Factors affecting the sexual reproduction of diatoms, with emphasis on *Pseudo-nitzschia* spp.

STEPHEN S. BATES[1] AND NICKOLAI A. DAVIDOVICH[2]
[1]*Fisheries and Oceans Canada*
Gulf Fisheries Centre, Moncton, New Brunswick, E1C 9B6 Canada
[2]*Diatom Biology Laboratory, Karadag Natural Reserve of National Academy of Sciences Feodosiya, Ukraine*

Introduction

Diatoms have the peculiar predicament that every time they divide vegetatively, they decrease in cell size. This is due to their unique construction of having two rigid silicon thecae that compose the diatom frustule. When the diatom cell reaches a threshold size, it becomes physiologically capable of undergoing sexual reproduction to produce auxospores which then develop into large initial cells (Geitler 1932, 1935; Drebes 1977; Round *et al*. 1990; Mann 1993). Most diatoms rejuvenate their original large cell size in this way, according to the scheme developed independently by MacDonald (1869) and Pfitzer (1869) in 1869 (Fig. 5). If the cells do not undergo auxosporulation, they will continue to decrease in size until they eventually die. Details of how sexual reproduction is carried out are different, depending on if the diatom is pennate or centric (Drebes 1977; Round *et al*. 1990).

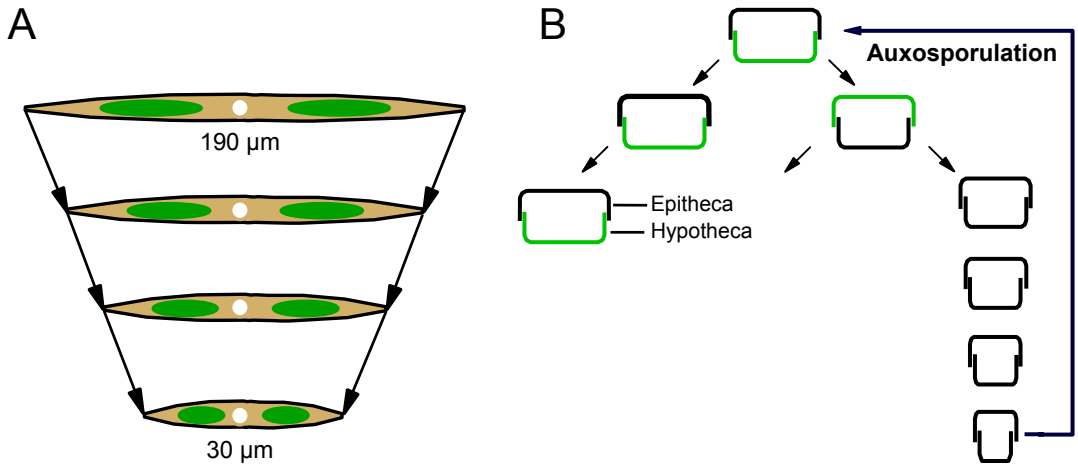

Fig. 5. (A) Decrease in cell apical length of *Pseudo-nitzschia.* (B) MacDonald-Pfitzer scheme for cell size reduction, and the restitution of large cell size via auxosporulation, for a generic centric diatom.

This presentation outlines the main factors that are responsible for affecting the sexual reproduction of diatoms. It will focus mainly on pennate diatoms of the genus *Pseudo-nitzschia* (Bates 2000). In 1987, *P. multiseries* was identified as the source of the neurotoxin, domoic acid, that contaminated blue mussels at aquaculture sites in eastern Prince Edward Island, Canada (Bates 1998; Bates *et al*. 1998). It was the first time that a diatom was shown to produce a phycotoxin. Indeed, diatoms form only a small minority of harmful or toxigenic phytoplankton

species, the majority belonging to the Dinophyceae. Several species of toxigenic *Pseudo-nitzschia* are present in European waters (e.g. Lundholm *et al*. 1994; 1997; Fraga *et al*. 1998; Amzil *et al*. 2001; Sarno & Dahlman 2000; Gallacher *et al*. 2001).

Toxic and harmful diatoms

All of the diatoms known to have produced a toxin or to have caused harm are shown in (APPENDIX 1) (cf. Hasle & Fryxell 1995; Fryxell & Hasle 2002). Curiously, many of the harmful, but non-toxic, diatoms are all fish-killers; they are also all centrics. Two species of *Chaetoceros* are characterized by barbed setae which can become lodged between the secondary lamellae of the gills of salmonids, causing mucus overproduction and eventual death by suffocation (Rensel 1993). Another diatom, that resembled *Corethron* sp., was associated with the mortality of coho salmon smolts (Speare *et al*. 1989). The cell has an apical corona of spiny setae that may become embedded in the fishes' gills. A centric, *Leptocylindrus minimus*, was implicated in mortalities of aquacultured salmonids in southern Chile (Clément & Lembeye 1993); the mode of death is not certain. In Asia, *Skeletonema costatum* and *Coscinodiscus wailesii* are considered "red tide" species (Yamochi & Joh 1986; Nagai & Imai 1999). They form intense blooms which can deplete the water of nutrients and decrease the light available for the cultivation of the red macroalga "nori" (*Porphyra*) for human consumption. The epiphytic *Tabularia affinis* causes problems by forming ribbon colonies that adhere to the surface of cultured *Porphyra* in Japan (Nagai *et al*. 1996).

Except for *Pseudo-nitzschia multiseries*, *P. pseudodelicatissima*, and *Coscinodiscus wailesii*, the sexual reproduction of the other harmful diatoms has not been studied specifically. It is possible that the centric diatoms are monoecious, each clone being able to produce both "male" and "female" gametes, since this is the pattern that has often been assumed to be characteristic of centrics (Drebes 1977); but this needs to be checked for each species. The resting stage is another aspect of the life history of diatoms for which there is little information (Mann; Diatom Discussion Group Report, this volume). Resting stages are known for some of the centrics listed in APPENDIX 1 (see also Hargraves & French 1975; French & Hargraves 1980; 1985; Garrison 1984; McQuoid & Hobson 1996; Nagai & Imai 1999).

Interest in the mode of sexual reproduction of *Pseudo-nitzschia* arose because clonal cultures continued to diminish in size and eventually died, without undergoing sexualization. Experiments that mixed together different clones documented that these pennate species are dioecious, i.e., are characterized by cells that produce either "male" or "female" gametes by separate gametangia in different clones (Davidovich & Bates 1998a, b; Kaczmarska *et al.* 2000). Originally, the sexual reproduction of *P. multiseries* was incorrectly described by Subba Rao *et al*. (1991), for the reasons given in Fryxell *et al.* (1991) and Rosowski *et al.* (1992). Clonal cultures of toxigenic *Pseudo-nitzschia* sp. cf. *pseudodelicatissima* were recently described as undergoing "enlargement", without producing any sexual cells (Pan *et al.* 2001).

Factors inducing sexualization

Cell size. A key condition to be met before diatoms become sexualized is that they must first decrease to a threshold size, known as the first cardinal point (Geitler 1932, 1935). It has sometimes been suggested that "only relatively small cells, measuring generally about 30-40% of maximal valve diameter within a species-specific size range, prove to be capable of sexualization" (e.g. Drebes 1977, p. 271).

However, our experience with *Pseudo-nitzschia multiseries*, at least, is that the largest sexually inducible cells thus far are about 120 µm long and that the largest vegetative cells are about 190 µm long (Fig. 6), which is 63% of the largest cells. A value of 70% was found in other experiments (Hiltz *et al.* 2000). The range of 30-40% is therefore clearly an underestimation; the window of opportunity for sexualization is larger than previously reported. Such information is relevant for determining how long it takes for *P. multiseries* cells to decrease to the size allowing for sexual reproduction. Given a size reduction of ~2.5 µm per month, or ~30 µm per year under constant growth conditions in culture, it was estimated that initial cells must grow for ~3 years to reach the sexually inducible size, assuming that the suitable length corresponds to 40% of the maximal size (Davidovich & Bates 1998b). In reality, recent experiments showed that one pair of clones produced initial cells after only 7 months, when they were ~120 µm long (pers. observ.). No information is available about the rate of cell size reduction of *Pseudo-nitzschia* spp. in the field, but changes in cell division rate over the long term must be taken into account. A knowledge of cell size may also be important if there is a relationship between cell size and toxicity, as has been observed in most of our cultures of *P. multiseries* (Bates *et al*. 1999).

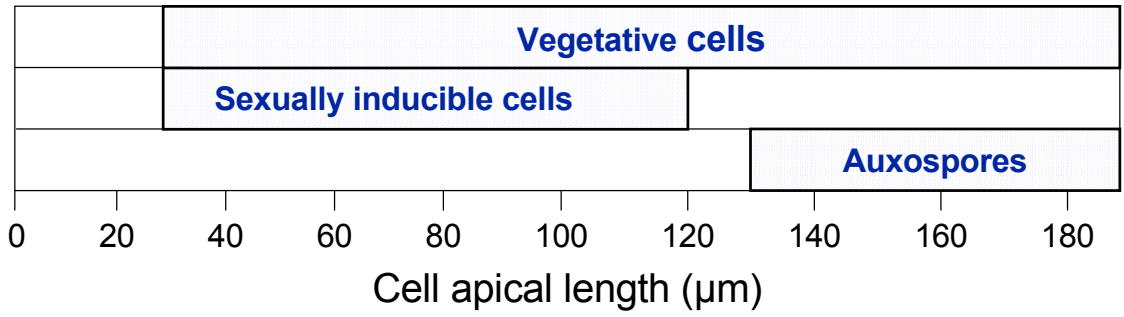

Fig. 6. Cell apical length of *Pseudo-nitzschia multiseries* at various stages in the life cycle (data from pers. observ.; Davidovich & Bates 1998b; Hiltz *et al*. 2000).

Growth phase. Once in the appropriate size window, it is essential that *Pseudo-nitzschia* spp. cells be in good physiological condition for them to be capable of reproducing sexually. This means that they must be in the exponential growth phase (e.g. days 3-6 after inoculation in batch culture). In nature, one would therefore expect to find sexualized cells only when conditions are favourable for growth, although this may not have to be during a bloom.

Irradiance and photoperiod. Being in "good physiological condition" also implies that the cells are exposed to sufficient light energy during a 24-h period. The absolute irradiance level required to initiate sexual reproduction has not been studied for *P. multiseries*. However, a study of different photoperiods at an irradiance level of ~100 µmol photons m^{-2} s^{-1} showed an increased production of gametes per vegetative cell with an increase in photoperiod length, up to the maximum studied of 16:8 h Light:Dark (Hiltz *et al*. 2000). Davidovich (1998) likewise found that the production of gametes, auxospores, and initial cells of *Nitzschia lanceolata* increased with the total light energy received by the parent cells. Thus, photosynthesis provides energy for sexual reproduction; stored energy is not sufficient to complete all the stages of sexual reproduction, including initial cell formation.

Water motion. All phytoplankton cells experience some movement due to currents within the water column. Such water motion may be important for spreading vegetative cells to new locations. For sexually reproducing cells, it may be both helpful and detrimental. Pennate diatom cells of opposite sex must come into contact with each other in order to mate. This may occur by the bi-directional movement of the cells over a substrate (e.g. suspended particulate material or directly on the sediments). Wind- or heat-induced water motion may also increase the chance of cell-to-cell encounter. Above a certain threshold, however, water motion may become a disadvantage. For example, even if the cells manage to touch one other, the hydrodynamic force may be too great for them to remain attached long enough to initiate the sexual process. Also, such forces may cause already formed gametes to become detached from the "mother" gametangial cell. Once they lose contact with the gametangium, it is unlikely that they will find another gamete with which to fuse to form a zygote.

A preliminary experiment showed that a mixture of *P. multiseries* clones of opposite sex grown in duplicate flasks containing 1.5 L of f/2 medium failed to produce gametes for four days while on an orbital shaker at 170 rpm; stationary control cultures produced gametes as usual during that same period. Once the water movement was stopped, however, there was a rapid, massive production of gametes and the subsequent formation of initial cells. A second experiment employed small volume (5 mL) cultures in petri plates (Gordon 2001). Results showed a significant effect of water motion (again, created by orbital shakers at 170 rpm), in both delaying and decreasing zygote production. In contrast to the large volume flask experiment, however, gametes were produced in the shaken treatments, a finding that may be related to the different culture volumes used and to the amount of water movement thus created. One may nevertheless conclude that still conditions favour *Pseudo-nitzschia* sexual reproduction. In high water motion, fertilization may still be successful if the cells reach a high density, when cell-to-cell contact is facilitated. If this were to be followed by a period of calm, then one may expect the highest success for producing auxospores.

Stimulants of gametogenesis (Pheromones?). Pennate diatom cells of opposite sex have the challenge of finding each other in order to mate. Although this is made easier when the cell number is high, the presence of a chemical signal (a pheromone) would be a further advantage. Because pennates are motile, they may take advantage of changes in chemical gradients of any such pheromone, in order to find a mate. Pheromones have been found in brown macroalgae for gamete

attraction (Maier 1995). However, it has also been debated whether or not pheromones would be advantageous for mate location in microalgae (Dusenbery & Snell 1995).

To look for possible chemical signals, pairs of clones of opposite sex were exposed to filtrates that may contain such a signal (Haché 2000). The filtrates were derived from cultures in which a pair of clones was allowed to grow for 48 h in order to initiate the sexual process, and in so doing, release possible pheromones. Different proportions of each test clone were used in order to determine if any effect on gametogenesis would be more apparent in clone mixtures that contained more "male" or more "female" cells. Control cultures contained the clone mixtures at these same proportions, but made in fresh f/2 medium instead of filtrates derived from sexually reproducing mixtures. The results showed a significantly greater production of gametes when the clone mixtures were made in filtrates compared to fresh f/2 medium. Moreover, the effect was most pronounced when the proportion of "female" cells was greatest. In that case, some "female" cells were also observed to produce gametes, even when they were not in contact with a "male" cell. The results suggest that the filtrates contained some type of compound (a pheromone?) that stimulated gametogenesis, but did not necessarily aid in mate location.

Bacteria. Bacterial-phytoplankton interactions are an intrinsic component of harmful algal bloom (HAB) ecology and physiology (Doucette *et al.* 1998). Bacteria have been shown to directly or indirectly take part in biotoxin production, promote or inhibit the growth of HAB species, and stimulate or inhibit phytoplankton sexual reproduction (perhaps via excretion of certain "promoter" organics). For example, the bacterium *Alcaligenes* sp. promoted spermatogenesis (but not oogenesis) in *Coscinodiscus wailesii* (Nagai *et al*. 1999). Experiments were therefore carried out to investigate the possible role of bacteria in mediating the sexual reproduction of *P. multiseries*, by mating pairs of axenic or non-axenic clones (Thompson 2000). A mixture of bacteria, isolated from each pair of non-axenic clones, was also reintroduced into the corresponding mixture of axenic clones. The control non-axenic clones retained their natural composition of bacteria. Results showed no sexual reproduction (i.e. no gametes or auxospores) in mixtures of one pair of axenic clones. Moreover, the reintroduction of bacteria to axenic mixtures restored sexual activity. In contrast, sexual reproduction did occur in axenic mixtures of another pair of clones. Both gametes and auxospores were observed in the axenic mixtures of this pair, as well as in the mixtures to which bacteria were added (as expected). It is possible that viable extracellular or intracellular bacteria remained in this particular pair of antibiotic-treated clones, accounting for the observed sexual activity. Additional experiments are required to unambiguously determine whether or not bacteria are required for *P. multiseries* sexual reproduction.

Clonal variability in domoic acid toxicity

Our ability to mate different clones of *P. multiseries* and to isolate individual initial cells into clonal culture has enabled us to look at differences in toxicity among sibling clones derived from the same parents. We have found both considerable variability among clones, as well as a decrease in toxicity as the clonal cultures age and the cells become smaller. The variations among sibling clones from common parents are caused in part by genetic variability. However, it is also possible that the presence of different types and numbers of bacteria in the individual cultures also accounts for these differences (see Bates [1998] for a summary of differences in

toxicity between axenic and non-axenic cultures). Free-living as well as attached bacteria have been found in the diatom cultures (Kaczmarska *et al.* 2000). The diversity and numbers of bacteria attached to *P. multiseries* cells increase over time after inoculation in batch culture. How the bacterial composition and concentration change over time after the diatom cells are first isolated has not yet been established. The extent to which the different types and numbers of bacteria affect toxicity and sexual reproduction also remains to be determined.

Remaining questions and gaps

Research on diatom sexuality will improve our ability to predict the occurrence, periodicity, and toxicity of *Pseudo-nitzschia* blooms. For example, years in which blooms are most intense and toxic may be associated with a particular stage in the cells' life history. However, the following gaps must first be filled before we can successfully apply our knowledge about the sexual reproduction of diatoms:

- Identification of the sexual stages of harmful and toxic diatoms other other than those mentioned in this report.
- Molecular techniques to identify sexual stages.
- Time required for *Pseudo-nitzschia* cells to reach the sexually inducible size in nature.
- Location of overwintering cells.
- Information on resting stages, if they exist, or of heavily silicified "winter growth stages".
- Information on asexual vegetative cell enlargement, especially for pennate diatoms.
- Genetic variability vs. biotic/abiotic factors in controlling sexual reproduction and toxicity.
- Location of mating (suspended within the water column, on the surface of a particle, on the bottom sediments?).
- Relationship between cell size and toxicity.
- Bloom intensity and toxicity in relation to the timing of sexual reproduction.
- Proportion of the population undergoing sexual reproduction at one time.
- Information on mating systems in and compatibility among morphologically distinct or cryptic *Pseudo-nitzschia* species.
- Study of cell cycle progression, including volumetric growth (and girdle development) and the mechanism by which the characteristic stepped colony is formed.

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Note: The references for this article can be found in the full document, which can be downloaded from: http://www.icm.csic.es/bio/projects/lifehab/ (an 8 MB file).