded. In all empirical models more than 89% of variance was attributable to significant terms of the equations.

Growth. Division rates increased with salinity (S), initial number of cells inoculated (I), and the interaction between temperature and salinity ($T \times S$). The response surfaces shown in Fig. 2 show this positive correlation of growth with salinity and size of the initial cell inoculum, according to the empirical equation $R \text{ (cells ml}^{-1}\text{)} = 14304 + 3535S + 5844I + 2054TS$, which was statistically consistent and significant (Table 3). This can be easily observed in the 2D-contour plots with an increment in the gradient of red colour (growth of *Alexandrium minutum*) to high salinities and initial cell inoculum.

Encystment. To account for growth and cyst production, cyst yield was defined as cysts produced per number of cells (i.e. cysts. cells⁻¹). This variable described the relationship between the number of cysts and the number of cells 18 days after sexual induction. The response of the cyst yield (the ratio cysts/cells) in the experiment was complex. T and the interaction $S \times I$ had positive effects on cyst yield, whereas the effects of first-order S and I , and the interaction $T \times S$ were negative. In Figure 4 these results are clarified, which are summarized by the following empirical equation: $R \text{ (cysts cells}^{-1}\text{)} = 83 + 59 T - 89 S - 40 I - 59 TS + 37 SI$. The 2D plots presented in the figure show the clearest effect, which is how decreasing salinity and increasing temperature promote an increase in the number of cysts per cell. Also, in these plots it can be observed the similarity in the results obtained when the most different initial inoculums employed in the analysis ($I=700 \text{ cells ml}^{-1}$ and $I=300 \text{ cells.ml}^{-1}$) are compared.

Photosynthetic efficiency. Chlorophyll fluorescence was measured and expressed as the ratio of variable fluorescence (Fv) to maximum fluorescence (Fm). The Fv/Fm ratio is used to indicate the physiological conditions of plants under various circumstances, although the values obtained also depend on different parameters of the studied species. In *A. minutum* cultures grown in suitable conditions is around 0.5-0.8 (unpublished data) and decreases towards 0 under stress conditions. The values obtained during the experiment indicated that the efficiency of photosynthesis contrasted with the trend in cyst production, with greater efficiency at higher salinities and lasting longer at lower temperatures (Fig. 5). In general, the values obtained confirmed the good physiological condition of the cultures in the temperature and salinity ranges assayed.

Experiment 2.

As in experiment 1, significant coefficients from the equations had the capability to explain more than 91% of experimental variance.

Planozygote formation. Planozygote formation was promoted by increasing temperatures (T), decreasing salinity (S), and the interaction $T \times S$. After 18 days, the formation of diploids followed the equation: $R (\% \text{ of diploids}) = 4.10 - 3.07S + 2.93 T - 3.37 ST$. The mathematical simulation of this model is graphically described in Fig. 6 and a similar pattern to previous case is shown. Thus, the highest percentage of planozygotes was produced with the lowest salinity and the highest temperature tested. This finding is clearly defined by the gradient of colour represented in the contour plot.

DISCUSSION

Since early studies, nutrient deficiency was considered the only essential trigger to induce sexuality in dinoflagellate cultures, whereas other factors were usually thought to play secondary roles. However, field observations suggest that the real picture has to be more complex than this. Here, we present some evidence of this complexity; given that the most important result of the

present study is that other factors than nutrients are playing the main role inducing the shift from asexual to sexual reproduction and cyst formation in *Alexandrium minutum* cultures. Specifically, our work shows that low salinities, in the presence of an appropriate temperature and reduced phosphate levels, may constitute essential conditions for promoting the massive formation of planozygotes and resting cysts in natural populations of this species.

To begin with, it is important to underline the significance of the statistical approach used to get our results. The commonly used one-factor-at-a-time approach to study, describe and optimise a biological process is time consuming and ignores interactions-dependent effects amongst variables. Optimal conditions and quantitative joint effects of variables are difficult to obtain with one-way or two-ways analysis of variance (ANOVA), general linear models methodology or multiple regression analysis without controlled variance. However, a combination of relevant variables (and their interactions) affecting the response can be identified and evaluated through experimental design methodology also known as response surface methodology (RSM) (Box et al. 2005). Although little used in the field of the marine cultures (Planas et al. 2004; Paz et al. 2006), the empirical models obtained by means of orthogonal or rotatable factorial designs have proved to be very useful tools in the quantitative description of mainly microbial systems (for ex. Vázquez et al. 2003; Vázquez et al. 2004) as a function of environmental conditions or independent variables of the system.

Our experiments were conducted with a high cyst producer cross of *A. minutum* clones, which nonetheless produced no resting cyst at all under some of the studied conditions. We first studied if the initial level of nutrients were affecting planozygote formation. We knew that this factor was important to explain resting cyst appearance in *A. minutum* (Figuerola et al. 2007), but the behaviour of the planozygotes can be complex and methods triggering planozygote formation may also prevent encystment due to low nutritional reserves in the planozygotes (Anderson et al. 1985, Anderson 1998). It is also known that planozygotes of *A. minutum* divide instead of encysting under certain conditions (Figuerola et al. 2007). As it was found to be the case in

Chlamydomonas (Goodenough et al. 2007), in all dinoflagellates species studied thus far (Figuerola and Bravo 2005a,b; Figuerola et al. 2006a,b, Figuerola et al. 2007), planozygotes switch their fate from encystment to division when conditions suddenly change and once again become favorable for growth. In other cases, only a small percentage of the planozygotes were observed to undergo the transition to resting cyst (Anderson and Lindquist 1985, Anderson 1998, Nagai 2004, Figuerola et al. 2005), which argues that nutritional or density-related factors prevent the encystment of planozygotes, which then die or divide when either nutrients or the appropriate encystment signals (cell contact) are lacking (Anderson and Lindquist 1985, Uchida 2001).

In this sense, our results clearly indicate that the absence of phosphates plays a role in triggering the formation of *A. minutum* planozygotes, which is coincident with the culture conditions in which more cysts are also produced (Figuerola et al. 2007). From this result we may conclude that the present and previous works done with this species were not limiting the ability of planozygotes to encyst due to low nutritional levels.

But our main result is that using the same level of initial nutrients, either large amounts or no resting sexual cysts nor planozygotes were produced depending on the salinity and temperature values. This finding suggests that these latter two factors play major roles in promoting the sexual differentiation of natural populations of *A. minutum*. Salinity levels not favorable for resting-cyst formation were, however, conducive to vegetative growth and resulted in higher photosynthetic efficiency (Fv/Fm). This, in turn, implied that the non-encysting cultures were in good physiological condition. Therefore, it seems that there is a genuine shift in the behavior of the population, from asexual to sexual reproduction, under low salinity and high temperature conditions, whereas nutritional stress plays a secondary role in the encystment process (assuming that growing cultures consume more nutrients).

The relationship between salinity levels and growth has been studied in several species. For example, populations of *Heterocapsa circularisquama* are mainly found in areas exposed to changes in salinity, which suggests that salinity can be an important factor for growth and the

outbreak of a bloom (Leong et al. 2006). Also, freshwater inputs have been related to the enhancement (i.e. vegetative growth) of *A. fundyense* (Anderson et al. 2005) and *A. minutum* (see for example Bravo et al. 2009) blooms in the field, although this fact may be a secondary effect given that these inputs are associated to the entrance of macro- and micro- nutrients and the stratification of the water column (Anderson et al. 2005). This effect of nutrient loading in estuaries is for instance also crucial to blue-green algal bloom formation (Mallin 1994)

A switch to sexual reproduction in response to a decrease in salinity, e.g., because of an influx of freshwater from a river in an estuarine system, may be very useful to avoid cell dispersion. Under these conditions, the population can wait in the cyst form until vegetative growth is possible, before they revert to the vegetative stage and re-colonize the water column. Our study shows that high salinity favors growth, which is thus perfectly concordant with this hypothesis. Given that the natural habitat of *A. minutum* ranges from estuarine to marine environments, the present results may reflect a species-specific adaptation strategy.

However, the real scenario must be much more complex for several reasons. First, sexuality in dinoflagellates is thought to be likely favoured by stable environments that allow for gamete recognition and mating, a particularly low encystment being found in Erlenmeyer flasks when the container is shaken (Anderson and Lindquist 1985) or in big volume containers (Persson et al. 2008). Secondly, a faster response to dispersion would be the formation of pellicle (asexual) cysts, described for this species as for other *Alexandrium* species as a fast answer to rapid environmental changes in both cultures (Figueroa et al. 2007) and in the field (Bravo et al. 2009). However, no significant formation of temporary cysts was observed in our experiments.

Because resting cysts are designed to last longer than pellicle cysts, resting cysts are more commonly related to resistance to seasonal changes rather than to short-term fluctuations. Specifically, 1.5-2 months was the minimum dormancy period (i.e, the minimum time before which germination is possible) estimated for *A. minutum* by Garcés et al. (2004) and Figueroa et al. (2007), whereas pellicle cysts usually germinate in a couple of days (Bravo et al. 2009). If this

is the case, our results may suggest that the timing of mating could be regulated by a multitude of different signals that together constitute a “mating-season” signal (Persson et al. 2008), among which low salinity may play a leading, although not exclusive, role in the case of *A. minutum*.

It is interesting that the temperature range studied did not cause differences in growth whereas the higher temperatures tested did have a significant effect on the formation of planozygotes and cysts. This result contrast with the observed by Ichimi et al (2001) in a bloom of *A. tamarense*, a species reported to have both higher growth rates and transformed to cysts in higher numbers in low water temperatures (7.5-9°C). A decrease of temperature also increases cyst formation in *Lingulodinium polyedrum* (Hardeland 1994), but again, species-specific adaptation strategies may explain these different patterns.

Because nutrient depletion has been shown to be a critical factor in the encystment of dinoflagellates in culture, we could hypothesize that freshwater inputs and sexuality may be inversely related in nature. For example, nitrogen content and temperature were identified as the most important factors controlling the encystment of *Peridinium cinctum* (Grigorszky et al. 2006), and nutrient depletion has been shown to be essential to achieving significant levels of sexual induction in many other species (for ex. von Stosch 1973, Binder and Anderson 1987, Blackburn et al. 1989, Blanco 1995, Ellegaard et al. 1998, Sgroso et al. 2001). In agreement with these experiments, our preliminary results indicate that not only encystment, but also the very beginning of the sexual cycle, i.e. planozygote formation, is triggered by the lack of phosphates in culture. Accordingly, encystment in nature seems to be also triggered by lack of phosphates in *A. tamarense* (Ichimi et al. 2001).

However, our two factorial experiments clearly show that low salinities (as low as 18psu) and high temperatures (23 °C) trigger sexuality at both planozygote formation and encystment levels. The other factor tested, the density of the initial inoculum, may not play a relevant role in determining the size of the fraction of the population undergoing encystment.

We were interested in checking if the density of the initial inoculum was important to explain the success of mating due to an increase in the numbers of cell contact and chemical cues. This factor was suggested by Uchida (2001) as potentially important for the encystment of *Gyrodinium instriatum* planozygotes, which did not encyst when they were individually isolated to a medium with low number of cells. This observation suggests that a similar mechanism as the bacterial quorum sensing (ability to sense the presence of nearby cells) may be present in dinoflagellates (Persson et al. 2008), explaining for example, the active cell aggregation in *Alexandrium* (Wyatt and Jekinson 1997). In a similar way, *Alexandrium* resting cysts often appear forming clumps whereas big spaces of the culture plates remain empty with no obvious reason while many planozygotes are still swimming in the culture and never encyst (Figueroa et al. 2006). However, the effect of the inoculum, although significant for growth and cyst production, was not very important to explain cyst yield (i.e. cyst production when corrected for the number of cells present in the culture) in our experiments. If our initial hypothesis was right, given that the cultures with bigger initial inoculum produced more planozygotes, planozygote encystment should have been triggered. Because this effect was not observed, it may exist a minimum level of chemical signal for encystment already achieved in the lowest inoculum tried, and thus, an increment in the number of planozygotes will not result in a better cyst yield because no planozygotes were limited by this factor.

Summarizing, our results show that sexual-cyst formation by *A. minutum* is a complex response, one that likely involves the interaction of several environmental factors, specifically, phosphate depletion, temperature and, even more importantly, salinity.

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Figure legends:

Fig. 1. Flow cytometry diagram shows the percentage of planozygotes formed in a sexual cross of *Alexandrium minutum*, carried out following the method described by Figueroa et al. (2007), under nutrient limited (-P) conditions.

Fig. 2. Combined effect of temperature (T), salinity (S), and inoculum density (I) on the production of cells and cysts of *Alexandrium minutum* 18 days after culture inoculation (using as response R). Contour lines, defining the intervals of values of the response, are shown in the 2D-coloured graphs.

Fig. 3. Micrographs showing the vegetative (a) and resting stage (b) of *Alexandrium minutum*. Scale bars 10 μm .

Fig. 4. Combined effect of temperature (T), salinity (S), and inoculum density (I) on cyst yield (cysts. cells⁻¹) of *Alexandrium minutum* 18 days after culture inoculation (R). Contour lines, defining the intervals of values of the response, are shown in the 2D-coloured graphs.

Fig. 5. Photosynthetic efficiency (Fv/Fm) at different temperatures and salinities depending on the sampling day. Mean values and standard deviation.

Fig. 6. Combined effect of temperature (T) and salinity (S) in the percentage of *Alexandrium minutum* diploids formed 18 days after culture inoculation (R). Contour lines, defining the intervals of values of the response, are shown in the 2D-coloured graphs.

TABLES

Table 1: Statistical calculation of sum of squares, degrees of freedom and mean squares for ANOVA analysis (Fisher's F -test).

	Sum of squares	Degrees of freedom
Model (SSM)	$\sum (R_i^m - \bar{R})^2$	$p_s - 1$
Error (SSE)	$\sum (R_i - R_i^m)^2$	$n - p_s$
Experimental error ($SSEe$)	$\sum (R_i - \bar{R}_i)^2$	$n_0 - 1$
Lack of fit ($SSLF$)	$\sum (\bar{R}_i - R_i^m)^2$	$n - p_s - (n_0 - 1)$
Total (SST)	$\sum (R_i - \bar{R})^2$	$n - 1$
	Mean squares	
Model (MSM)	$\sum (R_i^m - \bar{R})^2 / (p_s - 1)$	
Error (MSE)	$\sum (R_i - R_i^m)^2 / (n - p_s)$	
Experimental error ($MSEe$)	$\sum (R_i - \bar{R}_i)^2 / (n_0 - 1)$	
Lack of fit ($MSLF$)	$\sum (\bar{R}_i - R_i^m)^2 / [n - p_s - (n_0 - 1)]$	
Model + Lack of fit ($MSMLF$)	$\frac{\sum (R_i^m - \bar{R})^2 + \sum (\bar{R}_i - R_i^m)^2}{(n - n_0)}$	
<p>n is the total number of data. Initially, they are distributed in g-groups of replicates, with $n_1, n_2, \dots, n_g$ replicates per group ($n_1 + n_2 + \dots + n_g = n$). If replicates are only carried out in the center of domain (n_0), it is obtained that $n - g = n_0 - 1$; p_s is the number of significant parameters from the mathematical model. R_i are the experimental observations; R_i^m are the predicted values from the proposed model; $\bar{R}$ is the total average of the n-observations, $\bar{R}_i$ is the average of the replicates of each group. The isolated observations are like an average with null number of degrees of freedom.</p>		

Table 2: Experimental domain and codification of independent variables in factorial experiment (first-order design).

Coded values	Natural values in °C (<i>T</i>), psu (<i>S</i>) and cells mL ⁻¹ (<i>I</i>)		
	<i>T</i>	<i>S</i>	<i>I</i>
-1	15	18	300
0	19	27	500
+1	23	36	700

Codification: $V_c = (V_n - V_0) / \Delta V_n$; Decodification: $V_n = V_0 + (\Delta V_n \times V_c)$
 V_n =natural value in the center of the domain; ΔV_n = increment of V_n per unit of V_c .

Table 3: Results of factorial design and tests of significance for model ($R=14304+3535S+5844I+2054TS$). R : values of global production of cells (cells mL ⁻¹) and cysts at 18 days; RM : values of global production of cells (cells+2×cysts) and cysts estimated from the above equation; NS: not significant coefficient; SS: sum of squares; ν : degrees of freedom; MS: mean squares; MSM: mean squares model; MSE: mean squares for error; MSMLF: mean squares model for lack of fit; MSEe: mean squares for experimental error.								
T	S	I	R	RM	Coefficients	t	p -value	Model
-1	-1	-1	6439	6978	14303.6	43.0	0.000028	14304
-1	1	-1	9742	9941	1039.5	2.6	0.083650	NS T
-1	-1	1	17507	18666	3535.5	8.7	0.003211	3535 S
-1	1	1	20130	21629	5843.8	14.4	0.000732	5844 I
1	-1	-1	5176	2870	2054.0	5.1	0.015006	2054 TS
1	1	-1	13244	14049	479.8	1.2	0.323484	NS TI
1	-1	1	14712	14558	692.8	1.7	0.187298	NS SI
1	1	1	29002	25737	862.8	2.1	0.124213	NS TSI
0	0	0	13748	14304	Average value			14303.64
0	0	0	14173	14304	Expected average value			13922.92
0	0	0	15278	14304	Var(Ee)			1325236.93
0	0	0	12493	14304	t($\alpha<0.05$; $\nu=3$)			3.18
SS ν MS					MSM/MSE= 43.19		F_8^3 ($\alpha=0.05$)= 4.07	
Model	406944722.5	3	135648240.8		MSMLF/MSM= 0.39		F_3^8 ($\alpha=0.05$)= 8.85	
Error	25125069.7	8	3140633.7		MSE/MSEe= 2.37		F_3^8 ($\alpha=0.05$)= 8.85	
Exp. Error	3975710.8	3	1325236.9		MSLF/MSEe= 3.19		F_3^5 ($\alpha=0.05$)= 9.01	
Lack of fit	21149359.0	5	4229871.8				$r^2=$ 0.942	
Total	432069792.2	11					r^2 adjusted= 0.920	

Table 4: Experimental domain and codification of independent variables in the second factorial experiment (first-order design).

Coded values	Natural values in °C (T) and psu (S)	
	T	S
-1	15	18
0	19	27
+1	23	36

Codification: $V_c = (V_n - V_0) / \Delta V_n$; Decodification:
 $V_n = V_0 + (\Delta V_n \times V_c)$
 V_n = natural value in the center of the domain;
 ΔV_n = increment of V_n per unit of V_c .

Table 5: Results of factorial design and tests of significance for model ($R=747-507S+456T-535ST$). R : values of diploids concentration at 18 days; RM : values of diploids concentration estimated from the above equation; NS: not significant coefficient; SS: sum of squares; ν : degrees of freedom; MS: mean squares; MSM: mean squares model; MSE: mean squares for error; MSMLF: mean squares model for lack of fit; MSEe: mean squares for experimental error.							
S	T	R	RM	Coefficients	t	p -value	Model
-1	-1	404	262.6	747	16.5	0.00049	747
-1	1	2387	2245.3	-507	7.9	0.00422	-507 S
1	-1	462	320.1	456	7.1	0.00572	456 T
1	1	303	160.9	-535	8.4	0.00360	-535 ST
0	0	603	747.2	Average value		747.2	
0	0	505	747.2	Expected average value		605.5	
0	0	787	747.2	Var(Ee)		16435	
0	0	527	747.2	$t(\alpha<0.05; \nu=3)$		3.1824	
SS		ν	MS	MSM/MSE= 19.09		$F_4^3 (\alpha=0.05)= 6.59$	
Model	3005149	3	1001716	MSMLF/MSM= 0.790		$F_3^4 (\alpha=0.05)= 9.12$	
Error	209905	4	52476	MSE/MSEe= 3.19		$F_3^4 (\alpha=0.05)= 9.12$	
Exp. Error	49305	3	16435	MSLF/MSEe= 9.77		$F_3^1 (\alpha=0.05)= 10.13$	
Lack of fit	160600	1	160600	$r^2= 0.935$			
Total	3215054	7		r^2 adjusted= 0.886			

Table 6: Percentage (%) of planozygotes formed 3, 4 and 5 days after crossing the parental *A. minutum* strains (650x651) in media with different nutritional modifications.

* Days after the sexual cross. CV: coefficient of variation.

Media	Planozygotes (%)					
	Day* 3	CV	Day*4	CV	Day*5	CV
L-P (n=6)	70	7.1	77	8.2	78	7.0
L-N (n=6)	62	7.9	61	8.3	65	8.7
L/20 (n=6)	41	6.8	46	7.3	32	7.2

FIGURES

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5

Figure 6