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The dynamics of sexual phase in the marine diatom *Pseudo-nitzschia multistriata* (Bacillariophyceae)

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Abstract

Sexual reproduction represents a fundamental phase in the life cycle of diatoms, linked to both the production of genotypic diversity and the formation of large-sized initial cells. Only cells below a certain size threshold can be sexualized, but various environmental factors can modulate the success of sexual reproduction. We investigated the role of cell density and physiological conditions of parental strains in affecting the success and timing of sexual reproduction in the marine heterothallic diatom *Pseudo-nitzschia multistriata*. We also studied the dynamics of the sexual phase in still conditions allowing cell sedimentation and in gently mixed conditions that keep cells in suspension. Our results showed that successful sexual reproduction can only be achieved when crossing parental strains in the exponential growth phase. Evidence was provided for the fact that sexual reproduction is a density-dependent event and requires a threshold cell concentration to start, although this might vary considerably amongst strains. Moreover, the onset of the sexual phase was coupled to a marked reduction of growth of the vegetative parental cells. The crosses carried out in physically mixed conditions produced a significantly reduced number of sexual stages as compared to crosses in still conditions, showing that mixing impairs sexualisation. The results of our experiments suggest that the signalling that triggers the sexual phase is favoured when cells can accumulate, reducing the distance between them and facilitating contacts and/or the perception of chemical cues. Information on the progression of the sexual phase in laboratory conditions help understanding the conditions at which sex occurs in the natural environment.

Key words:

Density-dependent; diatoms; heterothallic; mixing; *Pseudo-nitzschia multistriata;* sexual reproduction.

Introduction

Sexual reproduction represents a fundamental phase in the life cycle of diatoms: it provides genotypic diversity through meiotic recombination occurring when gametes are produced (Tesson et al. 2013), and restores the maximum size through the formation of a specialized zygote, called the auxospore (Chepurnov et al. 2004). In fact, diatoms have a distinctive life cycle characterized by a progressive cell size reduction as vegetative division proceeds. This is due to the architecture of diatom cells that are surrounded by a rigid siliceous cell wall constituted by two unequal halves fitting together as a box and its lid. Upon mitotic division, the new hypovalves of the daughter cells are synthesized within the mother cell, thus producing one cell with the size of the mother cell and a slightly smaller one (Round et al. 1990).

In the ancestral centric diatoms, male (motile sperm) and female (immotile egg) gametes can be produced within the same clonal strain (homothallic life cycle), whereas in the vast majority of pennate diatoms strains of opposite mating type have to be co-cultured to induce the sexual phase (heterothallic life cycle). The sexual phase can be induced only in cells below a species-specific cell size threshold (Chepurnov et al. 2004). However, the reach of a critical cell size threshold is an obligate requirement but it is not the sole factor necessary for the induction of sex, because external cues can further regulate the process. Changes in salinity, light quantity and quality, or shifts in the composition of the growth medium, have been shown to induce sexualisation in centric diatoms (e.g., Schultz and Trainor 1968, Drebes 1977, Schmid 1995, Godhe et al. 2014), while the mixing of two sexually compatible strains seems to be sufficient for the induction of sexuality in heterothallic pennate diatoms (e.g., Davidovich and Bates 1998, Amato et al. 2005, Mann and Pouličkovà 2010, Fuchs et al. 2013). Nevertheless, the success of the sexual phase in terms of production of gametes, auxospores and initial cells can be tuned by daylength, irradiance or temperature in pennate diatoms, and the effect of these environmental factors varies considerably amongst species (Mizuno

and Okuda 1985, Davidovich 1998, Hiltz et al. 2000, Mouget et al. 2009). To our knowledge, little or no information is available on the link between cell concentration and the onset of the sexual phase in planktonic diatoms and generally in other unicellular microalgae (e.g., Sandgren and Flanagin 1986 in Chrysophyceae).

A density dependent mechanism could explain the production of sexual pheromones recently reported for two benthic diatoms (Sato et al. 2011, Gillard et al. 2013). In the araphid pennate *Pseudostaurosira trainorii*, sexualization is a two-step process in which female cells secrete a sex pheromone that induces the formation of gametes in the male strain, which in turn, secretes a sex-pheromone that induces gametogenesis in the female strain. A sex pheromone has been identified in *Seminavis robusta,* where MT^- cells release l-diproline that is capable of attracting MT^+ cells (Gillard et al. 2013).

Most of our knowledge on life cycle features of diatoms derives from detailed observation of strains in laboratory conditions and from observations carried out on natural population of freshwater, mostly benthic, species (reviewed in Edlund and Stoermer 1997, Chepurnov et al. 2004). In fact, notwithstanding the key-role of sexual reproduction in the life cycle of diatoms, there are only a handful of reports of sexual stages for planktonic species in the marine environment (Crawford 1995, Assmy et al. 2006, Holtermann et al. 2010, Sarno et al. 2010). These findings have been gained in markedly different environmental conditions, spanning from open and well mixed waters in the Southern Ocean (Crawford 1995, Assmy et al. 2006), to the surface layer of coastal waters (Sarno et al. 2010), to the very shallow surf zone (Holtermann et al. 2010). The frequency, success and environmental conditions that might regulate the occurrence of sexual reproduction are important factors to consider for explaining and eventually modelling population dynamics, genetic

structure and persistence of diatom species in the natural environment. As an example, if the conditions for sex are not met for consecutive years, a heterothallic diatom species might risk local extinction due to the fact that the population is not rejuvenated by the formation of large-sized cells (D'Alelio et al. 2010).

We present the results of experiments aimed at elucidating the dynamics of sexual reproduction in the marine planktonic diatom *Pseudo-nitzschia multistriata* (Takano) Takano. This species has been recorded in different coastal sites worldwide (reviewed in Lelong et al. 2012) and is capable of producing the neurotoxin domoic acid (Orsini et al. 2002). *Pseudo-nitzschia multistriata*, as the vast majority of congeneric species (Lelong et al. 2012), has a heterothallic life cycle and sexual reproduction is induced when cells are below 55 µm in apical length (D'Alelio et al. 2009). We tested the role of physiological conditions and the density of parental strains in affecting the success and timing of sexual reproduction. We also assessed the success and timing of sexual reproduction in time course experiments carried out in still conditions and under gentle constant mixing, with the hypothesis that physical mixing would impair the sexual phase by increasing the distance between cells.

Material and methods

Culture isolation and maintenance

Single cells or short chains of *Pseudo-nitzschia multistriata* were isolated with a micropipette from net samples collected at the Long-Term Ecological Research station Mare Chiara (LTER-MC) in the Gulf of Naples, Mediterranean Sea (40°47' 33"N, 14°11' 18"E; Table S1 in the Supporting Information). The cultures were grown in f/2 culture medium (Guillard 1975) at a temperature of 18°C, a photoperiod of 12:12 h L:D, and a photon flux density of 60 µmol photons $\cdot$ m^{-2} $\cdot$ s^{-1} provided

by cool white fluorescent tubes (Philips TLD 36W/950). Before carrying out each experiment, the apical axis of 20 cells per strain were measured at 400x magnification using a Zeiss Axiophot light microscope (Carl Zeiss, Oberkochen, Germany) equipped with an ocular micrometre.

Crosses of parental strains at different cell densities and growth phases

Experiments were carried out with different pairs of strains of compatible mating type (Table S1). The mating type of the strains used for the experiments was assessed by crossing them with reference strains of known mating type. The strain that was bearing the auxospores was defined 'Pm– ', i.e., female, and the other one 'Pm+', i.e., male.

A first experiment (Experiment #1) was aimed at estimating the timing and success of sexual reproduction in crosses carried out with strains at different growth phases and cell densities (Fig. S1 in the Supporting Information). Two flasks, one per parental strain, containing 700 mL of f/2 medium were inoculated with cells at a final concentration of about 300 cells · mL^{-1} and placed at a temperature of 18°C, a photoperiod of 12:12 h L:D and an irradiance of 110 µmol photons · m^{-2} · s^{-1}. Cell concentration of the two parental strains was estimated on day 0 (T0), 2 (T2), 4 (T4), 5 (T5), 6 (T6) and 7 (T7). At each time point, 50 mL of culture for each parental strain were mixed in a flask and aliquots of 4 mL were dispensed, after careful mixing, in two 6-wells culture plates (Fig. S1a). Plates were incubated at 18°C, a photoperiod of 12L:12D h and an irradiance of 60 µmol photons · m^{-2} · s^{-1}. Culture plates were inspected every day at an inverted microscope to check for the presence of gametes. Starting from the day at which gametes were first observed, and for four consecutive days, the content of three wells was fixed with formaldehyde solution at a final concentration of 1.6%. One mL of each triplicate was placed in a Sedgewick-Rafter counting slide and vegetative cells, gametes or zygotes (the two stages cannot always be differentiated in light microscopy), auxospores,

initial cells (large cells still surrounded by the perizonium), and F1 generation cells (long vegetative cells) were enumerated. A control was run with the parental strains in monoculture. Each strain was inoculated in a 500 mL Costar culture flask containing 200 mL of f/2 to reach a concentration of about 300 cells · mL^{-1}. Four mL aliquots were dispensed into 6-wells culture plates and grown at the experimental temperature and light conditions illustrated above for the crosses. Cell concentration was estimated every two days as illustrated above.

The highest percentage of sexual stages was obtained in crosses started with parental strains in exponential growth phase, at a cell concentration of about 5,000 cells · mL^{-1}. A second experiment was designed to test if parental strains in late- or post-exponential phase could become again competent for sex (Experiment #2; Table S1, Fig. S1b). The set up was the same as Experiment #1, with the only difference that parental strains collected at times 4 (T4), 5 (T5), 6 (T6), and 7 (T7) of the growth curve were diluted back, when co-cultures were started, to about 5000 cells · mL^{-1} with fresh f/2 medium. A third experiment was conducted to assess the concentration of parental strains at which sexual stages were first produced in co-cultures started from exponentially growing parental strains collected at the same time point of their growth curve (Experiment #3; Table S1). The experimental set up used for Experiments # 1 and 2 was followed, with the difference that parental strains were collected on day 3, when in exponential growth phase, centrifuged at 1,200 r.p.m. and re-suspended in 15 mL of medium. This concentrated stock was used to inoculate a series of culture plates at cell concentration (values for both parental strains) of 400 (1), 4000 (2), 6,000 (3), 8,000 (4) cells · mL^{-1}. The concentration of vegetative parental cells, gametes/zygotes, auxospores and initial cells was monitored every day until the formation of initial cells was confirmed. To estimate growth rate of the individual parental strains and nutrient (NO_3, NO_2, NH_4, PO_4 and SiO_4) concentration, one flask for each parental strain, filled with 500 mL of f/2 medium, was inoculated with cells from the concentrated stock at a final concentration of 300 cells · mL^{-1}. Every

two days, 4 mL of culture were sub-sampled in duplicate and used to estimate cell concentration. On the same days, 20 mL of culture were sub-sampled in duplicate, filtered through a 0.22 µm pore size MILLEX-GS filter unit and stored at -20°C until the analysis. Nutrient concentration was analyzed with a Systea Flowsys autoanalyzer (AxFlow) equipped with five continuous flux channels, following Hansen and Grasshoff (1983).

In order to rule out the possibility that the change in growth dynamics observed between the mono-cultures of parental stains and the co-cultures undergoing sexual reproduction could depend on the interaction between different strains independently from their mating type, we tested the growth curve of co-cultures of *P. multistriata* strains of the same mating type (Experiment #4, Table S1). We used strains of different cell size to distinguish them when co-cultured. One culture flasks was inoculated with 150 cells · mL^{-1} for each Pm+ strain and the other with the same concentration of Pm- strains. Subsamples of 4 mL were placed in 6-well culture plates and incubated at the same experimental conditions illustrated above. Every two days and for a period of 10 days, the culture material of 2 wells for each couple were fixed with formaldehyde solution at a final concentration of 1.6 %. Cell concentration the two co-cultured strains was estimated using a Sedgewick Rafter counting slide.

Crosses carried out in still and mixed conditions

In this experiment (Experiment #5), parental strains differing in cell size were used to follow the growth and behaviour of the individual strains (Table S1). Two 500 mL flasks, one for each parental strain, were filled with 240 mL of f/2 medium and inoculated with cells at final concentration of about 3,000 cells · mL^{-1}. Aliquots of 30 mL were dispensed, after gentle mixing, in eight 70 mL culture flasks. The stock co-culture of the two parental strains was prepared in a flask filled with 800 mL of

f/2 filtered medium and inoculated with cells at final concentration of about 1,500 cells · mL^{-1} for each parental strain (3,000 cells · mL^{-1} in total). Aliquots of 30 mL were dispensed, after careful mixing, in 26 70 mL flasks. For each parental strain and for the crosses, half of the flasks were placed on a rotating wheel (RW) and the other half were placed on a shelf (SH). The rotating wheel and the shelf were located in a walk-in climatic chamber at a temperature of 18°C and a photoperiod of 12L:12D h. The integrated irradiance at which cultures were exposed on the rotating wheel was 60 µmol photons · m^{-2} · s^{-1} (110 µmol photons · m^{-2} · s^{-1} at the top; 35 µmol photons · m^{-2} · s^{-1} at the bottom), which was the same as the one on the shelf. The rotating wheel was set at a 0.1 rpm; this rotation velocity caused a gentle and constant mixing of the cultures.

To monitor the growth rate of the individual parental strains at the two different conditions (RW and SH), 2 mL of culture were sub-sampled from each parental strain every two days from one randomly selected flask on the rotating wheel and from one randomly selected flask on the shelf, they were placed in Eppendorf vials and fixed with formaldehyde at a final concentration of 1.6%. To monitor the growth rate and the production of sexual stages in the crosses, one flask from the RW and one from the SH were randomly collected every day. From each flask, two subsamples of 3.5 mL were fixed with formaldehyde solution at a final concentration of 1.6 % and stored at 4 °C.

For each parental strain, one mL of fixed culture was counted using a Sedgewick-Rafter counting slide and live vegetative cells (cells with cytoplasm content) were enumerated using an Axiophot light microscope. For the cross samples, one mL of fixed culture, in triplicate, was placed in a Sedgewick-Rafter counting slide and the following stages were enumerated: live vegetative cells (cells with cytoplasm content), gametes/zygotes, auxospores, initial cells, and large F1 generation cells. Growth rate, expressed as divisions · d^{-1}, was estimated by calculating linear regression over

the exponential portion of the curve (Guillard 1980). Statistical significance between the different treatments was estimated by using a Student's t-test.

The theoretical distance (D) between cells in flasks incubated on the rotating wheel was calculated applying the formula (1) and assuming that cell distribution in the flask was homogeneous:

(1) $D = \{1\ \mu m^3 – [v * (N* 30\ mL) / V]\} / [(N* 30\ mL)/ V]$

where N = cell concentration (cells · mL^{-1}); v = average volume of a single cell; V = volume of all cells in the 30 mL sample.

The theoretical distance (D) between cells incubated on the shelf was calculated applying the formula (2), assuming that cells were sinking in a thin layer at the bottom of the flask. This layer was considered 10 µm-high and estimates were calculated assuming variable percentages of sinking cells, i.e. 80%, 60% and 40% of the total, respectively.

(2) $D = \{1\ \mu m^3 – [v * (N* 30\ mL * (\%)) / V]\} / [(N* 30\ l * (\%))/ V]$

Where (%) = percentage of sinking cells

Results

The physiological state and the density of parental strains affect the success of sexual reproduction

To define whether the occurrence and success of sexual reproduction in *Pseudo-nitzschia multistriata* could be dependent on the physiological state of the cells, we carried out crosses with parental strains collected at different time points of the growth curve, from day 0 (T0) to day 7 (T7) (Experiment #1, Fig. S1). Sexual reproduction occurred in all crosses but with variable timing and success (Fig. 1). In *P. multistriata,* the sexual phase starts with the pairing of cells of the opposite mating type along their longitudinal axis (Fig. S2a in the Supporting Information). Each gametangium produces two gametes (Fig. S2b). Upon fertilization of the Pm- gametes by the Pm+ ones, two auxospores (the zygotes) develop attached to the empty frustule of the Pm- gametangium (Fig. S2c). A large-size initial cell is produced within each auxospore (Fig. S2d). In the very initial phase of development, auxospores are round and can be confused with gametes. Because it was not always possible to discriminate between gametes and early-stage auxospores (the zygotes), we reported their sum in our graphs. The average maximum percentage of gametes and early stage zygotes was comprised between 13.5% ± 4.37 and 28.1% ± 3.01 st.dev in crosses carried out with parental strains in the exponential growth phase (i.e., before day 5), when the inoculum concentration was comprised between 300 and 100,000 cells · mL^{-1} (Fig. 1a). In crosses started with parental strains in post exponential or stationary growth phase, i.e., on day 6 and 7, the percentage of gametes and early stage auxospores decreased considerably to 4.3% ± 1.63 and 1.2% ± 0.29 st.dev., respectively (Fig. 1a). Gametes were first recorded on day 6 in crosses started at T0, and their timing of appearance gradually decreased in the crosses carried out in the following days, which were started with progressively higher cell concentrations. In crosses started on T2, gametes were detected on day 4 and in all the other crosses on day 3. In crosses carried out from T0 to T4, sexual reproduction was successful. Auxospores and large initial cells were produced with percentages ranging between 6.3% ± 0.46 and 23.6% ± 2.28 st.dev (Fig. 1b). Very few auxospores and no initial cells were instead

retrieved in the crosses started with parental strains inoculated in the late and post-exponential growth phase (Fig. 1b). No sexual stages were observed in the control test, where parental strains were grown in monoculture.

A second experiment was carried out with a similar set up but, starting from the inoculum carried out on day 4 (T4), the co-cultures of parental strains were diluted to < 5,000 cells · mL^{-1} in order to bring them in exponential growth (Experiment #2, Fig. S1). The maximum percentage of gametes and early stage zygotes produced in all crosses carried out between T0 and T7 was very similar (Student's t-test: t_3 = 6.2 to 7.6 for the 15 combinations, $p > 0.1$) and was ranging between 7.6% ± 5.84 and 14.1% ± 1.99 st.dev. (Fig. 2a). Sexual reproduction was successful in all crosses, where auxospores and initial cells were produced in percentages spanning from 5.6% ± 1.20 to 12.5% ± 4.02 st.dev (Fig. 2b). Gametes were first recorded on day 6 in the crosses started at T0, on day 4 in those started at T2 and on the third day in all the other crosses. These results indicate that cells have to reach a minimum concentration before they can enter the sexual phase; the cell density at which gametes were first observed in Experiment #2 spanned between 2,989 ± 619 and 5,150 ± 220 cells · mL^{-1} (Fig. 3).

Experiment #3, conducted with parental strains collected at the same time point of the growth phase, was carried out with a different pair of strains that produced a lower number of sexual stages as compared to the previous experiments. Also in this experiment, sexual stages were produced with a different timing, when cell concentration was at about 8,000 cells · mL^{-1} (Fig. S3 in the Supporting Information). Gamete production lasted for 2-3 days and was followed by the formation of auxospores and initial cells (Fig. S3). The maximum percentage of gametes and early stage zygotes was comprised between 1.4 and 15.25%.

In Experiment #3, nutrient concentration along the growth curve of the parental strains was measured (Table S2 in the Supporting Information). The average nutrient consumption over the exponential growth phase was $4.2 \cdot 10^{-4}$ µmol · cell^{-1} for total nitrogen, $1.0 \cdot 10^{-4}$ µmol · cell^{-1} for phosphate, and $0{,}0003.3 \cdot 10^{-4}$ µmol · cell^{-1} for silicate. Applying these values to the cross experiments started with parental cell inocula of different concentration (Fig. S3), the amount of nutrients consumed over the duration of the experiment ranged between 20.52 and 22.62 µmol · L^{-1} for total nitrogen, between 4.83 and 5.33 µmol · L^{-1} for phosphate and between 16.21 and 17.87 µmol · L^{-1} for silicate, thus showing that nutrient limitation was not occurring.

Vegetative growth is reduced when sexual reproduction takes place The two parental strains grown in monoculture reached similar maximum cell concentration (121,420 ± 4,900 cells · mL^{-1} for the Pm+ strain and 131,562 ± 562 cells · mL^{-1} for the Pm- strain), with maximum growth rates of 1.52 and 1.50 divisions·d^{-1} for the Pm+ and Pm- strains, respectively (Fig. 3). When comparing the time course of cell concentration of monocultures of parental strains with that of cells and sexual stages recorded in the crosses performed in Experiment #2, a much lower cell number was detected in the latter experimental set up, suggesting that an arrest of cell growth occurred in concomitance with the sexual phase. Crosses started with a low inoculum of parental cells (experiments at T0 and T2, Fig. 3a) had an increase in cell numbers up to the day in which gametes were first recorded, but the number of cells leveled out in the subsequent three days in which their concentration was monitored. The formation of sexual stages does not account for the observed lack of increase in cell number, since they represented only 3-20% of the total (Fig. 2, a and b). In the crosses started with higher cell concentration, when gametes were recorded on the third day, a similar trend was observed (Fig. 3, b and c). Vegetative growth was observed only up to the day in which sex started and then cell number did not increase at a rate comparable to that recorded in the clonal strains. A similar trend was observed also in Experiment #3, where co-cultures at different concentration of

parental strains were started with inocula collected at the same time point of the growth curve (Fig. S4 in the Supporting Information).

We tested the growth curve of co-cultures of *P. multistriata* strains of the same mating type, to rule out the possibility that the change in growth dynamics could depend from the interaction between different strains, independently from their mating type (Experiment #4). To this end, we used strains of different cell size, so to distinguish them when co-cultured. The two pairs of co-cultured strains of identical mating type had similar growth curves and growth rates (0.99 and 0.9 divisions·day^{-1} for the two Pm- strains and 1.33 and 1.49 divisions · d^{-1} for the two Pm+ ones), thus showing that there was no interaction between them (Fig. 4).

Mixing affects cell growth and patterns of sexual reproduction

The results of the previous experiments showed that a threshold concentration has to be reached in order to allow the onset of sex in the planktonic species *P. multistriata*. We thus designed an experiment in which the time course of parental cells and sexual stages was monitored in two different settings that reproduce i) a condition in which cells are allowed to aggregate and/or sink and ii) a condition in which cells are kept in continuous suspension. In the latter setting, a set of replicate bottles with either parental strains in monoculture or crosses was incubated on a rotating wheel that kept the cultures in constant slow mixing, while a second set of replicate bottles was incubated on a shelf, without mixing. In this experiment we used parental strains of different cell size, so to monitor cell concentration of the two mating types in co-culture over time.

When co-cultures were started, parental strains showed a decrease in cell concentration during the first two days, more marked in the rotating wheel set up (Fig. 5). In the latter experimental condition, the Pm- strain grew exponentially from day 2 to day 8 (max growth rate 1.56 divisions · d^{-1} ± 0.12 st.dev.) reaching maximum cell concentration 543,613 ± 67,363 st.dev cells · mL^{-1} on day 10. The Pm+ strain showed a lower growth rate (1.15 divisions · d^{-1} ± 0.11 st.dev from day 2 to day 8) and reached a much lower cell concentration on day 10 (an average of 34,293 cells · mL^{-1}). In this set up, gametes were first recorded on the second day, auxospores on the third day, albeit at very low concentration (from an average of 20.6 to 4.1 cells · mL^{-1} on day 2 and 5, respectively) corresponding to extremely low percentages (0.04% and 5.2% over the total number of cells, Fig. 5a and Fig. S5a). Gamete production was followed by their transformation into auxospores. Initial cells, i.e. cells of the maximum size and still wrapped in the perizonium, were observed on day 4 and 5 (Fig. S5a in the Supporting Information). Large F1 cells increased their number exponentially (maximum growth rate 1.79 divisions · d^{-1} ± 0.14) till day 8, reaching a maximum average concentration of > 14,000 cells · mL^{-1} on day 10 (Fig. 5a).

In the co-cultures incubated on the shelf, a condition in which cells tend to sink and aggregate at the bottom of the flasks, the Pm- strain grew exponentially (max growth rate 0. 81 divisions · d^{-1} ± 0.07 st.dev) until day 8 and reached an average maximum cell concentration of 58,157 cells·mL^{-1} on day 12, at the end of the observation period (Fig. 5b). Also in this experimental set up, both maximum growth rate (0.57 divisions·day^{-1} ± 0.03 st.dev) and maximum cell concentration (5,051 divisions · d^{-1} ± 634 st.dev) of the Pm+ strain on day 7 were lower as compared to those of the Pm- strain. Gametes and auxospores were found starting from the 2nd day while initial cells were observed from day 3 until day 6 (Fig. S5b). The total number of sexual stages, i.e. gametes, auxospores and initial cells was higher as compared to the rotating wheel and reached values of 450 cells · mL^{-1} ± 218 st.dev, corresponding to an average percentage of 5.4% ± 2.5 over the

total number of cells on day 6. Large F1 cells appeared on day 2, increased their number exponentially until day 7 and reached a maximum cell concentration of 10,533 cells · mL^{-1} ± 1,290 st.dev on day 12 (Fig. 5b).

The two parental strains grown in monoculture showed a different growth dynamics. Cell concentration of the small Pm+ strain peaked on day 8 and maximum growth rate was 1.01 and 0.79 div•day^{-1} on the rotating wheel and in still conditions, respectively (Fig. 6). The larger Pm- strain reached the maximum cell concentration on day 4 in both conditions with maximum growth rate of 1.69 and 1.99 divisions · d^{-1} on mixed and still conditions, respectively (Fig. 6). When comparing the growth curves of the parental strains in mono-culture with the growth curve of vegetative cells and sexual stages in co-culture, it is evident that, also in this experiment, the growth dynamics was different (Fig. 6). This difference is particularly noticeable in the experiment carried out in still conditions, over the time interval in which sexual stages were produced.

We have estimated the theoretical distance between cells (Pm+, Pm- and F1 generation when present) in mixed and in still conditions assuming – in this latter setting – that either 40% or 60% of cells sink on the bottom of the culture flask, in a thin layer 10 µm high. The difference in distance was significant (Fig. S6 in the Supporting Information; Student's t-test: 40%, t_{18}=26.3, $p < 0.00005$; 60%, t_{18}=11.6, $p < 0.00005$). On the second day after the inoculum, when the first sexual stages were observed, the theoretical distance between parental cells was 1,354.3 µm ± 87.3 st.dev. in mixed conditions, while it was much lower (113 µm ± 6.3 st.dev.) in still conditions.

Discussion

The experiments carried out to elucidate the time-course of sexual reproduction in the marine planktonic diatom *Pseudo-nitzschia multistriata* provided novel and interesting results on the conditions at which sex occurs and on the progression of the sexual phase in laboratory conditions. Sexual reproduction is density-dependent and requires a threshold cell concentration to occur. The formation of sexual stages is reduced in mixed conditions, indicating that the cell-cell signalling that triggers the sexual phase is favoured when cells can accumulate, reducing the distance between them and facilitating contacts and/or the perception of chemical cues. We also provided experimental evidence for the fact that successful sexual reproduction can only be achieved when parental strains are in the exponential growth phase. Finally, we showed that the onset of the sexual phase is coupled to a significant reduction of growth of the vegetative parental cells.

Short-term dynamics

Although the mode of sexual reproduction has been studied in many pennate diatoms (reviewed by Chepurnov et al. 2004), detailed quantitative information on the time course progression of the sexual phase has been reported only for a few species (Gillard et al. 2013, Vanormelingen et al. 2013). Prolonged incubation in the dark, a treatment that in several species has been shown to synchronise cell cycle (e,g, Brzezinski et al. 1990, Gillard et al. 2008), was effective in synchronizing the progression of the sexual phase of both *Seminavis robusta* (Gillard et al. 2013) and *Cylindrotheca closterium* (Vanormelingen et al. 2013), with the formation of high percentages of gametes within a short time interval. In *S. robusta,* DNA quantification proved that, after a prolonged dark treatment, cells were arrested in the G1 phase of the cell cycle (Gillard et al. 2008), a phase in which cells are prone to the reception of the pheromone that induces the attraction of cells of the opposite mating type (Gillard et al. 2013). In both *S. robusta* and *C. closterium* higher sexualization percentages were

reported as compared to the values recorded for *P. multistriata.* This difference might be due to the fact that we did not apply a prolonged dark treatment to synchronize the cells, but carried out our experiments under the same photoperiod conditions – 12:12 h light:dark - at which strains were routinely maintained in culture. However, preliminary experiments suggest that a prolonged dark treatment, while effective in blocking cells in G1 phase, is not effective in significantly increasing gamete production in *P. multistriata* (M. I. Ferrante et al. in preparation). In *P. multistriata* we do not observe an obvious attractive behaviour of one strain upon the other, rather cells from both mating types move actively and explore the environment until they find a cell to pair with (E. Scalco, unpublished data). These observations suggest that differences between the two species might be substantial, and this makes sense considering the different habits –benthic and planktonic, respectively – of the two species.

Gametogenesis appears as a rather synchronous process in *P. multistriata* and gametes are detected in the co-cultures only for a couple of days and immediately followed by the formation of auxospores and large initial cells. In the dark-synchronized *C. closterium,* a massive gamete production was largely confined within the first 14 hours after re-exposure to the light cycle (Vanormelingen et al. 2013) and a synchronous production of auxospores was observed also in the centric diatom *Skeletonema marinoi* within 3-4 days from the application of the environmental cue inducing gametogenesis (Godhe et al. 2014). This suggests that the production of gametes is linked to the perception of a chemical cue that synchronizes the process once cells of opposite mating type perceive each other.

An interesting result of our experiments was the different growth dynamics observed in monocultures of single strains and in co-cultures of strains of opposite mating type in which sexual

reproduction was in progress. In the latter conditions, the progression of sexual reproduction was coupled to a decrease in vegetative growth. This was evident in both the experiments carried out in small-volume culture plates (Experiments #2 and #3) and when comparing the growth dynamics of monocultures of the individual parental strains and the crosses in Experiment #5 (comparison between still and mixed conditions). In this latter experiment, where significantly (Student's t-test: $t_3 = 23$, $p < 0.001$) higher growth rates of parental strains were detected in the co-cultures with extremely low percentages of sexual stages, i.e., in the mixed condition. This result, together with the more marked reduction of growth in the co-cultures of Experiment #2, might suggest that the reduction of growth is proportional to the percentage of gamete formation. The arrest of the cell cycle in correspondence with the onset of the sexual phase mediated by sex pheromones has been reported for unicellular fungi (Bardwell 2004, Cote and Whiteway 2008) and the finding of a similar response in *P. multistriata* could indicate that a similar signalling mechanism also occurs in diatoms. This endogenous control of cell growth linked to the interaction of cells of different mating type might play an important role in regulating bloom dynamics in the natural environment. Phytoplankton blooms are accumulation of biomass due to rapid asexual cell division. The increase in cell concentration will facilitate encounter rates and/or the perception of chemical signals between cells. If gametogenesis – induced during exponential growth – is accompanied by a reduction of growth of the parental cells, the growth dynamics of the population will be negatively impaired. This peculiar aspect of diatom life cycle, might in fact further amplify the 'cost of sex' for these unicellular microalgae (Lewis 1983), which would not only be due to the investment of biomass into gamete formation and the risk of finding the partner, but would also have the counter-effect of impacting the growth of vegetative cells.

Cell density threshold for sex

Although the need to reach a threshold cell density should be a fundamental requirement to allow the perception of chemical signals and/or encounter and conjugation of gametes, this aspect has been seldom addressed in planktonic microalgae and – to our knowledge – never in diatoms. In the heterothallic chrysophyte *Synura petersenii,* sexual reproduction has been shown to be density-dependent (Sandgren and Flanagin 1986) and in dinoflagellates the formation of gametes and subsequent encystment were not observed when containers were shaken, thus inhibiting cell clustering at a microscale (Persson et al. 2008). The fact that a threshold cell concentration has to be reached in the planktonic *P. multistriata* for sex to occur supports the fact that chemical cues are responsible for the induction of sexuality, as recently reported for two benthic diatoms (Sato et al. 2011, Gillard et al. 2013). Preliminary experiments show that gamete formation is indeed induced in *P. multistriata* strains when exposed to the culture medium conditioned by the growth of strains of the opposite mating type (E. Scalco, unpublished data). Moreover, the fact that a higher percentage of sexual stages was recorded in the experiments carried out in physically undisturbed conditions, as compared to the set up in which bottles were constantly mixed, provides additional evidence that cell aggregation is needed to trigger sexual reproduction. Visual inspection of undisturbed culture vessels indeed shows that a variable percentage of cells is not suspended in the water but sinks to the bottom of the vessel. The different results obtained in the two experimental settings with and without mixing can thus be attributed to the fact that cells exchange diffusible signals and the perception of these signals increases when cells reach a threshold density. We have calculated the theoretical distance between cells growing in mixed and still conditions and found that the estimated distance between cells is one order of magnitude lower in the thin layer at the bottom of the culture flask (about 100 µm when gametes were first detected) than in mixed conditions. The markedly reduced distance between cells favors the perception of chemical signals and facilitates encounters between gametangia of opposite mating type. An alternative or complementary hypothesis is the presence of a quorum sensing-like mechanism that activates the production of sex

pheromones only beyond a critical cell threshold. This mechanism has been reported for bacteria, where two antagonistic molecules regulate the transfer of antibiotic resistance through conjugation only within defined threshold concentrations of the donor cells (e.g., Lyon and Novick 2004, Chatterjee et al. 2013), This would optimize the investment of energy in the production of chemical cues to the conditions in which they will have the maximum chance of being effective.

The cell density at which gamete production was first detected varied between experiments carried out with different pairs of *P. multistriata* strains. All strains used in the present investigation have been isolated in the same geographic area and have been characterized with microsatellite markers proving that they belong to the same genetic population (data not shown). These results thus suggest that there is a notable intraspecific difference in the competence for sex. Similar results have been obtained for the centric diatom *Skeletonema marinoi,* where both sexual and asexual auxospores production differed amongst strains (Godhe et al. 2014). A considerable intraspecific diversity in other life cycle traits, such as production of resting stages and their dormancy length has been reported in dinoflagellates (e.g., Figueroa et al. 2005, Figueroa et al. 2006).

Two out of the few reports of sexual reproduction in the natural environment deal with *Pseudo-nitzschia* species. In both cases, the sexual event involved two different species at the same time: *P. australis* and *P. pungens* along the NW Pacific coast (Holtermann et al. 2010), and *P.* cf. *pseudodelicatissima* and *P.* cf. *calliantha* in the Gulf of Naples, Mediterranean Sea (Sarno et al. 2010). The density of vegetative cells during the sexual event ranged from 187-929 cells · mL^{-1}, the cumulative cell concentration of both *P. australis* and *P. pungens* (Holtermann et al. 2010), to 700 cells · mL^{-1} for *P.* cf. *calliantha,* up to $9.1 \cdot 10^3$ cells · mL^{-1} for *P.* cf. *pseudodelicatissima* (Sarno et al. 2010). The range of cell concentrations spanned over one order of magnitude, but it is in the range

recorded in the laboratory experiments carried out with *P. multistriata*. We have shown that in our experimental model species the formation of gametes started when parental cell concentration was comprised between 2,600 and 5,000 cells · mL^{-1} in the experiments carried out using small volume culture plates, between 1,199 and 1,666 cells · mL^{-1} in the experiment conducted at still conditions, and between 325 and 450 cells · mL^{-1} in the experiment at constantly mixed conditions. While the values recorded in mixed bottles might be assimilated to a well-mixed water column, the values recorded in still conditions might be assimilated either to very high cell concentrations during a bloom or to values recorded in accumulation layers along the water column. The high concentration of *P. cf. delicatissima* (millions of cells · L^{-1}) might represent an example of the first case, when sexual stages were indeed recorded in correspondence with one of the highest concentrations of this species ever recorded at the LTER-MC station (D. Sarno, pers. comm.). The formation of thin layers (TLs) due to physical processes such as vertical gradients of horizontal velocity due to shear, advection, or gradients in temperature and/or salinity can be produced along the water column (Durham and Stocker 2012). Thin layers can have a thickness spanning from centimetres to a few meters, can extend horizontally for kilometres and persist for days. The small vertical structure that characterizes TLs makes them difficult to detect by conventional sampling and profiling instruments (McManus et al. 2008). In several cases, *Pseudo-nitzschia* species have been recorded in TLs, with cell concentrations comprised between $5 \cdot 10^4$ and $3.3 \cdot 10^6$ cells · L^{-1}, which were about 3 times higher than outside the accumulation layers (Rines et al. 2002, Ryan et al. 2005, McManus et al. 2008, Velo Suárez et al. 2008). It is thus reasonable to assume that the short term TLs represent a place where sexual reproduction in *Pseudo-nitzschia* species can occur at sea. Model simulations have also shown that the formation of clusters of cells can also occur along the water column as a result of passive sedimentation under calm hydrodynamic conditions (Botte et al. 2013). Our time-course experiments, in which non-synchronized strains were used, showed that the formation of gametes can occur within a very short time interval (a few days), which is compatible with the life-time of TLs (Durham and Stocker 2012). The clustering of phytoplankton cells in TLs or their

clustering due to passive sinking in low turbulence environments can facilitate the perception of chemical signals and/or increase the probability of mating success, due to higher cell concentration.

Sex in optimal conditions

The observation that optimal growth conditions are required for the onset of the sexual phase in pennate diatoms has been reported in several publications (e.g., Davidovich and Bates 1998, Amato et al. 2005, Chepurnov et al. 2005), but this statement was not supported by an experimental approach. We have provided quantitative experimental evidence showing that the timing and success of sexual reproduction in *P. multistriata* is related to the physiological condition of parental strains. Successful completion of the sexual phase with formation of auxospores and initial cells was in fact obtained only when crossing parental strains in their exponential growth phase. When crossing strains in the late exponential or in the post-exponential phase, the formation of a few gametes can still be recorded but the production of auxospores and initial cells does not take place. Sex is a costly event requiring energy for meiosis, production of gametes, synthesis of the auxospore, *de novo* synthesis of the frustule of initial cell (Lewis 1983). Moreover, initial cells give rise to new cohorts of cells in the population, which require energy to increase in number. From a 'demographic' perspective, it is thus expected that sex is related to positive growth conditions in the natural environment (Flatt and Heyland 2011). Plants and animals invest in offspring production when environmental conditions are favourable for growth and when food is available. However, in other microalgae such as dinoflagellates and chlorophytes, sex is generally induced by environmental conditions unfavourable for growth. Either phosphorous or nitrogen deficiency are required to induce the sexual phase in dinoflagellates (Pfiester and Anderson 1987), while shifts to considerably high temperature trigger sexualisation in *Volvox* (Nedelcu and Michod 2003) and nitrogen starvation induces gamete differentiation and subsequent syngamy in *Chlamydomonas* (Beck and Haring 1996). Amongst diatoms, a link between low nutrient concentration and auxosporulation has been

demonstrated only for *Leptocylindrus danicus* (French and Hargraves 1985). In the above mentioned taxa, sexual reproduction is generally followed by the transformation of the zygote into a resting stage, which might explain the role of adverse exogenous cues in triggering sex. Nevertheless, in chrysophyceans - where the sexual phase is also followed by the formation of a resting statospore - sexual reproduction takes place during periods of active population growth (Sandgren and Flanagin 1986). Although information on the proximate role of external cues in controlling sexual reproduction in different microalgal taxa is still too limited to allow generalizations, it is evident that different selective pressures shaped the structure of life cycle in these unicellular organisms.

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References

Amato, A., Orsini, L., D'Alelio, D. & Montresor, M. 2005. Life cycle, size reduction patterns, and ultrastructure of the pennate planktonic diatom *Pseudo-nitzschia delicatissima* (Bacillariophyceae). *J. Phycol.* 41:542-56.

Assmy, P., Henjes, J., Smetacek, V. & Montresor, M. 2006. Auxospore formation in the silica-sinking oceanic diatom *Fragilariopsis kerguelensis* (Bacillariophyceae). *J. Phycol.* 42:1002-6.

Bardwell, L. 2004. A walk-through of the yeast mating pheromone response pathway. *Peptides* 25:1465-76.

Beck, C. F. & Haring, M. A. 1996. Gametic differentiation of *Chlamydomonas*. *Int. Rev. Cytol.* 168:259-302.

Botte, V., Ribera D'Alcalà, M. & Montresor, M. 2013. Hydrodynamic interactions at low Reynolds number: an overlooked mechanism favouring diatom encounters. *J. Plankton Res.* 35:914-8.

Brzezinski, M. A., Olson, R. J. & Chisholm, S. W. 1990. Silicon availability and cell-cycle progression in marine diatoms. *Mar. Ecol. Prog. Ser.* 67:83-96.

Chatterjee, A., Cook, L. C. C., Shu, C. C., Chen, Y. Q., Manias, D. A., Ramkrishna, D., Dunny, G. M. & Hu, W. S. 2013. Antagonistic self-sensing and mate-sensing signaling controls antibiotic-resistance transfer. *Proc. Natl. Acad. Sci. USA* 110:7086-90.

Chepurnov, V. A., Mann, D. G., Sabbe, K., Vannerum, K., Casteleyn, G., Verleyen, E., Peperzak, L. & Vyverman, W. 2005. Sexual reproduction, mating system, chloroplast dynamics and abrupt cell size reduction in *Pseudo-nitzschia pungens* from the North Sea (Bacillariophyta). *Eur. J. Phycol.* 40:379-95.

Chepurnov, V. A., Mann, D. G., Sabbe, K. & Vyverman, W. 2004. Experimental studies on sexual reproduction in diatoms. *Int. Rev. Cytol.* 237:91-154.

Cote, P. & Whiteway, M. 2008. The role of *Candida albicans* FAR1 in regulation of pheromone-mediated mating, gene expression and cell cycle arrest. *Mol. Microbiol.* 68:392-404.

Crawford, R. M. 1995. The role of sex in the sedimentation of a marine diatom bloom. *Limnol. Oceanogr.* 40:200-04.

D'Alelio, D., Amato, A., Luedeking, A. & Montresor, M. 2009. Sexual and vegetative phases in the planktonic diatom *Pseudo-nitzschia multistriata*. *Harmful Algae* 8:225-32.

D'Alelio, D., Ribera d'Alcalà, M., Dubroca, L., Sarno, D., Zingone, A. & Montresor, M. 2010. The time for sex: a biennial life cycle in a marine planktonic diatom. *Limnol. Oceanogr.* 55:106-14.

Davidovich, N. A. 1998. Transition to sexual reproduction and control of initial cell size in *Nitzschia lanceolata*. *Diatom Res.* 13:29-38.

Davidovich, N. A. & Bates, S. S. 1998. Sexual reproduction in the pennate diatoms *Pseudo-nitzschia multiseries* and *P. pseudodelicatissima* (Bacillariophyceae). *J. Phycol.* 34:126-37.

Drebes, G. 1977. Sexuality. *In* Werner, D. [Ed.] *The Biology of Diatoms.* Blackwell Scientific Publications, Oxford, pp. 250-83.

Durham, W. M. & Stocker, R. 2012. Thin phytoplankton layers: characteristics, mechanisms, and consequences. *Ann. Rev. Mar. Sci.* 4:177-207.

Edlund, M. B. & Stoermer, E. F. 1997. Ecological, evolutionary, and systematic significance of diatom life histories. *J. Phycol.* 33:897-918.

Figueroa, R. I., Bravo, I. & Garcés, E. 2005. Effects of nutritional factors and different parental crosses on the encystment and excystment of *Alexandrium catenella* (Dinophyceae) in culture. *Phycologia* 44:658-70.

Figueroa, R. I., Rengefors, K. & Bravo, I. 2006. Effects of parental factors and meiosis on sexual offspring of *Gymnodinium nolleri* (Dinophyceae). *J. Phycol*. 42:350-62.

Flatt, T. & Heyland, A. 2011. Mechanisms of Life History Evolution: The Genetics and Physiology of Life History Traits and Trade-Offs. Oxford University Press, Oxford,

French III, F. W. & Hargraves, P. E. 1985. Spore formation in the life cycles of the diatoms *Chaetoceros diadema* and *Leptocylindrus danicus*. *J. Phycol*. 21:477-83.

Fuchs, N., Scalco, E., Kooistra, W. C. H. F., Assmy, P. & Montresor, M. 2013. Genetic characterization and life cycle of the diatom *Fragilariopsis kerguelensis*. *Eur. J. Phycol.* 48:411-26.

Gillard, J., Devos, V., Huysman, M. J. J., De Veylder, L., D'Hondt, S., Martens, C., Vanormelingen, P., Vannerum, K., Sabbe, K., Chepurnov, V. A., Inze, D., Vuylsteke, M. & Vyverman, W. 2008. Physiological and transcriptomic evidence for a close coupling between chloroplast ontogeny and cell cycle progression in the pennate diatom *Seminavis robusta*. *Plant Physiol.* 148:1394-411.

Gillard, J., Frenkel, J., Devos, V., Sabbe, K., Paul, C., Rempt, M., Inze, D., Pohnert, G., Vuylsteke, M. & Vyverman, W. 2013. Metabolomics enables the structure elucidation of a diatom sex pheromone. *Angew. Chem.* 52:854-7.

Godhe, A., Kremp, A. & Montresor, M. 2014. Genetic and microscopic evidence for sexual reproduction in the centric diatom *Skeletonema marinoi*. *Protist* 165:401-416.

Guillard, R. R. L. 1975. Culture of phytoplankton for feeding marine invertebrates. *In* Smith, W. L. & Chanley, M. H. [Eds.] *Culture of Marine Invertebrate Animals.* Plenum Press, New York, pp. 29-60.

Guillard, R. R. L. 1980. Division rates. *In* Stein, J. E. [Ed.] *Handbook of Phycological Methods.* Cambridge University Press, Cambridge, pp. 300-11.

Hansen, H. P. & Grasshoff, K. 1983. Automated chemical analysis. *In* Grasshoff K, Ehrhardt M, & Krernling K [Eds.] *Methods of Seawater Analysis*. Verlag Chemie, Weinheim, pp 347–379

Hiltz, M., Bates, S. S. & Kaczmarska, I. 2000. Effect of light:dark cycles and cell apical length on the sexual reproduction of the pennate diatom *Pseudo-nitzschia multiseries* (Bacillariophyceae) in culture. *Phycologia* 39:59-66.

Holtermann, K. E., Bates, S. S., Trainer, V. L., Odell, A. & Armbrust, E. V. 2010. Mass sexual reproduction in the toxigenic diatoms *Pseudo-nitzschia australis* and *P. pungens* (Bacillariophyceae) on the Washington coast. *J. Phycol.* 46:41-52.

Lelong, A., Hégaret, H., Soudant, P. & Bates, S. S. 2012. *Pseudo-nitzschia* (Bacillariophyceae) species, domoic acid and amnesic shellfish poisoning: revisiting previous paradigms. *Phycologia* 51:168-216.

Lewis, W. M. J. 1983. Interruption of synthesis as a cost of sex in small organisms. *Am. Nat.* 121:825-33.

Lyon, G. J. & Novick, R. P. 2004. Peptide signaling in *Staphylococcus aureus* and other Gram-positive bacteria. Peptides 25:1389-403.

Mann, D. G. & Pouličkovà, A. 2010. Mating system, auxosporulation, species taxonomy and evidence for homoploid evolution in *Amphora* (Bacillariophyta). Phycologia 49:183-201.

McManus, M. A., Kudela, R. M., Silver, M. W., Steward, G. F., Donaghay, P. L. & Sullivan, J. M. 2008. Cryptic blooms: Are thin layers the missing connection? *Estuaries and Coasts* 31:396-401.

Mizuno, M. & Okuda, K. 1985. Seasonal change in the distribution of cell size of *Cocconeis scutellum* var. *ornata* (Bacillariophyceae) in relation to growth and sexual reproduction. *J. Phycol.* 21:547-53.

Mouget, J. L., Gastineau, R., Davidovich, O., Gaudin, P. & Davidovich, N. A. 2009. Light is a key factor in triggering sexual reproduction in the pennate diatom *Haslea ostrearia*. *FEMS Microbiol. Ecol.* 69:194-201.

Nedelcu, A. M. & Michod, R. E. 2003. Sex as a response to oxidative stress: the effect of antioxidants on sexual induction in a facultatively sexual lineage. *Proc. R. Soc. Lond. Ser. B. Biol. Sci.* 270:S136-S39.

Orsini, L., Sarno, D., Procaccini, G., Poletti, R., Dahlmann, J. & Montresor, M. 2002. Toxic *Pseudo-nitzschia multistriata* (Bacillariophyceae) from the Gulf of Naples: morphology, toxin analysis and phylogenetic relationships with other *Pseudo-nitzschia* species. *Eur. J. Phycol.* 37:247-57.

Persson, A., Smith, B. C., Wikfors, G. H. & Alix, J. H. 2008. Dinoflagellate gamete formation and environmental cues: Observations, theory, and synthesis. *Harmful Algae* 7:798-801.

Pfiester, L. A. & Anderson, D. M. 1987. Dinoflagellate reproduction. *In* Taylor, F. J. R. [Ed.] *The Biology of Dinoflagellates.* Blackwell Sci. Publ., Oxford, pp. 611-48.

Rines, J. E. B., Donaghay, P. L., Dekshenieks, M. M., Sullivan, J. M. & Twardowski, M. S. 2002. Thin layers and camouflage: hidden *Pseudo-nitzschia* spp. (Bacillariophyceae) populations in a fjord in the San Juan Islands, Washington, USA. *Mar. Ecol. Prog. Ser.* 225:123-37.

Round, F. E., Crawford, R. M. & Mann, D. G. 1990. *The Diatoms. Biology and Morphology of the Genera.* Cambridge University Press, Cambridge, 747 pp.

Ryan, J. P., Chavez, F. P. & Bellingham, J. G. 2005. Physical-biological coupling in Monterey Bay, California: topographic influences on phytoplankton ecology. *Mar. Ecol. Prog. Ser.* 287:23-32.

Sandgren, C. D. & Flanagin, J. 1986. Heterothallic sexuality and density dependent encystment in the crysophycean alga *Synura petersenii* Korsh. *J. Phycol.* 22:206-16.

Sarno, D., Zingone, A. & Montresor, M. 2010. A massive and simultaneous sex event of two *Pseudo-nitzschia* species. *Deep-Sea Res Pt II* 57:248-55.

Sato, S., Beakes, G., Idei, M., Nagumo, T. & Mann, D. G. 2011. Novel sex cells and evidence for sex pheromones in diatoms. *PLoS ONE* 6:e26923.

Schmid, A. M. M. 1995. Sexual reproduction in *Coscinodiscus granii* Gough in culture: a preliminary report. *In* Marino, D. & Montresor, M. [Eds.] *Proceedings of the 13th international diatom symposium 1994.* Biopress, Bristol, pp. 139-59.

Schultz, M. E. & Trainor, F. R. 1968. Production of male gametes and auxospores in the centric diatoms *Cyclotella meneghiniana* and *C. cryptica*. *J. Phycol.* 4:85-88.

Tesson, S. V. N., Legrand, C., van Oosterhout, C., Montresor, M., Kooistra, W. H. C. F. & Procaccini, G. 2013. Mendelian inheritance pattern and high mutation rates of microsatellite alleles in the diatom *Pseudo-nitzschia multistriata Protist* 164:89-100.

Vanormelingen, P., Vanelslander, B., Sato, S., Gillard, J., Trobajo, R., Sabbe, K. & Vyverman, W. 2013. Heterothallic sexual reproduction in the model diatom *Cylindrotheca*. *Eur. J. Phycol.* 48:93-105.

Velo Suárez, L., González-Gil, S., Gentien, P., Lunven, M., Bechemin, C., Fernand, L., Raine, R. & Reguera, B. 2008. Thin layers of *Pseudo-nitzschia* spp. and the fate of *Dinophysis acuminata* during an upwelling-downwelling cycle in a Galician Ría. *Limnol. Oceanogr.* 53:1816-34.

Figure 1. Success of sexual reproduction in *Pseudo-nitzschia multistriata* as related to the physiological state of parental strains (Experiment #1). Crosses were started with parental strains at different time points of their growth curve. Percentage of gametes and zygotes (a) and percentage of auxospores and initial cells (b) recorded when starting the co-cultures with parental strains at different inoculum concentrations (cells · mL^{-1}), i.e., at different phases of their growth curve. Each bar represents the average of 3 replicate values; vertical lines represent st. dev.. The time points of the growth curve at which parental strains were collected to start the co-cultures are reported above the bars.

Figure 2. Success of sexual reproduction in *Pseudo-nitzschia multistriata* as related to the inoculum cell density of parental strains (Experiment #2). Crosses were started with parental strains collected at different time points of their growth curve, but diluted back to the exponential growth phase (see text for explanation). Percentage of gametes and zygotes (a) and percentage of auxospores and initial cells (b) recorded when starting the co-cultures after dilution. Each bar represents the average of 3 replicate values; vertical lines represent st. dev.. The time points of the growth curve at which parental strains were collected to start the co-cultures are reported above the bars.

Figure 3. Growth curves of co-cultures of *Pseudo-nitzschia multistriata* started with parental strains collected at different time points along their growth curve (T0→T7), but diluted back to the exponential growth phase (data of Experiment #2 illustrated in Fig. 2). Circles represent the total number of cells: parental vegetative cells, sexual stages, large F1 cells. For comparison, the growth curve of the monoclonal parental strains (Pm+, grey up-triangles; Pm-, white down-triangles) is included in each panel. (a) Crosses carried out at T0 (black circles) and T2 (white circles). (b) Crosses carried out at T4 (black circles) and T5 (white circles). (c) Crosses carried out at T6 (black circles) and T7 (white circles). For each cross are represented: the initial cell concentration (cells · mL^{-1}) and the total cell concentration estimated for four days, starting from the day in which gamete production was first observed. Symbols represent the average value of duplicate (parental strains) or triplicate (crosses) counts; vertical bars represent maximum and minimum values (parental strains) or standard deviation (crosses).

Figure 4. Growth curve of co-cultures of *Pseudo-nitzschia multistriata* strains of the same mating type (Experiment #4). (a) Co-culture of two male Pm+ strains: large strain Sy668 (white up-triangles)

and small strain Sy373 (black up-triangles). (b) Co-culture of two female Pm- strains: large strain Sy800 (white down-triangles) and small strain Sy379 (black down-triangles).

Figure 5. Dynamics of sexual reproduction in *Pseudo-nitzschia multistriata* co-cultures grown in mixed (a) versus still (b) conditions (Experiment #5). Cell concentration (cells · mL^{-1}) of the two parental strains (grey up-triangles for Pm+ and white down-triangles for Pm-), sexual stages (white circles for the sum of zygotes, auxospores, and initial cells), and large F1 cells (black circles). Each symbol represents the average value of triplicate counts; st.dev. is represented with vertical lines.

Figure 6. Growth curves of *Pseudo-nitzschia multistriata* parental strains in monoculture and in co-cultures grown in mixed (a) *versus* still (b) conditions (Experiment #5). Cell concentration (cell·mL^{-1}) of the two parental strains (grey up-triangles for Pm+ and white down-triangles for Pm-) and the average cumulative abundance of cells (parental vegetative cells, sexual stages, F1 cells; data of Fig. 5) in co-culture (black circles).

Supporting information

Figure S1. Schematic drawings illustrating the experimental set up of crosses carried out with *Pseudo-nitzschia multistriata* parental strains collected at different cell densities and growth phases. (a) At each time point (T0→T7), a subsample of the two parental strains (Pm+ and Pm-) was mixed and inoculated into culture plate wells (Experiment #1). (b) At time points between T4 and T7, when cell concentration of the parental strain was > 3,000 cells · mL^{-1}, the co-culture was diluted before the inoculum into culture plate wells (Experiment #2). In this way, all co-cultures started with cell densities comparable to those of the exponential growth phase.

Figure S2. Light micrographs of different life cycle stages of *Pseudo-nitzschia multistriata*. (a) Pairing cells of opposite mating type (Pm+ and Pm-); (b) each gametangium (Pm+ and Pm-; out of focus) produced two gametes, which are approaching conjugation; (c) two auxospores produced by the fusion of gametes; (d) initial cell inside the auxospore (arrow). Scale bars = 10 µm.

Figure S3. Growth curves of *Pseudo-nitzschia multistriata* co-cultures started with parental strains collected at the same time points of their growth curve and diluted at different inoculum concentration (Experiment #3). Cell concentration (cells · mL^{-1}) of the parental vegetative cells (black squares), gametes (yellow circles), auxospores (green circles) and initial cells (green circles) in crosses started with a parental strain inoculum of 400 cells · mL^{-1} (a), 4,000 cells · mL^{-1} (b), 6,000 cells · mL^{-1} (c) and 8,000 cells · mL^{-1} (d). Each symbol represents the average value of duplicate (parental strains) or triplicate (crosses) counts; vertical lines represent maximum and minimum values (parental strains) or standard deviation (crosses).

Figure S4. Growth curves of *Pseudo-nitzschia multistriata* co-cultures started with parental strains collected at the same time points of their growth curve and diluted at different inoculum concentration (Experiment #3). Circles represent the total number of cells (parental vegetative cells, sexual stages, large F1 cells). For comparison, the growth curve of the monoclonal parental strains (Pm+, grey up-triangles; Pm-, white down-triangles) is included in each panel. (a) Crosses carried out at 400 cells · mL^{-1} (black circles) and 4,000 cells · mL^{-1} (white circles) and (b) Crosses carried out at 6,000 cells · mL^{-1} (black circles) and 8,000 cell·mL^{-1} (white circles). Each symbol represents the average value of duplicate (parental strains) or triplicate (crosses) counts; vertical bars represent maximum and minimum values (parental strains) or standard deviation (crosses).

Figure S5. Cell concentration (cells · mL^{-1}) of *Pseudo-nitzschia multistriata* parental strains (black squares), the sexual stages (yellow circles: gametes; green circles: auxospores; violet circles: initial cells) and the large F1 cells (light blue circles) in co-cultures incubated in mixed (a) and in still (b) conditions (Experiment #5). Each point represents the average of triplicates counts; st.dev. is represented with vertical lines.

Figure S6. Estimated distance between adjacent *Pseudo-nitzschia multistriata* cells (Pm+, Pm-, and F1 generation are included) when cultures were incubated in mixed (a) and still (b) experimental conditions; note the different scale between (a) and (b). In (b) two different assumptions were made: 40% (black dots) and 60% (grey dots) of cells sink in a 10 µm thin layer at the bottom of the culture plate.

Table S1. Strains used in the different experiments: strain code and mating type (in parenthesis), isolation date and the average length of the apical axis (n=20 cells) when experiments were carried out.

Table S2. Nutrient concentrations (µmol · L^{-1}) measured at different time points along the growth curve of Pm- strain MVR171.8 and Pm+ strain MVR171.1. For each time point, two replicate measurements are reported.