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# Dinoflagellate-Cyanobacterium Communication May Determine the Composition of Phytoplankton Assemblage in a Mesotrophic Lake

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## Summary

**The reasons for annual variability in the composition of phytoplankton assemblages are poorly understood but may include competition for resources and allelopathic interactions [1–4]. We show that domination by the patch-forming dinoflagellate, *Peridinium gatunense*, or, alternatively, a bloom of a toxic cyanobacterium, *Microcystis* sp., in the Sea of Galilee [5] may be accounted for by mutual density-dependent allelopathic interactions. Over the last 11 years, the abundance of these species in the lake displayed strong negative correlation. Laboratory experiments showed reciprocal, density-dependent, but nutrient-independent, inhibition of growth. Application of spent *P. gatunense* medium induced sedimentation and, subsequently, massive lysis of *Microcystis* cells within 24 hr, and sedimentation and lysis were concomitant with a large rise in the level of McyB, which is involved in toxin biosynthesis by *Microcystis* [6]. *P. gatunense* responded to the presence of *Microcystis* by a species-specific pathway that involved a biphasic oxidative burst and activation of certain protein kinases. Blocking this recognition by MAP-kinase inhibitors abolished the biphasic oxidative burst and affected the fate (death or cell division) of the *P. gatunense* cells. We propose that patchy growth habits may confer enhanced defense capabilities, providing ecological advantages that compensate for the aggravated limitation of resources in the patch. Cross-talk via allelochemicals may explain the phytoplankton assemblage in the Sea of Galilee.**

## Results and Discussion

Annual blooms of *P. gatunense* have dominated the phytoplankton assemblage in the Sea of Galilee for many years. Deterioration of this stability during the last decade (Figure 1A) was indicated by delays, or even absence, of *P. gatunense* patches. Examination of the abundances of *P. gatunense* and *Microcystis* sp. in the Sea of Galilee over the last 11 years (Figure 1A) provided the first indication that these disturbances were correlated with the appearance of *Microcystis* sp. Statistical analysis (nonparametric Spearman's rank correlation coefficient [see the Supplementary Experimental Procedures in the Supplementary Material available with this article online]) of these data revealed a strong negative correlation between the abundance of these two organisms, suggesting allelopathic interactions. Inhibition of photosynthesis in *P. gatunense* by *Microcystis* sp. [7] supported this notion. This was further confirmed in long-term growth experiments that indicated reciprocal inhibition of *P. gatunense* growth in the presence of *Microcystis* sp. and vice versa. When the initial *P. gatunense* inoculum was 200 cells/ml, its growth was completely inhibited by the presence of *Microcystis* sp. MG ($10^6$ cells/ml), even when amply supplemented with nutrients. In similar experiments in which the initial *P. gatunense* inoculum was raised to 560 cells/ml, growth was 60% inhibited. In contrast, when the initial density of *P. gatunense* was 2300 cells/ml, growth was scarcely affected by *Microcystis* sp. (not shown). Conversely, growth of *Microcystis* sp. was hardly affected by the presence of *P. gatunense* as long as the initial inoculum of the latter did not exceed 1000 cells/ml, but growth was severely depressed when higher cell densities of *P. gatunense* were applied (Figure 1B), even in the presence of an adequate nutrient supply. These data suggested that allelopathic interactions between these organisms were density dependent and affected their long-term growth.

### Induction of Oxidative Stress in *Peridinium* by *Microcystis*

To examine more immediate responses of *P. gatunense* to *Microcystis* sp., we measured the accumulation of reactive oxygen species (ROS), which reflect stress responses in *P. gatunense* [8] and other aquatic photosynthetic organisms [9, 10]. The fraction of *P. gatunense* cells that contained ROS increased significantly within 0.5 hr after the addition of *Microcystis* MG (Figures 2A and 2B). The level of ROS-positive cells reached a first maximum in about 1 hr, followed by a secondary oxidative burst that reached a maximum within 24 hr and then declined to the initial level (Figure 2D). Application of microcystin-LR, a hepatotoxin that inhibits protein phosphatases types 1 and 2A [11, 12], the main toxin produced by *Microcystis* MG, also resulted in a typical biphasic pattern, but the second maximum was reached considerably faster (Figure 2D). By contrast, the addition of *Microcystis* MB, which does not produce detectable amounts of microcystins, scarcely affected the initial ROS level in *P. gatunense* and did not induce a secondary oxidative burst. The amplitude and the exact timing of the biphasic responses observed were affected by various growth conditions. For example, the ratio between the primary and the secondary ROS-positive peaks increased with the age of the culture (compare the data in Figure 2D with Figure 2E). Further, while the *Microcystis* treatment of older cultures such as those used in Figure 2E resulted in approximately 30% sytox-

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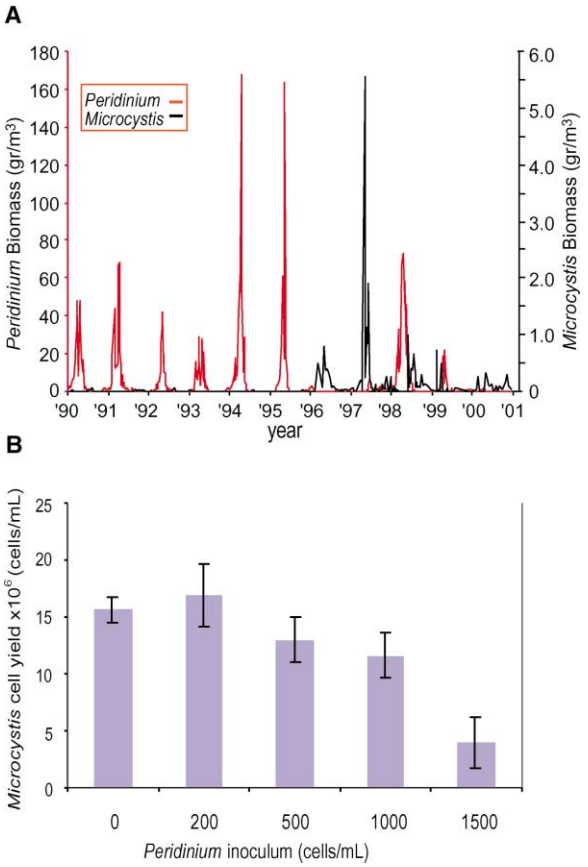


Figure 1. Growth of *Microcystis* sp. and *P. gatunense* under Natural and Laboratory Conditions

(A) Annual variations in the abundance of *P. gatunense* and of *Microcystis* in the Sea of Galilee over the period of 1990–2001. As described in [30], biomass was calculated in samples collected weekly from the 1 m top layer at a central site in the Sea of Galilee. Biomass was used rather than cell counts since, under natural conditions, *Microcystis* tends to aggregate.

(B) Growth of *Microcystis* sp. KLL MG is affected by the initial density of *P. gatunense* inoculum. The data show cell yield after 21 days. The initial *Microcystis* density was $2 \times 10^5$ cells/ml.

positive (dead) cells within 24 hr, it hardly affected the extent of cell death in the younger cultures. An oxidative burst following microcystin or okadaic acid treatments (which inhibits protein phosphatases) was also reported for plant and animal cell cultures [13, 14]. Biphasic ROS production has also been observed in cultured plant cells treated with an avirulent bacterium that triggered hypersensitive response resulting in cell death, but it has not been observed in those treated with a virulent strain [15, 16].

Protein kinases, and in particular MAP kinases (MAPK), are known to mediate cellular responses to biotic and abiotic signals in mammalian [17] and plant cells [18]. We employed specific inhibitors to examine whether protein kinases are involved in the *Microcystis* MG-induced ROS accumulation in *P. gatunense*. Preincubation of *P. gatunense* with PD98059 (PD) and SB203580 (SB), specific inhibitors of the ERK1 (extracellular regulated kinase 1) [19] and p38 [20] pathways, respectively, severely depressed the fraction of ROS-positive cells during the 24 hr exposure to *Microcystis* MG (Figure 2F). Treatments with LY294002 (LY), a specific inhibitor of protein phosphatidylinositol 3-kinase [21], or H7, a broad-based serine/threonine kinase inhibitor [22], also halted the accumulation of ROS during the first 8 hr; however, in contrast to SB and PD, these treatments stimulated the formation of ROS thereafter (Figure 2F). These data suggested a role for the MAPK family in the perception of the cyanobacterium (or its toxin) by the dinoflagellate and in the signal transduction process leading to the accumulation of ROS.

### Cell Death and Thymidine Incorporation in *Peridinium* in Response to *Microcystis*

Earlier results demonstrated that accumulation of ROS induced a process that resembled programmed cell death in *P. gatunense* [8]. The *Microcystis* MG treatment that resulted in ROS accumulation (Figure 2) also promoted death of *P. gatunense* cells within 24 hr, as determined by the fraction of sytox-positive cells (Figures 3A–3D). Preincubation of *P. gatunense* with SB (which depressed the accumulation of ROS, Figures 2C and 2F) prior to the addition of *Microcystis* prevented cell death (Figures 3C and 3D). Interestingly, this inhibitor is known to specifically block the p38 MAPK and thereby the apoptotic pathway [23]. In contrast to the effect of SB, the addition of LY or H7, which inhibited the initial ROS formation but not the secondary burst of ROS (Figure 2F), resulted in a percentage of dead cells similar to or even higher than that observed in their absence (Figure 3D).

In addition to promoting cell death, *Microcystis* MG stimulated division of the surviving *P. gatunense* cells, as indicated by cell counts (not shown). Incorporation of $^3$H-thymidine increased by approximately 8-fold in cells pretreated with spent *Microcystis* MG medium, but not with microcystin-LR (Figure 3E), reflecting enhanced DNA synthesis by a component present in this medium. Both MAPK inhibitors, PD and SB, attenuated this rise (the former was somewhat more effective). The extent of thymidine incorporation observed here could result from both cell division and DNA repair. Indeed, DNA fragmentation was observed in *P. gatunense* cells, which accumulated ROS due to $CO_2$ limitation [8]. Notably, promotion of *P. gatunense* division by *Microcystis* was mainly pronounced in young cultures in which cell death was not promoted, despite the accumulation of ROS (Figure 2D).

### Protein Kinase Activity, Level, and Expression in *Peridinium* in Response to *Microcystis*

Data presented in Figures 2 and 3 suggest that specific protein kinase families might be involved in the response of *P. gatunense* to the presence of *Microcystis* MG. We took several approaches to examine whether changes in the activity, level, and expression of protein kinases in *P. gatunense* occur in response to the presence of *Microcystis*, its toxin, and $H_2O_2$. The activity of protein kinases at the time of harvesting was assessed by analyses of in-gel kinase activities [24]. In Figures 4A–4D, we show that the activity of certain protein kinases was strongly affected by the various treatments imposed;

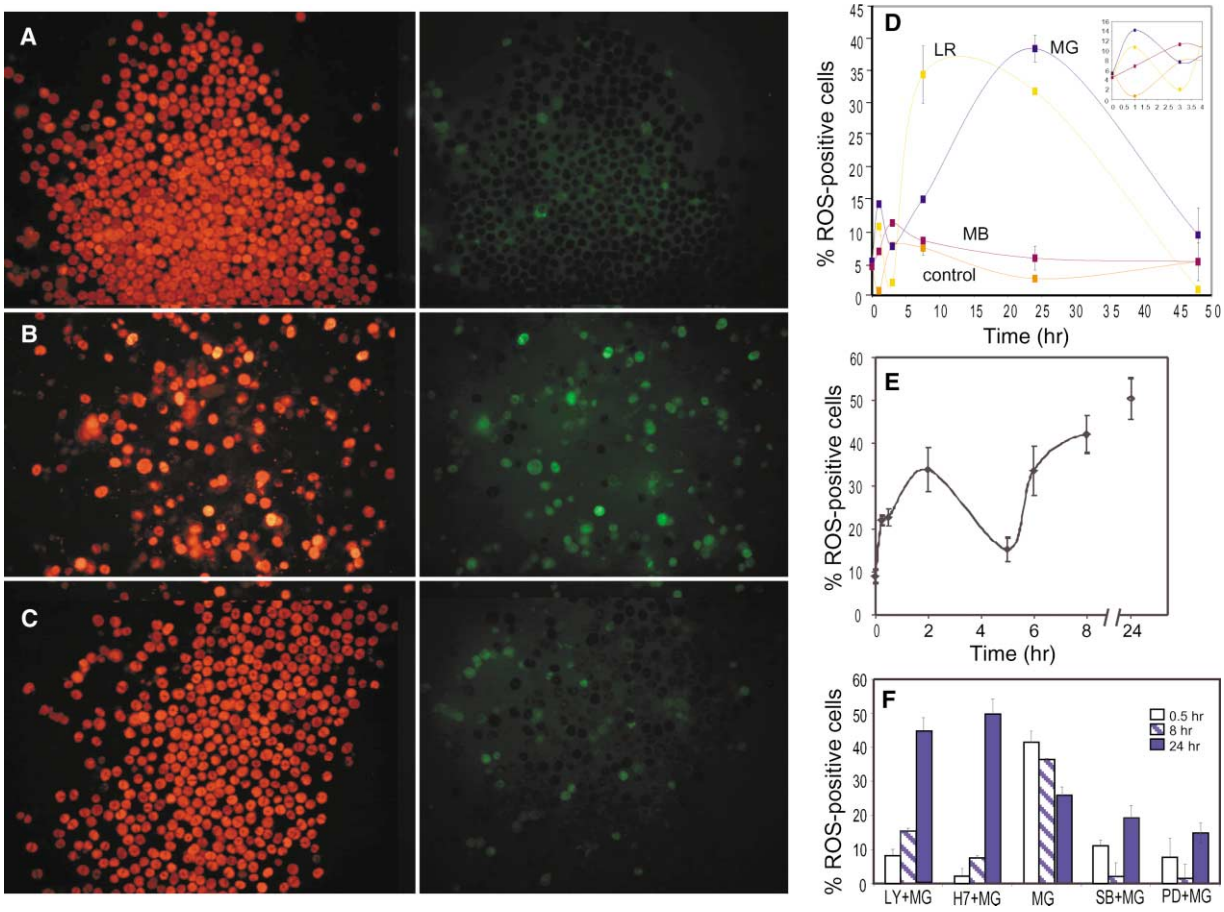


Figure 2. Induction of Reactive Oxygen Species Formation in *P. gatunense* by *Microcystis*

Reactive oxygen species (ROS)-positive cells are indicated by green fluorescence; the red images show chlorophyll autofluorescence.

(A) Control cells.

(B) Cells exposed to *Microcystis* MG for 0.5 hr.

(C) Cells preincubated with SB203580 (SB, 10 μM) prior to the addition of *Microcystis* MG.

(D) Time course for the accumulation of ROS-positive cells in cultures at mid logarithmic growth phase. Curves are shown for control *P. gatunense* cells and cells responding to *Microcystis* MG, *Microcystis* MB, and microcystin-LR (50 μg/L). The inset depicts the first 4 hr and shows the primary oxidative burst.

(E) ROS-positive cells in cultures at late logarithmic growth phase treated with *Microcystis* MG.

(F) The fraction of ROS-positive cells after preincubation with kinase inhibitors H7 (50 μM), LY294002 (LY, 20 μM), PD98059 (PD, 50 μM), or SB and subsequently exposed to *Microcystis* MG for 0.5, 8, or 24 hr.

The data points provided in (D)–(F) are the average ratio estimates ± the SE, calculated as in [34]; the number of cells per field was larger than 500.

also, specific kinases responded differently to these treatments. A 72 kDa protein kinase was activated within 0.5 hr by both *Microcystis* strains (MG and MB) as well as by spent *Microcystis* MG medium and its toxin (microcystin-LR), but not by the $H_2O_2$ treatment (Figure 4A). The activity of a 38 kDa protein kinase was sharply increased by *Microcystis* MG, but not by its toxin microcystin-LR (Figure 4B). Unlike the 72 kDa kinase, the activity of this kinase declined following treatments with *Microcystis* MB or $H_2O_2$. In Figure 4C, we provide an example in which changes in the protein kinase activities (that of a 20 kDa protein kinase) were transient. Its activity increased strongly within 0.5 hr of exposure to *Microcystis* MG but declined thereafter and reached a level close to the control within 22 hr. Treatments with microcystin-LR, *Microcystis* MB, or $H_2O_2$ scarcely affected the activity of this kinase. Notably, even though ROS accumulated in *P. gatunense* cells treated with *Microcystis* MG (Figure 2), the $H_2O_2$ treatments did not bring about changes in the activities of the 72, 38, and 20 kDa protein kinases (Figures 4A–4C) similar to those observed following the addition of *Microcystis* MG.

In sum, the activities of certain protein kinases in *P. gatunense* were strongly affected by exposure to *Microcystis* in a strain-specific manner (cf. Figures 4B and 4C). While the exact nature of the kinases recognized here is yet to be established, we postulate that alterations in the activity of specific kinases preceded the accumulation of ROS. Further, the adverse responses to microcystin-LR, as compared with *Microcystis* MG or its spent medium, suggested that the toxin was necessary and sufficient to induce changes in the activity

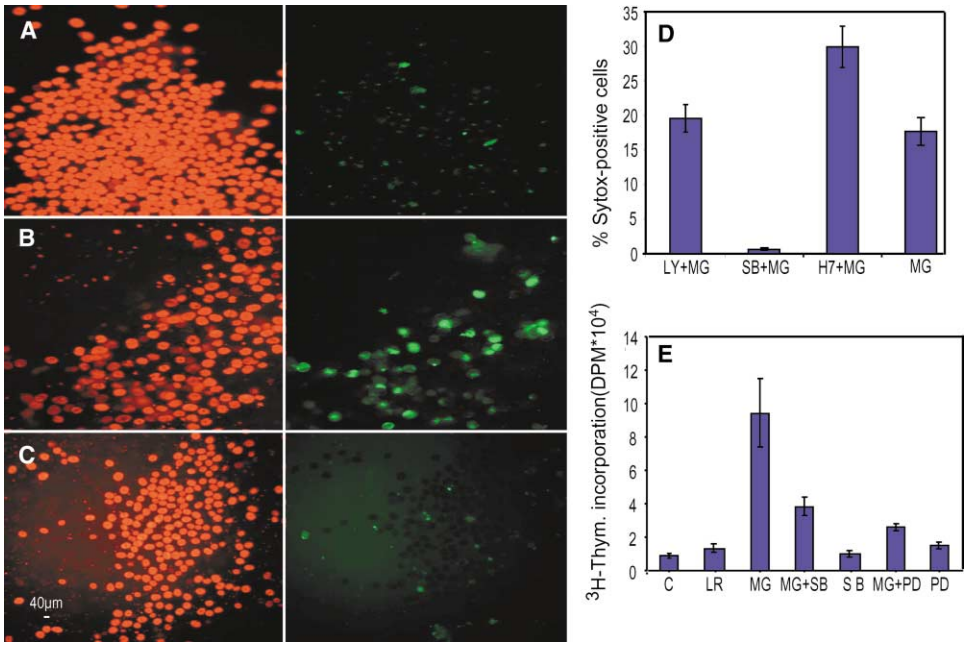


Figure 3. Cell Death and Thymidine Incorporation in *P. gatunense* following Exposure to *Microcystis* With or Without Protein Kinase Inhibitors

Cell death was indicated by sytox fluorescence (green); the red images show chlorophyll autofluorescence.

(A) Control *P. gatunense* cells.

(B) Cells exposed to *Microcystis* MG for 24 hr.

(C) Cells incubated with 10 μM SB for 2 hr prior to 24 hr with *Microcystis* MG.

(D) Extent of cell death in cultures treated with protein kinase inhibitors as in Figure 2F, 2 hr prior to exposure to *Microcystis* MG. Data presented here show the percentage of cell death in excess of that observed for the control (5%). The data points provided are ratio estimates ± the SE, calculated as in [34]; the number of cells per field was larger than 500.

(E) $^3$H-thymidine incorporation into *P. gatunense* during 72-hr treatments with spent medium of *Microcystis* MG, microcystin-LR (50 μg/L), or kinase inhibitors.

of certain kinases (such as the 72 kDa), whereas the activation of other kinase pathways required the presence of the whole organism or an unknown component in its spent medium.

The presence and level of the phosphorylated (active) form of certain protein kinases was examined by Western analyses with an antibody raised against the activated form of mammalian p38 MAPK. Notably, the amount of a 38 kDa protein increased significantly in response to exposure to *Microcystis* MG or its toxin (Figure 4D). To the best of our knowledge, this is the first demonstration of the presence of homologs of such stress-activated kinases in a dinoflagellate. These proteins have been assigned a major role in the response of various organisms to stress conditions [23, 25]. Taken together, the results presented in Figure 4 suggest that the perception of *Microcystis* sp. by *P. gatunense* is mediated by changes in the activity, abundance, and expression (see the Supplementary Material) of specific protein kinase families that are part of a signal transduction pathway.

## Response of *Microcystis* to *Peridinium*

The growth experiments demonstrated nutrient-independent inhibition of *Microcystis* MG growth by the presence of *P. gatunense*, but only when the initial *P.*

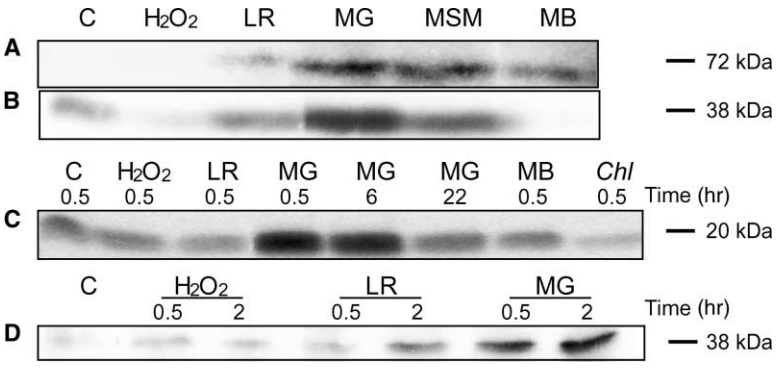


Figure 4. Activities, Level, and Expression of Kinases in *P. gatunense*

(A–C) In-gel kinase activities in protein extracts from *P. gatunense* exposed to the following treatments: C, control; H, 0.5 mM $H_2O_2$; LR, 50 μg/L microcystin-LR; MG, *Microcystis* MG ($10^6$ cells/ml); MSM, *Microcystis* MG spent medium; MB, *Microcystis* MB ($10^6$ cells/ml); Chl, *Chlorella* ($10^6$ cells/ml). The duration of the treatments was 0.5 hr in (A) and (B) and 0.5–22 hr in (C).

(D) Western analysis of total proteins extracted from *P. gatunense* using an antibody raised against the phosphorylated mammalian p38. Treatments and exposure times are indicated in the figure.

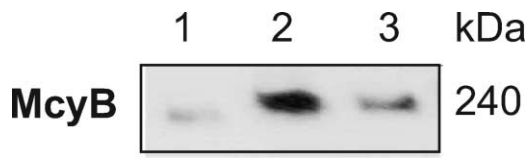


Figure 5. Abundance of McyB in *Microcystis* in Response to Spent Media from *P. gatunense* and *C. reinhardtii*

Western blot of *Microcystis* MG proteins using an antibody against McyB. Lane 1, dilution with equal volumes of fresh *P. gatunense* medium; lane 2, spent *P. gatunense* medium; lane 3, spent *C. reinhardtii* medium. Initial *Microcystis* density was $8 \times 10^6$ cells/ml. Final cell counts in these samples were $1 \times 10^7$, $2.4 \times 10^6$, $6.6 \times 10^6$ cells/ml in lanes 1–3, respectively. Samples were taken after 24 hr of treatment.

*gatunense* inoculum was above 1000 cells/ml (Figure 1B). To further examine the possibility that *Microcystis* recognized the presence of *P. gatunense*, we applied spent *P. gatunense* medium on *Microcystis* MG cultures. This treatment resulted in loss of buoyancy of the *Microcystis* cells, followed by massive lysis of approximately 75% of the cells within 24 hr. Despite the substantial *Microcystis* MG cell death observed here, the level of McyB, a 240 kDa subunit of the peptide synthetase complex involved in microcystin biosynthesis [6], detected in the remaining cells increased dramatically (Figure 5). The extent of cell lysis was only about 15% when spent medium from *Chlamydomonas reinhardtii* was applied, and the level of McyB was far less affected (Figure 5).

The observation that spent *Peridinium* medium caused lysis of the *Microcystis* cells and enhanced the cultures' potential for toxin production may help to elucidate the biological role and regulation of microcystin production. Earlier studies [26] showed that the media of stationary-phase *C. reinhardtii* cells inhibited formation of this toxin in *Anabena flos-aquae*. In our study, which focused on the interactions between two naturally co-occurring organisms, toxin production capability was stimulated rather than inhibited. The difference between these results highlights the complexity of the biotic interactions in water bodies.

## Conclusions and Perspectives

The results presented here provide the first report on mutual allelopathic interactions between a dinoflagellate and a toxic cyanobacterium and on some of the cellular responses involved. The statistical analyses indicated that delays in the succession (or even absence) of the annual *P. gatunense* blooms during the last decade (Figure 1) were correlated with increased abundance of *Microcystis* in the lake. Laboratory experiments showed that allelochemical(s) excreted by *Microcystis* MG to its medium affected both growth and mortality of *P. gatunense* via species-specific, density-dependent signal transduction pathways. Certain MAPKs and cysteine proteases [8] appear to be involved in the regulation of the oxidative burst and of the fate of the *P. gatunense* cells. We suggest that the composition of the phytoplankton assemblage in the Sea of Galilee is strongly affected by allelochemicals. These substances could contribute to the dominance of *P. gatunense* during 30 years of monitoring, when the abundance of *Microcystis* was low. The nature of the cascade of events that determines the fate of an individual *P. gatunense* cell, i.e., death, division, or cyst formation [8], is not clear. Nevertheless, at the population level, communication via medium-mediated substance(s) may confer an ecological advantage by eliminating less fitted cells by PCD-like processes [27–29].

During its growth, *Peridinium* forms large and dense patches in the lake [30]. Growth in patches has been attributed to physical parameters [31], but the advantage to be gained by a motile organism, such as *P. gatunense*, from a patchy growth habit is poorly understood. Growth in patches of high cell densities could impose local unfavorable conditions for *P. gatunense*, including high pH and nutrient limitations [32]. In view of data obtained here, we propose that a density-dependent defense capability (arising from changes in the concentration of secreted factors) may provide the biological dimension to current physical models simulating patchy growth forms in phytoplankton [33]. The enhanced defense capability conferred by the high cell density may compensate for the aggravated nutrient limitation within the patch. Finally, the observation that *P. gatunense* strongly depressed the viability of *Microcystis*, but also raised its potential for toxin formation, may help to elucidate the ecological role of microcystin formation. This study may also open up the prospect of biological control of toxic *Microcystis* blooms that pose a serious threat to fresh-water lakes worldwide.

### Supplementary Material

Supplementary Material including the Experimental Procedures, which includes the protocol used to identify a clone encoding a receptor kinase from *Peridinium gatunense* (designated rkd1), is available at http://images.cellpress.com/supmat/supmatin.htm. We also show that the expression of rkd1 was strongly affected by the exposure to spent *Microcystis* medium

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