Density-dependent mechanisms regulate spore formation in the diatom *Chaetoceros socialis*

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**Scientific Significance Statement**

The interplay between environmental and biotic factors in regulating organisms’ life strategies is a frequently debated issue in plankton ecology. The prevalent view is that resource availability plays a major role, and nutrient starvation is considered the major trigger for the formation of resting spores in diatoms. By contrast, our study shows that the marine diatom *Chaetoceros socialis* forms spores also in nutrient replete conditions when cell concentration is high. Moreover, spore formation is induced by spent culture media obtained from healthy as well as from lysed cells, demonstrating that the transition from vegetative cells to spores is mediated by a chemical signal produced by the cells. Exploring the link between cell density and spore formation may elucidate mechanisms involved in species succession.

**Abstract**

Resting stages are reported for several unicellular eukaryotes including diatoms that can produce spores or resting cells. These stages represent a repository of diversity in sediments but the factors that induce their formation are elusive. We investigated spore formation in the marine diatom *Chaetoceros socialis*. Our results confirm that nitrogen starvation is an effective experimental trigger for spore formation. However, we observed that spores are also produced when the external pool of nutrients is not limiting, and by using a semicontinuous setup, we could show that they appear only when cell density is high. The presence of a chemical cue mediating spore formation was supported by experiments carried out incubating cells with culture media conditioned by both healthy and lysed cells. These findings shed new light on the mechanisms that regulate the transition between life cycle stages and represent an experimental baseline for the identification of the signaling molecules.

Diatoms are a prominent group of unicellular microalgae and their life strategy includes periods of rapid growth during which they can form extensive blooms followed by mass sinking (Smetacek 1985; Raven and Waite 2004). In many species, the end of the bloom is accompanied by the formation of...
spores or resting cells (Pitcher 1986; von Bodungen et al. 1986). Spores have thick siliceous cell walls and are morphologically different from the vegetative cells thriving in the water column, while resting cells are morphologically undistinguishable from vegetative cells (McQuoid and Hobson 1996). Both stages have high amounts of storage materials and reduced metabolism that allow them to survive in the absence of light and at cold temperatures, thus overcoming unfavorable environmental conditions and mortality by predators or pathogens (McQuoid and Hobson 1996; Raven and Waite 2004).

Resting stages sink to the sediments where they accumulate forming “seed banks,” especially in coastal surface sediments (McQuoid et al. 2002; Montresor et al. 2013), which can remain viable, and thus capable to germinate, for decades (Härnström et al. 2011). Thus, resting stages assure the persistence of the species over a wide time span and is a key trait of diatom evolutionary fitness. In addition, the high abundance of spores in sediment traps deployed in open oceanic waters demonstrates that they can considerably contribute to carbon export at depth (Rynearson et al. 2013).

Despite all this, the triggers and the mechanisms that drive the shift from vegetative cells to resting stages are not completely understood. Laboratory experiments showed that nitrogen limitation is the most effective factor inducing this life cycle transition (Kuwata et al. 1993; McQuoid and Hobson 1996 and references therein). In some species, however, spores are produced at low light conditions, or at low/high temperature, or when the concentration of other nutrients is low (Kuwata et al. 1993; McQuoid and Hobson 1996; Sugie et al. 2010), or even in control conditions when nutrients were available (Oku and Kamatani 1995). This latter intriguing result suggests that formation of resting stages in diatoms may be driven by more sophisticated mechanisms than those presently assumed.

We addressed the issue using the marine colonial diatom Chaetoceros socialis, which produces endogenous spores (Pelusi et al. 2019) and is abundant in many coastal areas (De Luca et al. 2019). We tested nitrogen depletion, traditionally the most effective environmental factor, but explored also the possible role of biological cues related to cell density indicative of a coordinated response at the level of the whole population. Density-dependent mechanisms, including the formation of dormant stages, have been investigated in unicellular organisms like bacteria, which can perceive their population density through the production and perception of signaling molecules (Keller and Surette 2006). Intraspecific communication mediated by chemical signals is known also for unicellular eukaryotes and includes, among others, the production of sexual pheromones and the induction of programmed cell death (reviewed by Venuleo et al. 2017) suggesting that such collective response might be widespread in nature.

**Methods**

**Strains and experimental conditions**

Strains of C. socialis (APC1, APC2, and APC12) were isolated from the germination of spores collected in surface sediments at station LTER-MareChiara in the Gulf of Naples. Strain isolation, maintenance, and genetic characterization are illustrated in Pelusi et al. (2019). Culture media were prepared with artificial seawater at salinity of 36 (Sea salts, Sigma-Aldrich; Pelusi et al. 2019); ventilated tissue culture flasks (Corning) were used. Experiments were carried out at controlled temperature (18°C ± 1°C) and irradiance (~ 90 μmol photons m⁻² s⁻¹ daily average) obtained with a sinusoidal cool-white illumination system at 12 : 12 h light : dark photocycle. Light and temperature were monitored with a HOBO Pendant Temperature/Light Data Logger (Onset Computer Corporation). To measure cell and spore concentration, 3 mL of culture were fixed with neutral formaldehyde at final concentration of 1.6% and counted with a Sedgewick-Rafter chamber on a Zeiss Axiophot microscope (ZEISS) at 400× magnification.

**Spore formation induced by nitrogen limitation**

Strains APC1 and APC2 were grown in a modified f/2 medium (Guillard 1975) (+N, control) and in N-limited medium (–N, treatment). The control had a nitrogen concentration of 580 μM, to obtain N : P close to Redfield ratio, and a silica concentration of 300 μM. The treatment had a much lower nitrate concentration (23 μM). Three replicates for treatment and control conditions were prepared by inoculating cells in exponential growth phase at a final cell concentration of ~ 3000 cells·mL⁻¹ in 750 mL flasks filled with 500 mL of culture medium. Concentration of vegetative cells and spores was assessed daily, while concentration of dissolved inorganic nutrients (SiO₂, PO₄, and NO₃) and intracellular nitrogen and carbon on days 0, 2, 4, and 7. To measure intracellular nitrogen and carbon, three samples were collected from each flask, filtered on pre-combusted (450°C, 4 h) 1.2 μm pore sized glass-fiber filters (Whatman GF/C, Merck) and immediately stored at −20°C. The analyses were performed with a Thermo Scientific FlashEA 1112 automatic elemental analyzer (Thermo Fisher Scientific) following (Hedges and Stern 1984). Cyclohexanone-2,4-dinitrophenylhydrazone was used as standard.

To measure the concentration of dissolved inorganic nutrients, 50 mL of sample were collected from each flask. Samples were filtered on 0.45 μm pore-sized Whatman Cellulose Acetate Membranes in two high-density polyethylene vials (20 mL each) and immediately stored at −20°C. The analyses were carried out using FlowSys Systea Autoanalyzer equipped with five continuous flux channels and following (Hansen and Grasshoff 1983).
Spore formation in semicontinuous cultures at different cell density

Experiments were carried out with strain APC12 and three cell densities (cell concentration at day 0) were tested: (1) high: $3 \times 10^4$ cells mL$^{-1}$, (2) medium: $3 \times 10^3$ cells mL$^{-1}$, and (3) low: $3 \times 10^2$ cells mL$^{-1}$. Strain APC12 was inoculated in a 250 mL flask filled with 100 mL of modified f/2 medium (control medium of the previous experiment). The culture was acclimated at the semicontinuous setup (transfers every 2 d) and, after 8 d, aliquots were used to inoculate flasks at the different cell concentrations. Every day, cell and spore concentration was estimated and cell concentration was brought back to the initial values every 2 d. The pH was measured in the high-density setup with full strength medium using a Corning 240 pH meter.

To rule out possible effects related to higher pH values in batch cultures, we carried out a parallel experiment in culture flasks bubbled with air filtered through sterile, 0.22 μm filter units. Three replicate flasks inoculated with strain APC12 at an initial cell concentration of $3 \times 10^4$ cells mL$^{-1}$ were grown with the semicontinuous setup illustrated above. The pH was measured before and after dilution and vegetative cells and spores were counted daily.

Spore formation induced by culture medium conditioned by high cell density

For the preparation of the experimental culture media, strain APC12 was grown in 500 mL of f/2 medium with a semicontinuous setup. After four transfers, when cell density was about $5 \times 10^5$ cells mL$^{-1}$, that is, the value reached before dilution in the semicontinuous experiment at high cell density, four 100 mL subsamples were processed as follows: (1) gentle filtration with a syringe on a 1.2 μm pore size filter (filtered medium); (2) filtration as above and addition of concentrated nutrient stock equivalent to f/20 (filtered medium + nutrients); (3) sonication for three times, 30 s each, with a

![Graphs](image-url)

**Fig 1.** Time course of vegetative cells and spores, and percentage of spores in *C. socialis* strains APC1 (A, B) and APC2 (C, D) in nitrogen-deplete medium (A, C) and nitrogen-replete medium (B, D). Values shown as average + SD ($n = 3$).
Branson Sonifer 250 and filtration as in (1) (sonicated medium); (4) sonication and filtration as in (3) and nutrient addition as in (2) (sonicated medium+nutrients). All media were immediately used to carry out the experiments. Triplicate flasks for each of the four treatments were filled with 30 mL of the specific conditioned culture media illustrated above and inoculated with an exponentially culture of strain APC12 at an initial cell concentration of 1000 cells mL⁻¹. A control was setup with triplicate flasks filled with f/2 medium. Cells and spores were counted daily.

Statistical analyses
A multiple t-test was used to test differences of intracellular nutrients between treatment and control. Spore concentration and pH, measured in high-density cultures before each dilution in still and bubbled cultures, were compared with a paired t-test. Comparisons between spore percentage in control and conditioned media were performed using a Kruskal–Wallis followed by a Dunn’s multiple comparison test. Statistical analyses were performed using GraphPad Prism version 6.0 and values were defined significantly different when p < 0.05.

Results
Spore formation induced by nitrogen limitation
In cultures of *C. socialis* strains APC1 and APC2 grown in –N, total cell (vegetative cells + spores) concentration increased exponentially in the first 2 d and then leveled off, with

![Graph showing concentration of nutrients](Fig 2)

Fig 2. Concentration of dissolved nutrients: (A) nitrates (NO₃⁻), (B) silicates (SiO₂⁻), and (C) orthophosphates (PO₄³⁻) in cultures of *C. socialis* strains APC1 (solid line) and APC2 (dashed line) grown in nitrogen-deplete medium (blue) and in nitrogen-replete medium (orange). Data shown as average ± SD (n = 3).

![Graph showing intracellular content](Fig 3)

Fig 3. Intracellular nitrogen content (A), carbon content (B), and their molar ratio (C) in *C. socialis* strain APC1 (dark colors) and APC2 (light colors) grown in nitrogen-deplete medium (−N, blue) and in nitrogen-replete medium (+N, orange). Data shown as average ± SD (n = 3); **p < 0.01, ***p < 0.001.
average maximum value of $2.25 \times 10^5 \pm 1.43 \times 10^4$ cells mL$^{-1}$ for strain APC1 and $1.86 \times 10^5 \pm 3.15 \times 10^4$ cells mL$^{-1}$ for strain APC2 on day 7 (Fig. S1). Spore formation became evident on day 3 and more than 90% of the cells turned into spores within day 4 (strain APC1) or day 3 (strain APC2) (Fig. 1A,C).

In control conditions (+N), cultures kept growing after the initial exponential growth phase reaching, at the end of the experiment, values about one order of magnitude higher compared to those obtained in the treatment (Fig. S1). However, spores were recorded also in control conditions. Average

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**Fig 4.** (A) Total cell concentration (vegetative cells + spores) of *C. socialis* strain APC12 in semicontinuous growth with nitrogen-replete medium at three different cell densities (high, blue; medium, red; low, green). (B) Concentration of vegetative cells and spores in the high cell density setup. Data shown as average $\pm$ SD ($n$ = 3).

**Fig 5.** Total cell concentration (A) and percentage of spores (B) of *C. socialis* strain APC12 produced in experiments carried out with conditioned culture media (control, blue; filtered medium, dark red; filtered medium + nutrients, light red; sonicated medium, dark green; sonicated medium + nutrients, light green). Data shown as average $\pm$ SD ($n$ = 3).
maximum spore percentages at the end of the experiment were of 44% ± 4.5% and 27% ± 2.9% in strains APC1 and APC2, respectively (Fig. 1B,D).

In -N treatments, nitrate concentration reached almost undetectable values on day 4 in strain APC1 (0.27 ± 0.06 μM L⁻¹) and on day 2 in strain APC2 (0.56 ± 0.71 μM) (Fig. 2A). Still high concentration of silicates and orthophosphates were detected at the end of the experiment (Fig. 2B,C). In +N controls, high nitrate concentration of 279 ± 4 and 248 ± 15 μM L⁻¹ were still measured at the end of the experiment (day 7) in strain APC1 and APC2, respectively (Fig. 2A). Silicates reached almost undetectable concentrations in both strains on day 7, while orthophosphates were not limiting (Fig. 2B,C).

In both strains, intracellular nitrogen concentration increased in postexponential growth phase in the controls but it did not significantly change in the treatments (Fig. 3A). Intracellular carbon concentration remained constant in the controls, while it was significantly higher (ρ < 0.001) in treatments at days 4 and 7 (Fig. 3B). As a consequence, the C : N molar ratio significantly increased in the treatment on days 4 and 7 (Fig. 3C), when massive spore formation occurred, while it remained constant in cultures grown in nitrogen replete medium.

**Spore formation in semicontinuous cultures at different cell density**

The results of the previous experiment showed that spores were formed also in the controls, when total cell concentration was > 1 × 10⁵ mL⁻¹ but nutrients were not limiting. We thus investigated spore formation dynamics with a semicontinuous experimental setup in which three different cell concentrations were tested. Average absolute growth rates, estimated over the 2-d dilution intervals, were between 1.9 and 1.2 d⁻¹ (Fig. 4A; Table S1). Spores were detected in all dilution steps only in the high-density treatment, where their average abundance increased within each dilution interval, showing that new spores were consistently produced over a 2-d time interval (Fig. 4B). In the medium- and low-density treatments few spores were sporadically detected and their percentage was always lower than 1% (Fig. S2). Average pH values measured every second day before dilution in cultures grown at high density were between 8.39 (± 0.015) on day 0 and 8.62 (± 0.015) on day 2. In order to test possible effects of pH on spore formation, we ran a parallel semicontinuous experiment with high cell density but using air bubbling in the flasks (Fig. S3). The pH values measured before each dilution and the concentration of spores were not significantly different (ρ = 0.6831 and ρ = 0.2269, respectively) in the two experiments (Fig. S3; Table S2).

**Spore formation induced by culture medium conditioned by high cell density**

Density-dependent biological responses are mediated by chemical cues and we investigated the formation of spores using culture media obtained by filtration or sonication of high-density cultures. Two additional treatments were included in which nutrients were added to the conditioned media to completely rule out nutrient limitation. The total cell number increased only in the control over the first day, while it remained constant or even slightly decreased in all treatments (Fig. 5A). Positive growth was detected during the second day, with higher values for the control and the cultures incubated in filtered medium, with and without the addition of nutrients, while the lowermost values were measured for cultures grown in sonicated medium. Spores were not produced in the control but were detected already on the first day in all treatments and their percentages on day 2 were not statistically different among the four treatments (Fig. 5B).

**Discussion**

While nitrogen depletion was confirmed as an effective experimental treatment to induce the formation of resting stages in *C. socialis*, high numbers of spores were recorded also in nutrient replete controls when a threshold cell concentration was reached. The results of our study strongly suggest the presence of chemical-mediated intraspecific signals that induce the transition from vegetative cells to resting spores in this diatom. Experiments carried out with semicontinuous cultures showed that spores were consistently produced only in the high cell density treatment. These results were confirmed when inoculating cells in a medium conditioned by the growth of high-density cultures.

**Spore formation induced by nitrogen limitation**

The use of nitrogen-deplete culture media has been shown to induce spore production in various *Chaetoceros* species (French III and Hargraves 1985; Kuwata et al. 1993; McQuoid and Hobson 1996) including *C. socialis*, where different strains isolated from the Gulf of Naples responded in the same way, all turning into spores within a few days (Pelusi et al. 2019). We have now convincingly shown that spore formation occurs also in control conditions when nutrients are not limiting, and this suggests that their depletion in the medium does not represent the only trigger for this transition. Our results also show that the average, internal nitrogen pool did not decrease during starvation in *C. socialis*. It might be possible that the decrease in nitrogen availability causes a qualitative shift in intracellular nitrogenous compounds that elicits the formation of spores in this species. An induction of the tricarboxylic acid (TCA) cycle, which allows the recycling of nitrogen derived from the degradation of proteins, was in fact detected in nitrogen starved *Thalassiosira pseudonana* and *Phaeodactylum tricornutum* (Brembu et al. 2017 and references therein). However, during the process of spore formation, *C. socialis* cells keep assimilating carbon, likely to build metabolic energy reserves, and this further supports the idea that their physiology is not impaired but re-directed. The internal nitrogen pool instead decreased in nitrogen-limited cultures of *Chaetoceros pseudocurvisetus* (Kuwata et al. 1993), which suggests that the amount of internal nitrogen can trigger the life...
cycle transition in this species. However this interpretation is in apparent contrast with the fact that also *C. pseudocurvisetus* produces spores when cultures were not limited by nitrogen (Oku and Kamatani 1995).

The fact that spore formation is not necessarily linked to nutrient limitation is consistent with several observations carried out in the natural environment. A massive production of spores was recorded for *Chaetoceros* cf. *diadema* during the spring bloom in the North Atlantic Ocean when nutrient concentration was not limiting (Rynearson et al. 2013). Similarly, high fluxes of diatom spores were recorded in upwelling regions (Pitcher 1986), and in sediment traps deployed in the naturally iron fertilized waters downstream of South Georgia, an area with permanently high nutrient concentration (Rembauville et al. 2015).

**Cell density mediated by chemical cues induces the formation of spores**

In organisms that include different life cycle stages, the transition from one stage to another can be induced by the perception of an environmental signal, by a signal produced by another organism, or by a signal produced by cells of the same species. Our results suggest that the formation of spores in *C. socialis* is an example of the latter case, where the signal for the life cycle transition is produced by cells that have reached a threshold density. The experiments carried out with conditioned medium included a treatment in which cells were gently filtered and one in which cells were first sonicated and then filtered. The rationale was to test if the chemical compound/s inducing spore formation was produced during cell lysis, when some diatoms can produce secondary metabolites such as oxylipins (Whichard et al. 2005). Oxylipins include polyunsaturated aldehydes (PUAs) that have been shown to negatively affect growth and reproduction of various organisms, from metazoans to bacteria (Ianora and Miralto 2010) and have been also suggested to mediate intraspecific cell-to-cell communication in diatoms (Vardi et al. 2006). The results of our experiments showed that both conditioned media, that is, the one in which cells were gently filtered and the one in which they were first sonicated and then filtered, could induce the formation of spores. The sonicated medium induced also a reduction of growth within the first 24 h of incubation that recalls the results obtained on various phytoplankton species with PUAs (Ribalet et al. 2007). However, *C. socialis* does not produce PUAs but nonvolatile oxylipins (Fontana et al. 2007). High molecular weight oxylipins include members of the hydroxylated eicosapentaenoic acids (HEPEs) family, which are constitutively released by *Chaetoceros didymus* where they inhibit the growth of algicidal bacteria (Meyer et al. 2018). The results obtained in this study represent a starting point for further investigations aimed to identify the signaling molecules involved in spore formation and growth control.

**Ecological implications**

The adaptive value of resting stages is considered to be an escape from present or near-future unfavorable conditions until the occurrence of more favorable ones. Unfavorable conditions may be nutrient depletion, mortality risk from grazers or parasites (Rengefors et al. 1998), and reduced light availability (Eilertsen and Degerlund 2010). Our results showing that cell density, mediated by a still unknown chemical signal, is a trigger for the formation of spores suggest that there is an upper limit of abundance beyond which the population starts a process of self-removal from the water column. Spores and resting cells, on one side, increase sinking velocity and thus the removal of the population from the upper layers of the water column but, on the other side, can survive in the deeper layers or in the sediments. The formation of resting stages can thus have direct implications on successional patterns of diatoms and other unicellular microalgae where the selective removal of a population, when environmental conditions are still suitable for growth, leaves ecological space to other species.

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