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Research Article

Induced Bacteriovory in a Declining Culture of the Mixotrophic Dinoflagellate *Prorocentrum minimum* (Pavillard) Schiller

Gary H. Wikfors and Emilie Fernandez

NOAA, NMFS, Northeast Fisheries Science Center, 212 Rogers Avenue, Milford, CT 06460, USA

Correspondence should be addressed to Gary H. Wikfors; gary.wikfors@noaa.gov

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Bacteriovory was reported previously in the dinoflagellate, *Prorocentrum minimum*, but it was unclear if this is constitutive or induced under certain conditions. We tested the hypothesis that phosphate deficiency, or cessation of autotrophic growth for other reasons, would induce bacteriovory in a culture of *P. minimum* that is harmful to shellfish. Phosphate-starved cells did not ingest fluorescently labeled bacteria and died. In stationary-phase, full-enrichment cultures, more than half of viable *P. minimum* cells showed declines in chlorophyll that was coincident with incorporation of fluorescently labeled bacteria. Declining populations of *P. minimum* increase in toxicity to suspension-feeding shellfish; this suggests a possible association between bacteriovory and toxicity.

1. Introduction

Prorocentrum minimum (Pavillard) Schiller is a dinoflagellate with world wide distribution in coastal waters; blooms can have ecosystem-disrupting consequences [1]. Sporadic expression of toxicity to mammals [2] and to suspension-feeding molluscs [3–6] has been reported. Although toxicity in this species appears to be strain dependent [2], recently we demonstrated conclusively that senescent cultures of one strain are more bioactive against bivalves than actively growing cultures [7]. The chemical identities of toxic agents produced by this species are unknown, although the term "venerrupin" has been associated with it [1].

Prorocentrum minimum has been described as a mixotroph [8]. The species grows photosynthetically on inorganic nutrients with light as the sole energy source but has been shown to assimilate dissolved, organic nitrogen and carbon compounds [9]. Furthermore, phycoerythrin fluorescence within *P. minimum* cells growing together with cryptophytes, and incorporation of coincubated, fluorescently-labeled bacteria [10] indicate the potential for phagotrophy in this species.

Previous observations in our laboratory of lowered chlorophyll content in declining cultures of *Prorocentrum minimum* (unpublished microscope and flow-cytometer observations) suggested loss of photosynthetic capacity, yet cultures remain viable for months after nearly all pigments have disappeared. Previous studies have shown that *P. minimum* can release a bacteriostatic compound when cultured under phosphate-depleted conditions [11], which we confirmed with the JA-98-01 strain of *P. minimum* used in the present study (data not shown). In an initial experiment, we found no evidence that fluorescently labeled bacteria were incorporated within cells of actively growing cultures of the JA-98-01 strain, in contrast to the findings of Li et al. [10].

In the present study, we tested the hypothesis that phagotrophic ingestion of bacteria by *Prorocentrum minimum* strain JA-98-01 is initiated by either phosphate deficiency or other physiological changes associated with cessation of autotrophic growth.

2. Materials and Methods

Triplicate, 500 mL Erlenmeyer flasks were prepared with 250 mL of the following enriched-seawater media: (1) nutrient-replete, EDL7 [12], and (2) EDL7 with no phosphate enrichment. Each flask was inoculated with an EDL7-grown culture of the JA-98-01 strain of *Prorocentrum minimum* (Choptank, MD, USA isolate maintained in the Milford

Microalgal Culture Collection) at 5×10^3 cells mL^{-1} and incubated in a light room at 18°C with 12 : 12 hr illumination at 175 μE m^{-2} s^{-1} PAR. Cultures were sampled aseptically three days per week and counted with a FACScan flow cytometer (BD BioSciences, San Jose, CA, USA), identifying *P. minimum* cells based on side scatter and chlorophyll fluorescence (SSC/FL3).

On days 7 and 26, incorporation of fluorescently labeled bacteria within *P. minimum* cells subsampled from the cultures was tested. Bacteria for these tests were collected from another, full-nutrient, actively growing culture of JA-98-01 (the strain is bacterized) by selective filtration and stained with BacLight Green (Invitrogen, Carlsbad, CA, USA). Stained bacteria were washed twice in sterile-filtered (0.22 μm) seawater (FSW) and added to a sub-sample of *P. minimum* from each experimental flask at approximately the same number of stained bacteria ($1\text{-}2 \times 10^5$ cells mL^{-1}) as the density of unstained bacteria. After 30 min and 1, 24, and 48 hr of incubation in the dark at 18°C, samples were run on the flow cytometer; presence of fluorescently-labeled bacteria associated with the *P. minimum* cells was determined by FL1 (green fluorescence) of cells within the *P. minimum* SSC/FL3 region. In addition, samples were observed with epifluorescence microscopy (Zeiss AxioSkop 2) to determine if green-fluorescent bacteria were within *P. minimum* cells or were attached to the outside.

3. Results and Discussion

Division time of *Prorocentrum minimum* in both treatments was approximately 3 d for the first 14 d (Figure 1(a)). By day 21, the full-nutrient cultures had reached a stable, stationary phase of approximately 10^5 cells mL^{-1}, while the phosphate-deficient cultures fluctuated between 0.2 and 2×10^5 cells mL^{-1}. After 32 or 35 days, cells were alive and motile, but not increasing in number.

The test for incorporation of fluorescently labelled bacteria on day 7 showed no evidence, by flow cytometry or epifluorescence microscopy, of bacteriotrophy in any cultures. On day 26, green bacterial fluorescence associated with full-nutrient *P. minimum* cells incubated with fluorescently-labeled bacteria was observed with both flow cytometry and microscope observations (Figure 1(b)). In these full-nutrient cultures, the flow cytometer discriminated two distinct populations of *P. minimum* cells: one with relatively high chlorophyll fluorescence and the other with less red fluorescence (Figure 1(b)). Intensity of green fluorescence in low-chlorophyll cells increased from 30 min to 1 hr, decreasing thereafter, and was significantly higher than in chlorophyll-replete cells (Figure 1(c), t-test, $P < 0.05$). Thus, only the low-chlorophyll *P. minimum* cells in the full-enrichment medium were bacteriotrophic. Phosphate deprivation did not induce bacteriotrophy in this strain of *P. minimum* and did not result in a viable population of low-chlorophyll cells; instead, empty thecae of dead *P. minimum* cells were seen.

These findings suggest that bacteriostatic properties of phosphorus-deficient *Prorocentrum minimum* cultures are not involved in bacteriotrophy. Further, more-active bacteriovory in *P. minimum* cells with lower chlorophyll fluorescence than in high-chlorophyll cells suggests that ingestion of bacteria may replace the carbon fixed previously by photosynthesis [13].

Jeong and coauthors [14] reviewed ecological roles of mixotrophic dinoflagellates and used the list of prey ingested by *P. minimum* as an example of the diversity of prey that a mixotroph may feed on. In the section of this review devoted to light and nutrient effects, *P. minimum* prey-ingestion rates were reported to be independent of light; however, no reports on *Prorocentrum* spp. heterotrophy being affected by nutrient status are cited [14]. This review concludes, in part, that mixotrophy may have evolved to supplement autotrophic growth "when light and nutrient conditions are not favorable for photosynthesis." In our study, light remained sufficient throughout the incubation, but nutrients were removed from solution during growth.

More recently, Glibert and co-authors [15] reviewed existing knowledge of the ecological roles of planktonic and benthic *Prorocentrum* species. This review advanced a compelling conceptual model linking *P. minimum* autecology with nutrient availability (Figure 13 in [15]). Using the Redfield ration of N : P as a fulcrum, these authors generalize that autotrophic growth dominates at N : P below the Redfield ratio, with high growth rates supported by reduced N substrates, especially urea. Above the Redfield, the model characterizes a transition to a mixotrophic mode associated with increased allelopathy or toxicity, slower growth, and increased alkaline-phosphatase activity. Although we did not set out to study nutrient ratios in the present study, we note that the nutrient enrichment used has an N : P ratio of 25—above the Redfield. Our results, nevertheless are consistent with the Glibert et al. [15] conceptual model in that declining growth in cultures that had assimilated a portion of the nutrients was coincident with a change in a high proportion of the population to mixotrophy (phagotrophy). Our main intent in reporting this change in trophic status is to emphasize that this same strain becomes more bioactive against scallops as this change in trophic status occurs, possibly implicating enzymes associated with digestion of bacteria in effects on mollusks [7].

The present study was part of a long-term effort to understand variable "toxicity" in *Prorocentrum minimum* [4]. That bacteriovory and higher toxicity are coincident in "declining" cultures does not demonstrate cause and effect, but previous studies suggest a possible mechanism linking these cell properties. Zhou and Fritz [16] reported that organelles within *P. lima* and *P. maculosum* cells that stained positively with periodic-acid Schiff (PAS-positive) were "lysosomes." When *P. minimum* cells containing these PAS-positive bodies were fed to juvenile oysters, digestive gland absorptive cells became dysfunctional; accumulations of PAS-positive food vacuoles impeded further digestion [17]. The original interpretation of these observations was that lysosomes may release cytotoxic, autolytic enzymes. The present study presents the alternative interpretation that lysosomes in declining achlorotic *P. minimum* cells may be phagosomes wherein digestion of bacteria occurs. Possible

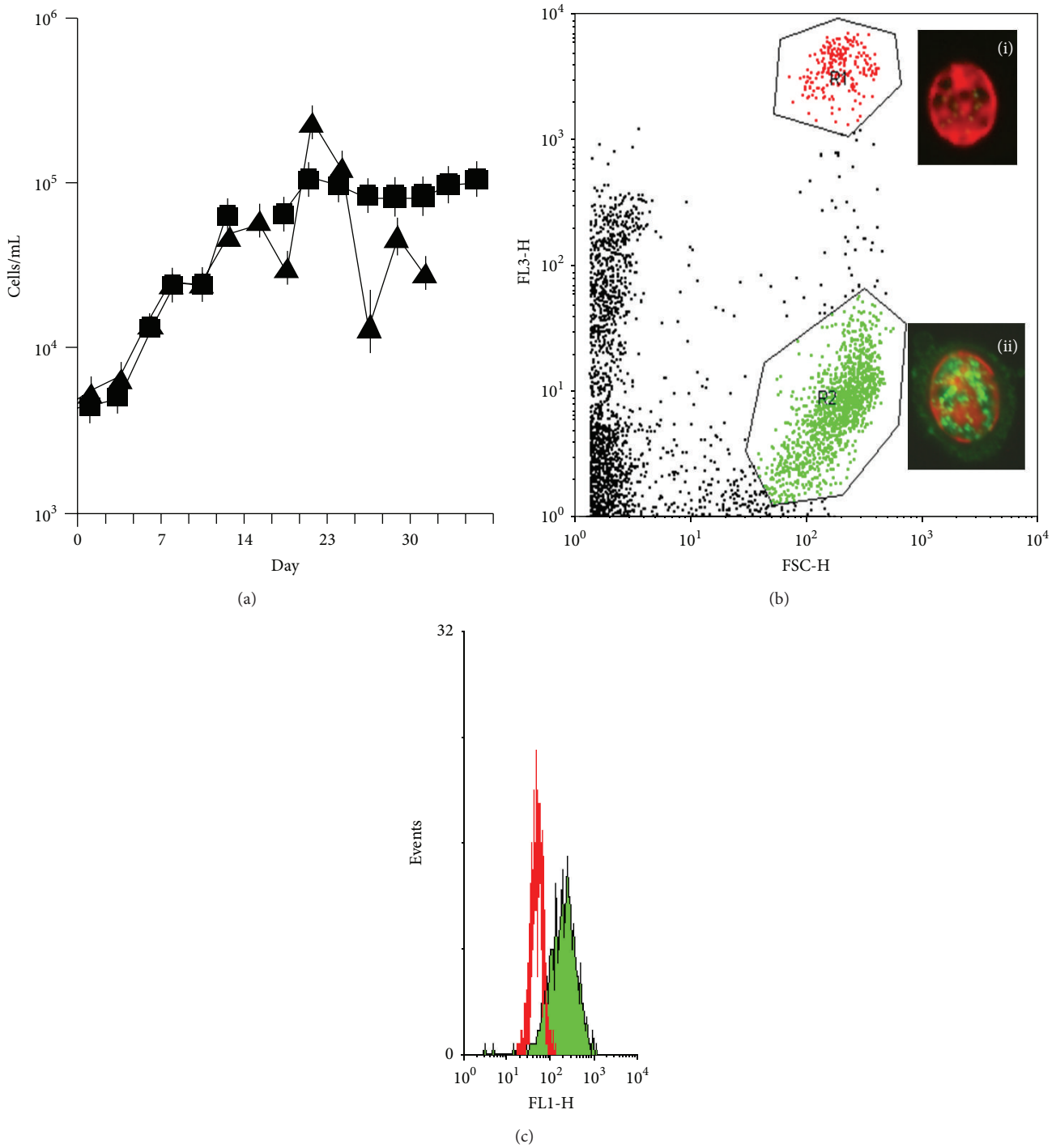

Figure 1: (a) Growth of *Prorocentrum minimum*, strain JA-98-01, in media with full enrichment (square symbols) or with no phosphate enrichment (triangle symbols); means of triplicate flow-cytometer counts ± SE. (b) Flow-cytometer plot and fluorescence photomicrographs of *Prorocentrum minimum* cultures sampled after 26 days in nutrient-replete medium. Biplot of relative cell size (FSC, in arbitrary detector units) and red chlorophyll fluorescence (FL3) showing two morphologies of cells within the sample: R1, high-chlorophyll cells, shown in photomicrograph (i) and R2, low-chlorophyll cells that had incorporated fluorescently labeled bacteria, shown as green-fluorescent inclusions in photomicrograph (ii). (c) Histogram plot showing intensity of green fluorescence in cells differentiated by R1 (red line) and R2 (green filled area), indicating relatively low prevalence of bacteriocytic cells in the high-chlorophyll cell population.

associations between toxicity and bacteriovory in *P. minimum* may help to direct biochemical investigations into the identity of toxic agents produced by this enigmatic dinoflagellate.

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