

Growth Tendencies in *Didymosphenia geminata*

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ABSTRACT

Didymosphenia geminata cells, when secreting excessive exopolysaccharide stalks, can coalesce and form blooms that affect their local community. It is unknown what causes *D. geminata* cells to produce stalk, as not all *D. geminata* cells do produce stalk. Natural growth patterns and the potential for *D. geminata* cells to utilize a population dynamic such as quorum sensing to begin to produce stalk was tested through placement of microscope slides in Copper Creek and the East River (to observe undisturbed growth over time) and observations of stalk production based on compartmentalized *D. geminata* populations of different cell densities (8, 80, 800, and 8000 cells/cm²), to monitor how cell density affects stalk production. Statistical analyses found that stalk production (measured by the frequency of cells with stalks, or FSC) was significantly different due to differing cell densities, and that after a threshold of approximately 15 cells/cm², the rate of change of FSC changed, indicating that cell density does affect stalk production.

INTRODUCTION

Didymosphenia geminata (Lyngbye) M. Schmidt is a benthic diatom that grows best in low-nutrient, unshaded, low temperature rivers (Bothwell and Kilroy 2011a). *D. geminata* has been researched heavily since the 1900's due to increased bloom formation in several areas, from New Zealand to Chile to the United States (Taylor and Bothwell 2014). The problem that comes with increased bloom formation, however, is not due to a higher *D. geminata* cell count, but is attributed to excessive stalk production and mat formation in oligotrophic fresh waters that impact invertebrates and fish (Jellyman and Harding 2016). Much research has been done on the causes of exopolysaccharide stalk production, most notably on characterization of the stalks and on what causes them to grow. The stalks, which make up most of the biomass of *D. geminata* algal mats, are made of exopolysaccharide (EPS) of which have an increased secretion rate under nutrient-limited conditions, a common characteristic of benthic diatoms (Whitton et al. 2009). Furthermore, it has been shown that both stalk production and mat formation are directly linked to the concentration of available dissolved phosphorus in surrounding water, and that decreased phosphorus levels are related to increased stalk growth and mat formation, and that increased phosphorus levels are related to increased cell division rates (Bothwell and Kilroy 2011a; Bothwell and Kilroy 2011b; Bothwell and Kilroy 2012).

Stalk production is of specific interest because of the tendency of individual stalk-producing cells to coalesce and form tufts, which can lead to the formation of blooms (Figure 1); however, the presence of *D. geminata* cells does not necessarily mean that there will be blooms (Kilroy and Unwin 2011). What is not known is if there is a group dynamic such as quorum sensing that specifically triggers stalk production. Quorum sensing (QS) is a method of cellular communication, primarily associated with bacteria, in which organisms communicate with each other via cell-to-cell signaling, coordinating gene expression based on population density (Miller and Bassler 2001). Although it is mostly found in within bacterial populations, QS also persists between bacteria and their hosts, commonly in symbiotic relationships (Freestone 2013). In marine diatoms, however, QS analogs have been observed to regulate reproduction through the secretion of pheromones fucoserratene, homosirene, and ectocarpene (Hombeck and Boland 1998; Pohnert and Boland 1996; Derenbach 1986). QS has not yet, however, been observed in freshwater diatoms. The propensity of *D. geminata* to utilize QS to regulate its own properties, such as stalk production, has been previously unexamined, but examination of QS in *D. geminata* is important in understanding the ways in which *D. geminata* cells do or do not communicate, allowing for the identification of mechanisms causing recent increases in blooms and thus the enactment of management and policy decisions.

The goal of my research was to test whether *D. geminata* population density affects EPS stalk formation. The objectives of my proposed research were twofold: (1) observe natural *D. geminata* cell densities and stalk production over time and at a high temporal frequency, and (2) determine if QS is linked to *D. geminata* stalk production. The main hypothesis of my proposed research was that *D. geminata* population density would be positively related to stalk formation. This hypothesis was tested using both observational and experimental techniques.

To test the main hypothesis, observational and experimental techniques were used to evaluate *D. geminata* per the following aims:

- 1) Observe changes in *D. geminata* growth dynamics over time. The working hypothesis for this aim was that the proportion of cells producing stalks would increase with cell density (cells/cm²).
- 2) Determine if QS is linked to *D. geminata* stalk production. The working hypothesis for this aim was that more cells would produce stalks when part of a larger population of cells. It is important to note that testing the effects of cell density on EPS stalk production is not a direct test of quorum sensing activity. Such a relationship could provide insight as to the possibility of existing quorum sensing activity, but QS can only be directly tested through genetic analysis.

METHODS

Natural growth observations

In situ growth observations. Data were collected in two rivers known to produce *D. geminata* blooms, in the East River and in Copper Creek within the Gothic, CO town site (Figure 2) to observe natural *D. geminata* cell growth tendencies. Glass microscope slides affixed to ceramic tiles were placed in shallow water near the riverbank at depths averaging 16.3 cm in Copper Creek and 35.8 cm in the East River (Figure 3). Tiles were removed from the river and counted under a compound microscope at a high temporal frequency, with tiles in the river for colonization from a span of two to forty-five days in Copper Creek, and two to thirty-seven days in the East River. On each removal date, three tiles (nine glass microscope slides) were removed and quantified. Glass microscope slides were analyzed for colonization rate (cell density, measured in cells/cm²), frequency of dividing cells (FDC) of both cells with stalks and cells without stalks, stage of cell division (Bothwell), and frequency of cells with stalks (FSC). A segmented regression was performed in R to determine a threshold after which stalk production, in terms of FSC, changed due to cell density.

Nutrient (NH_4 , NO_3 , and SRP) chemistry. Nutrient concentrations, notably nitrate nitrogen ($\text{NO}_3^- \text{N}$ mgL^{-1}), ammonium nitrogen ($\text{NH}_4\text{-N}$ μgL^{-1}), and soluble reactive phosphorus (SRP μgL^{-1} , also known as dissolved reactive phosphorus, or DRP) were measured at various time points spanning the time in which the glass microscope slides were in the two rivers. To measure $\text{NO}_3^- \text{N}$, $\text{NH}_4\text{-N}$, and SRP, water samples were collected and analyzed via the Thermo Fischer Scientific GENESYS™ 10s UV-Vis Spectrophotometer ($\text{NO}_3^- \text{N}$ and SRP) and the Turner Designs Trilogy® Laboratory Fluorometer ($\text{NH}_4\text{-N}$) via methods adapted from Crumpton et al. 1992; Murphy and Riley 1958; Murphy and Riley 1962; Ostrofsky and Rigler 1987; Patton and Truitt 1992; Solorzano and Sharp 1980; Strickland and Parsons 1972.

Data analysis. Linear regressions were completed in R to determine a) the effect of days of growth on cell density (cells/ cm^2), frequency of dividing cells (FDC) for both cells with stalks and cells without stalks, and frequency of stalked cells (FSC), b) the effect of cell density (cells/ cm^2) on FDC and FSC, and c) the effect of FDC on FSC and vice versa. Furthermore, linear regressions were completed to determine the effects of $\text{NH}_4\text{-N}$, $\text{NO}_3^- \text{N}$, and SRP on the same response variables: cell density (cells/ cm^2), frequency of dividing cells (FDC), and frequency of stalked cells (FSC).

Cell density experiment

Well plate setup. To test the effect of *D. geminata* cell density on stalk production, rocks showing visible *D. geminata* tufts in Copper Creek were scrubbed to remove the maximum possible number of cells (tufts were scrubbed to ensure that stalk-producing cells were collected). Collected samples were sieved, first in a 125 μm sieve and second in a 45 μm sieve to break cells from stalks. Sieved samples were quantified for cell counts under a compound microscope, and cell densities (cells/ cm^2) were calculated using the image processing program ImageJ. Four cell density solutions were created, each with a difference of one order of magnitude, resulting in four solutions of 8 cells/ cm^2 , 80 cells/ cm^2 , 800 cells/ cm^2 , and 8000 cells/ cm^2 (Figure 4a). Six Corning Costar® 12-well plates were filled with the four densities, with 1 mL of solution and 3 mL of chilled and filtered montane river water from the East River. Three replicates of each density were pipetted into each plate. Each well in three out of the six 12-well plates was spiked with 400 μg P to stimulate cell division. All six plates were placed in a flow-through channel in the East River to allow for growth in natural water conditions, specifically to maintain growth at a natural water temperature (Figure 4b). On each well plate collection date, all well plates were collected, and those that were not analyzed (and were being put back in the flow-through channel) were given fresh water and were re-spiked with 400 μg P, if applicable.

Data analysis. Two two-way ANOVA models were completed in R to determine the effects of cell density (cells/cm²) and the addition of P on FDC and FSC, as well as the impact of both treatments together. Following each two-way ANOVA, one-way ANOVA models were completed to test the effect of each of the two treatments (cell density and P-addition) individually, and pairwise t-tests (adjusted using the Bonferroni correction model) were performed to compare the means between the categories within each of the two treatments.

RESULTS

Natural growth observations in Copper Creek

Effect of days of colonization. Microscope slides placed in Copper Creek were colonized by *D. geminata* with a range of cell densities of 0 cells/cm² (after two days of colonization) to 321.2 cells/cm² (after forty-five days of colonization). Over said forty-five days of colonization, cell density increased exponentially with days of colonization, an overall significant increase (Figure 5). Overall frequency of dividing cells increased linearly with days of colonization, but not significantly, at a rate of change in overall FDC of 0.60% per day (Figure 6a); however, although overall FDC (both cells with stalks and cells without stalks) increased with days of colonization, FDC of cells without stalks had no significant change (Figure 6b), while FDC of cells with stalks increased significantly and linearly (Figure 6c). FSC increased significantly and linearly with days of colonization at a rate of increase of 1.8% per day (Figure 6d).

Effect of cell density (cells/cm²).

Cell density increased from 0 cells/cm² to 321.2 cells/cm² over forty-five days of colonization. As cell density increased, overall FDC increased, although not significantly, at a rate of change in FDC of 0.13% per cell (Figure 7a). FDC of cells without stalks encountered no significant change (Figure 7b). FDC of cells with stalks increased, but not significantly, at a rate of change in FDC of 0.11% per cell (Figure 7c). FSC increased significantly with a rate of change in FSC of 0.27% per cell increase in cell density (Figure 7d). A segmented regression was used to find a statistical threshold after which the rate of change of FSC changed, and found that the slope changed from 0.025 to 0.0013 at approximately 15 cells/cm² (Figure 7e).

Effect of FDC and FSC on each other. FSC increased largely as FDC increased, but not significantly, at a rate of 29% (Figure 8a). Similarly, FDC increased as FSC increased, but not significantly, and only slightly (Figure 8b).

Effect of nutrient changes. Changes in SRP, NO₃, and NH₄ (measured from 28 May 2017 through 20 July 2017) had no significant impacts on overall FDC, FSC, or cell density regarding the cells

counted on microscope slides from Copper Creek during the time in which water chemistry was analyzed.

Natural growth observations in the East River

Effect of days of colonization. Microscope slides placed in the East River were colonized by *D. geminata* with a range of cell densities of 0 cells/cm² (after two days of colonization) to 76.1 cells/cm² (after thirty-seven days of colonization). Over said thirty-seven days of colonization, cell density increased exponentially with days of colonization, an overall significant increase (Figure 9). Frequency of dividing cells also increased significantly with days of colonization at a rate of change in overall FDC of 1.2% per day, but at no specific pattern (Figure 10a). In addition to an increase in overall FDC (FDC of both cells with stalks and cells without stalks) with days of colonization, FDC of cells without stalks increased significantly at a rate of 3.1% per day (Figure 10b), and cells with stalks increased significantly at a rate of 1.7% per day (Figure 10c). FSC increased significantly and linearly with days of colonization at a rate of increase of 3.1% per day (Figure 10d).

Effect of cell density (cells/cm²). Cell density increased from 0 cells/cm² to 76.1 cells/cm² over thirty-seven days of colonization. As cell density increased, overall FDC significantly increased at a rate of change in FDC of 0.56% per cell (Figure 11a). FDC of cells without stalks increased significantly at a rate of change in FDC of 0.84% per cell (Figure 11b). FDC of cells with stalks also increased significantly at a rate of change in FDC of 0.17% per cell (Figure 11c). Furthermore, FSC increased significantly with a rate of change in FSC of 1.2% per cell increase in cell density (Figure 11d).

Effect of FDC and FSC on each other. FSC increased significantly as FDC, but once FSC reached approximately 0.7, the relationship remained relatively horizontal (Figure 12a). Similarly, FDC increased significantly as FSC increased, but after a FSC reached approximately 0.7, the relationship remained relatively vertical (Figure 12b).

Effect of nutrient changes. The effects of changes in SRP, NO₃, and NH₄ (measured from 28 May 2017 through 20 July 2017) were unable to be quantified due to a lack of colonization of *D. geminata* cells on the microscope slides in the East River at that time. *D. geminata* cells did not colonize the microscope slides in the East River until 27 July 2017.

Cell density experiment

Effects of cell density (cells/cm²) and P-addition on FSC. A two-way ANOVA completed in R found that cell density had a significant effect on FSC ($p = 3.35e-09$, $F = 99.3$), but P-addition did not ($p = 0.714$, $F = 0.138$), and that cell density and P-addition did not have a combined significant effect on FSC ($p = 0.760$, $F = 0.0960$). Therefore, a one-way ANOVA concerning the effect of cell density on FSC was completed in R and found that cell density (cells/cm²) had a significant impact on FSC for each of the four cell densities included: 8 cells/cm², 80 cells/cm², 800 cells/cm², and 8000 cells/cm² ($p = 5.96e-10$, $F = 108$), with FSC ranging from a mean of 0.143 (8 cells/cm²) to 0.473 (8000 cells/cm²) after only four days of growth in the well plates set in the East River (Figure 13). Furthermore, a pairwise t-test utilizing the Bonferroni correction model found that the difference between each of the FSC means for each of the four cell densities was statistically significant, with p-values ranging from 4.4e-08 to 0.020.

Effects of cell density (cells/cm²) and P-addition on FDC. A two-way ANOVA completed in R found that P-addition had a significant effect on FDC ($p = 0.083$, $F = 3.334$), but cell density did not ($p = 0.21$, $F = 1.705$), and that cell density and P-addition did not have a combined significant effect on FDC ($p = 0.425$, $F = 0.664$). Therefore, a one-way ANOVA concerning the effect of P-addition on FDC was completed in R and found that P-addition had a significant impact on FDC ($p = 0.084$, $F = 3.279$), with FDC of well plates that had been spiked with P being higher than that of well plates that had not been spiked with P in 75% of cell densities across all well plates (Figure 14).

DISCUSSION

Natural growth observations

Copper Creek. The results from the natural growth observations are consistent with the expectation that overall growth would increase with time, as *D. geminata* cells have more time to settle and attach to the microscope slide substrate, increasing cell density on the microscope slide. As cells attached and remained on the substrate longer, there was an increase in time available for cell division and/or stalk production to take place; overall FDC increased with time and, consequently, cell density. As FDC increased, however, FDC of cells without stalks decreased, while FDC of cells with stalks increased, likely due to an overall decrease in total cells without stalks and an overall increase in cells producing stalk. In addition to overall FDC, FSC increased with both time and cell density, supporting the premise that stalk production and cell density have a clear relationship. Moreover, a cell density threshold after which FSC begins to be seen at much higher levels is seen to exist at approximately 15 cells/cm² (Figure 7e). It is surprising that changes in SRP, NO₃, and NH₄ over time had no significant impact on FDC and/or FSC, as these nutrients, specifically P, are consistently linked to division stimulation and stalk

suppression (Bothwell and Kilroy 2011a; James et al 2015). These nutrient effects, however, are typically observed at bloom conditions, and because these growth observations were not performed in bloom conditions, they are not necessarily applicable.

East River. There was little to no growth (maximum of five *D. geminata* cells on a given microscope slide of area 10.5 cm²) seen on the slides placed in the East River until 27 July 2017, when there was a sudden increase to seventeen *D. geminata* cells seen on a slide of area 9.25 cm². In addition to an increase in cell density, there was a sudden increase in overall FDC and in FSC on this date that continued until the end of data collection on 11 August 2017. It is possible that *D. geminata* failed to colonize the slides placed in the East River due to weather events, including a nearby landslide on Bellview Mountain and several heavy rainfall events in mid-to-late July. However, *D. geminata* cells were able to attach to rocks in the East River, resulting in the presence of *D. geminata* blooms in the East River throughout this period. Still, it is possible that because the tiles with affixed microscope slides were placed on the stream bottom of the East River in a relatively smooth area (few large rocks), consistent weather events may have resulted in constant rapidly moving mud and sediment above and directly in contact with the microscope slides, preventing *D. geminata* attachment, despite the fact that *D. geminata* is able to attach to any texture substrate (Bergey et al. 2009).

Cell density experiment

P-addition. Increased FDC due to an increase in P was expected based on previous research indicating that P stimulates *D. geminata* cell division (Bothwell and Kilroy 2011a). Still, it is interesting to note that a change in P affected FDC in the well plate set up but not in the observational study as reported previously, even though neither *D. geminata* population was measured at bloom conditions; the significant impact of P in the well plate experiment could be due to the contained and concentrated sample within each well, so a change in P had more of an effect in the smaller sample area than it did on the microscope slides placed in the river, where changes in P were spread out over the whole area of the river and continued to flow past the plates, rather than staying in the vicinity where cell growth was being measured. *D. geminata* cells in the wells spiked with P had more time to take up the added P and utilize it, resulting in changes in FDC.

Cell density and stalk production. According to the natural growth observations in Copper Creek, the rate of change of FSC changed at a FSC of approximately 50% and a cell density of approximately 15 cells/cm² (Figure 13). These data are consistent with the results of the cell density experiment; the wells containing a density of 80 cells/cm² had a significantly higher FSC than that of the wells containing a density of 8 cells/cm². Therefore, after the 15 cells/cm²

threshold, the wells experienced a significant change in FSC. Such a threshold could be the result of interactions between *D. geminata* cells within the population in each well, or a communication mechanism relating cell density to stalk production, such as quorum sensing. Quorum sensing, however, cannot be tested directly without genetic analyses.

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FIGURES



Figure 1



Figure 2



Figure 3

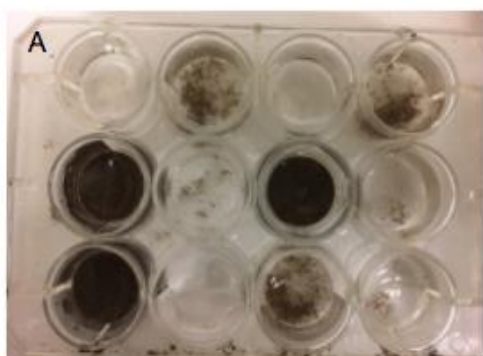


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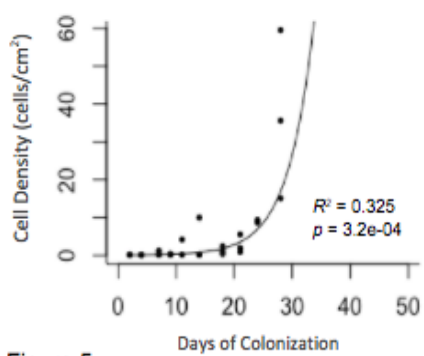


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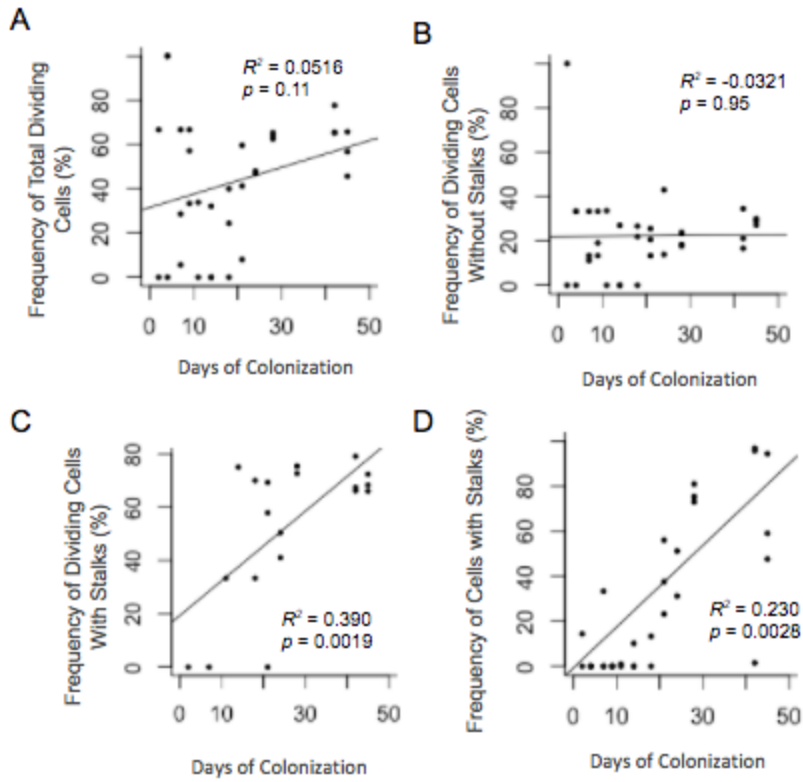


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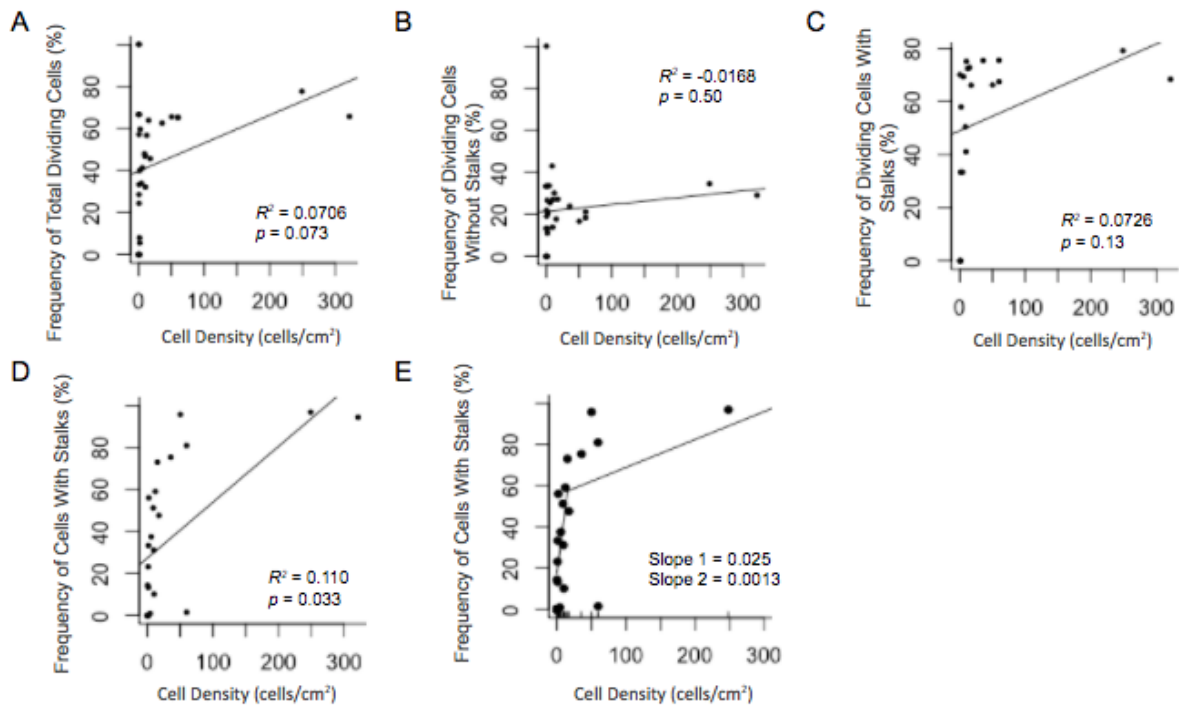
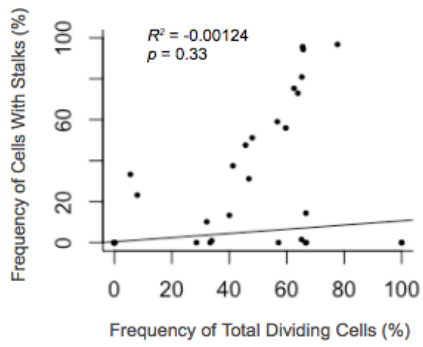


Figure 7

A



B

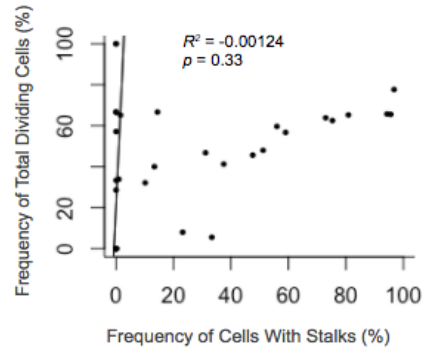


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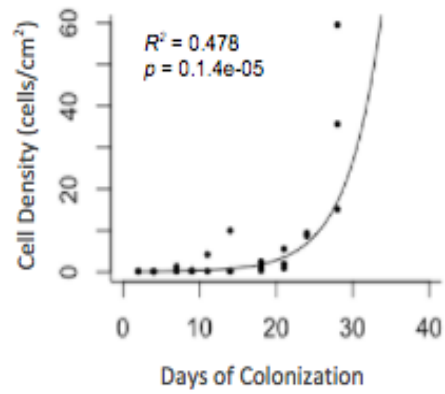


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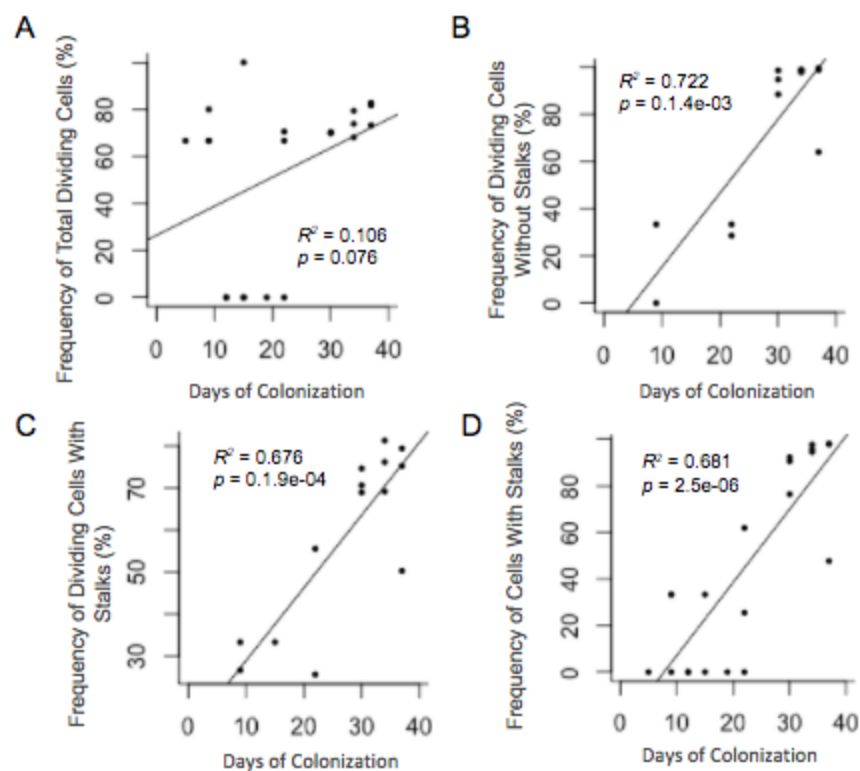


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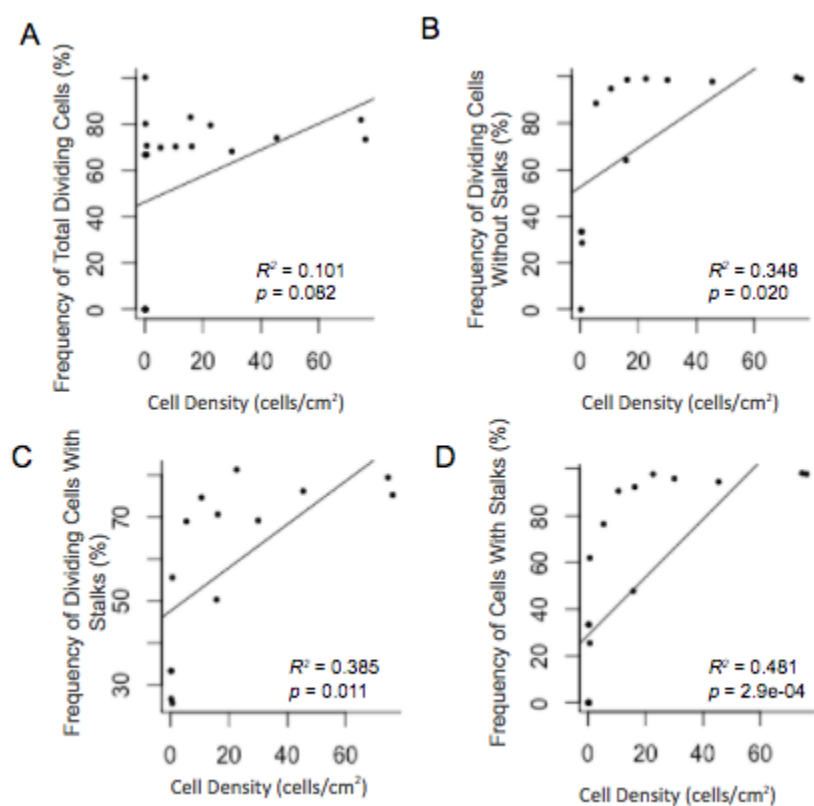


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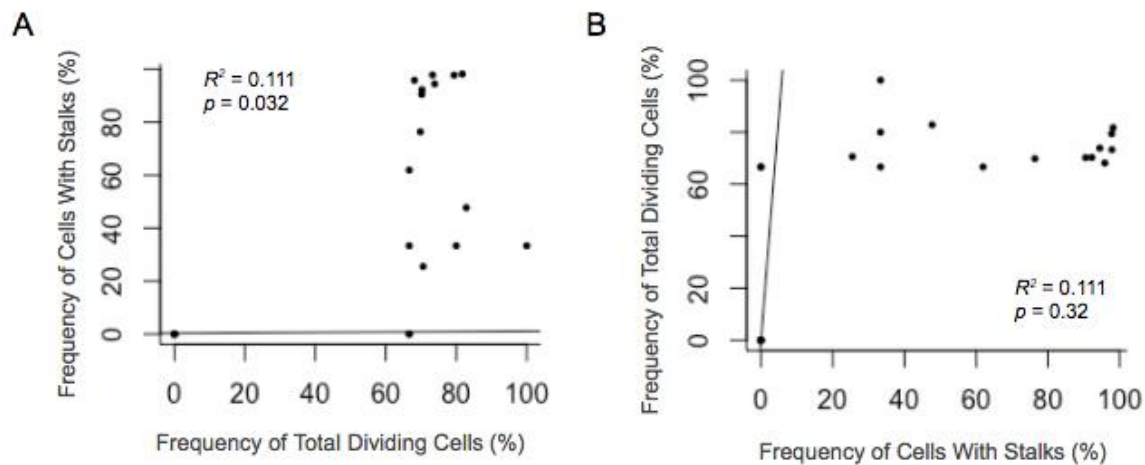


Figure 12

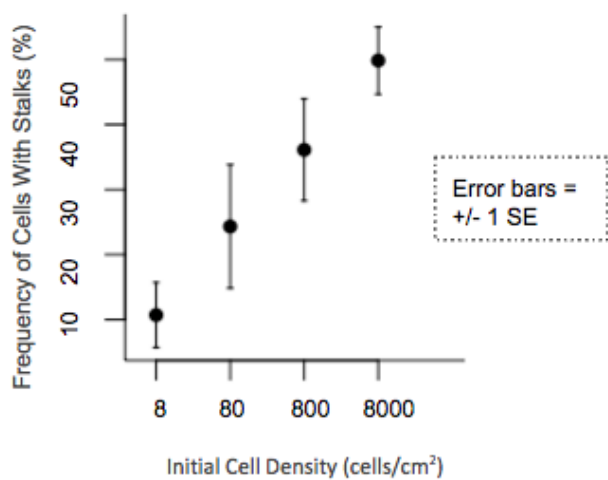


Figure 13

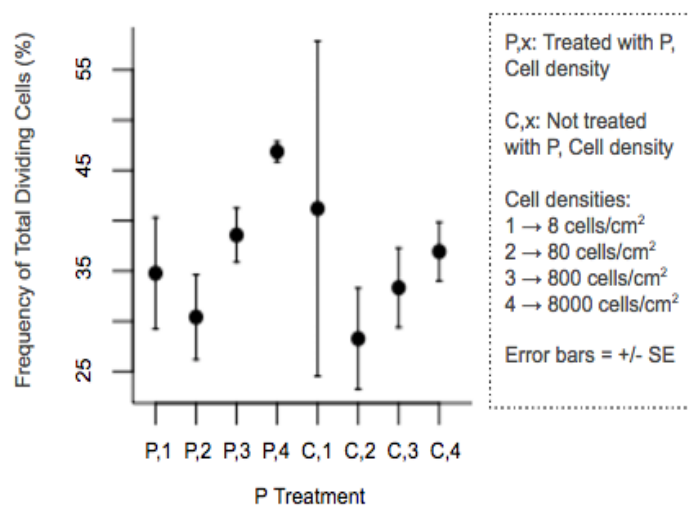


Figure 14

FIGURE LEGENDS

Figure 1 *D. geminata* tufts found on a rock in Copper Creek on 21 July 2017.

Figure 2 Copper Creek and the East River near the Gothic, CO town site were used as observational study sites for natural growth observations. A flow-through channel containing the well plates for the cell density experiment was placed in the East River at the Gothic, CO town site.

Figure 3 Glass microscope slides were affixed to ceramic tiles via electrical tape and were placed in shallow water in both Copper Creek and the East River. Tiles were removed from the river and the slides examined under a compound microscope to analyze *D. geminata* colonization rate (cell density, measured in cells/cm²), frequency of dividing cells (FDC) of both cells with stalks and cells without stalks, stage of division, and frequency of cells with stalks.

Figure 4 a) Each 12-well plate had three replicates of solutions containing four *D. geminata* cell densities (cells/cm²): 8, 80, 800, and 8000 b) Six 12-well plates were placed in a flow-through channel in the East River.

Figure 5 Time course observations of *D. geminata* cell density. Microscope slides placed in Copper Creek were collected periodically over forty-five days and analyzed for changes in cell density. Log10 of a linear regression was performed to find the best line of fit.

Figure 6 Time course observations of *D. geminata* a) total frequency of dividing cells b) frequency of dividing cells without stalk c) frequency of dividing cells with stalk and d) frequency of cells with stalk. Microscope slides placed in Copper Creek were collected periodically over forty-five days. Linear regressions were performed to determine statistical relevance.

Figure 7 *D. geminata* cell density observations in relation to a) total frequency of dividing cells b) frequency of dividing cells without stalk c) frequency of dividing cells with stalk d) frequency of cells with stalk and e) a stalk production threshold. Microscope slides placed in Copper Creek were collected periodically over forty-five days. Linear regressions were performed to determine statistical relevance. A segmented regression was performed to determine a stalk production threshold value of approximately 15 cells/cm².

Figure 8 A visual representation of the effect of *D. geminata* a) cell division rates on stalk production rates and b) stalk production rates on cell division rates in Copper Creek. Linear regressions were performed to determine statistical relevance.

Figure 9 Time course observations of *D. geminata* cell density. Microscope slides placed in the East River were collected periodically over thirty-seven days and analyzed for changes in cell density. Log10 of a linear regression was performed to find the best line of fit.

Figure 10 Time course observations of *D. geminata* a) total frequency of dividing cells b) frequency of dividing cells without stalk c) frequency of dividing cells with stalk and d) frequency of cells with stalk. Microscope slides placed in the East River were collected periodically over thirty-seven days. Linear regressions were performed to determine statistical relevance.

Figure 11 *D. geminata* cell density observations in relation to a) total frequency of dividing cells b) frequency of dividing cells without stalk c) frequency of dividing cells with stalk d) frequency of cells with stalk and e) a stalk production threshold. Microscope slides placed in the East River were collected periodically over thirty-seven days. Linear regressions were performed to determine statistical relevance.

Figure 12 A visual representation of the effect of *D. geminata* a) cell division rates on stalk production rates and b) stalk production rates on cell division rates in the East River. Linear regressions were performed to determine statistical relevance.

Figure 13 Significant differences among mean frequency of cells with stalks for four different cell densities (cells/cm²): 8, 80, 800, and 8000. *D. geminata* cells were grown in well plates in different densities to examine the relationship between cell density and stalk production. One-way ANOVA was performed to determine statistical relevance among the four densities.

Figure 14 Differences among mean frequency of total dividing cells among four different cell densities due to 400 ug P additions. *D. geminata* cells were grown in six well plates at four different cell densities. Three of the six well plates were spiked with 400 ug P at three different times. One-way ANOVA was performed to determine the impact of P-addition on frequency of total dividing cells. Frequency of total dividing cells was higher in P-spiked well plates in 75% of cell densities.