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Colony formation in *Scenedesmus:* No contribution of urea in induction by a lipophilic *Daphnia* exudate

Abstract—The common green alga *Scenedesmus* may respond morphologically to numerous environmental factors. The formation of colonies in *Scenedesmus* resulting from exposure to grazer (*Daphnia*) excreta is of particular interest since the induced colony formation may be an induced defense. Recent studies suggested that unicell-colony transformation in *Scenedesmus* could result from urea released by actively feeding *Daphnia* and/or by a more lipophilic substance that could be retained by lipophilic solid phase extraction (C18-SPE). We investigated the generality of the contribution of urea to the overall morphogenetic activity by separating urea and the more lipophilic infochemical using C18-SPE. No morphogenetic effect of urea was detected in two different algal growth media, whereas *Daphnia* water induced colony formation. After C18-SPE of active *Daphnia* water, the urea-containing run-through was inactive, whereas the desorbed eluate remained active. Thus, colony-inducing activity is more lipophilic than urea-related.

Recent studies indicate that detection of predators through chemical messengers (infochemicals sensu Dicke and Sabelis 1988) is extremely widespread in aquatic systems, with prey organisms reducing the risk of being eaten by behavioral or morphological responses to infochemicals (Larsson and Dodson 1993; Tollrian and Harvell 1999; Brönmark and Hansson 2000; Wisenden 2000). In contrast to the large number of studies on induced defenses in aquatic animals, only a limited number have addressed grazer-induced defenses among phytoplankton (Hansson 1996; Wolfe et al. 1997). Colony formation in the common freshwater green alga *Scenedesmus* has been the most examined subject in this regard (Hessen and Van Donk 1993; Lampert et al. 1994; Von Elert and Franck 1999; Wiltshire and Lampert 1999).

Hessen and Van Donk (1993) were the first to discover that a chemical substance released from the grazer *Daphnia* stimulated the formation of colonies in the otherwise unicellular strain of *Desmodesmus subspicatus* (formerly known as *Scenedesmus subspicatus* sensu Hegewald 2000). The phenomenon was confirmed by Lampert and coworkers (1994) using a nonspiny species, *Scenedesmus obliquus* (formerly known as *S. acutus* sensu Van Hannen et al. 2000), which showed a dramatic increase in the number of colonies when the initially unicellular culture was exposed for 2 d to medium in which *Daphnia* had been grown. In *Scenedesmus*, colony formation is not a lumping of individual cells, but a defense against grazing. For instance, the grazing rate of a 1.75 mm *Daphnia* was considerably reduced when the proportion of eight-celled colonies was high (Hessen and Van Donk 1993). Accordingly, the phenomenon of grazer-activated colony formation in *Scenedesmus* could be interpreted as an induced defense.

One of the important prerequisites for an adaptive inducible defense is that the information should be reliable and predictable and apprise the algae about the presence of active grazers (Tollrian and Harvell 1999). Especially for small aquatic organisms such as algae that lack sophisticated organs to detect acoustic and visual cues, chemicals may be important carriers of information. In order to advance this research, the chemical identity of the colony-inducing cues released by grazers is needed. Once it is known, the production and dispersal of infochemicals and the underlying sensory, physiological, and genetic capabilities in the receiving algae can be systematically investigated.

The colony-inducing compound has been characterized as a nonvolatile organic compound of low molecular weight, which is heat-stable, pH-resistant in the range 1–12, and probably not a protein (Lampert et al. 1994). Recent work of Von Elert and Franck (1999) has indicated that the biologically active component is moderately lipophilic and extractable by C18-solid phase extraction (SPE). Further chemical analyses showed that hydroxyl and amino groups could be excluded as moieties of the infochemical and that the major inducing factor is an olefinic carboxylic acid (Von Elert and Franck 1999). Wiltshire and Lampert (1999) have shown that urea produced by the zooplankter *Daphnia* may induce colony formation in the green alga *Scenedesmus;* urea is a polar molecule without lipophilic moieties. Thus, it seems that the *Daphnia* water functions as a carbon and salt cocktail where several single compounds such as the olefinic carboxylic acid and urea could work in synergy (Wiltshire and Lampert 1999). However, the absolute cell size differences induced with urea were rather low: the maximum increase was from 1.5 cells per colony to 2.5 cells per colony (Wiltshire and Lampert 1999). In contrast, in *Daphnia* water treatments an average of 5.8 cells per colony has been observed (Lampert et al. 1994). On account of its polar nature, urea should not be retained by C18-SPE. Hence C18-SPE provides a good way to investigate the contribution of urea to the overall morphogenetic activity by separating urea (run-through water) and the more lipophilic infochemical (methanolic eluate) investigated by Von Elert and Franck (1999) and Von Elert (in press). In this study, we investigate the generality of the small morphogenetic effect of urea and test whether urea significantly contributes to the biological activity released by *Daphnia.*

The Center for Limnology in Nieuwersluis (CL) experiment—The green alga used in this study, *Scenedesmus obliquus,* originated from the Max-Planck-Institute for Limnology (Plön, Germany) and is being maintained at the CL in 1-liter chemostats. In the CL experiment, the *S. obliquus* had been adapted to CHU12 medium (Müller 1972) by transferring an inoculum from the chemostat into CHU12 medium, in which the algae were grown for 2 weeks with CHU12

medium being renewed every third day. Inocula from this culture were then transferred into 50 ml fresh CHU12 medium in cellulose-plug stoppered 100-ml Erlenmeyer flasks and exposed to urea (Baker Chemicals) in the concentrations 0, 100, 200, 400, 800, and 1,600 μg L^{-1}. Five ml *Daphnia* water being added to *S. obliquus* in 45 ml fresh CHU12 medium served as positive controls, i.e., treatments in which colony formation was expected. The *Daphnia* water was obtained by incubating 25 *Daphnia magna* for 24 h in 100 ml RT medium (Tollrian 1993) with *S. obliquus* as food (~10 mg C L^{-1}), followed by filtration through a glass-fiber filter (GF52, Schleicher and Schuell). The initial algal density in the Erlenmeyer flasks was 1.6×10^4 particles ml^{-1}. The biotest was run in triplicate for 5 d in a climate-controlled cabinet at 20 °C on a rotating shaking device (80 rpm) in 16 : 8 h light : dark with an intensity of 175 μmol quanta m^{-2} s^{-1}. A subsample was taken daily from the incubations, and the algal density and size distribution were determined in the range from 3 to 25 μm equivalent spherical diameter (ESD) using a Coulter Multisizer II (100-μm capillary). After 2 d a subsample of 0.9 ml was preserved with 0.1 ml Lugol's fixative and the mean number of cells per colony was determined with a light-microscope by counting at least 100 particles (i.e., unicells as well as colonies). The mean particle volume as determined from Coulter analysis and the mean number of cells per colony after 48 h of incubation were statistically compared using one-way ANOVA followed by a Tukey's test.

The Limnological Institute in Konstanz (LI) experiment—In the LI experiment, *S. obliquus* was grown in a chemostat on WC (Woods Hole modified CHU 10) medium (Guillard 1975). Inocula were transferred into 100-ml Erlenmeyer flasks containing a total volume of 50 ml of WC medium modified by omitting TES and silica (WC*, Von Elert and Franck 1999). Controls were incubations in fresh WC*, as positive controls served incubations to which 5-ml filtered *Daphnia* water was added, whereas the treatments consisted of fresh WC* with urea added in the range 1.25–33 μM (75–2000 μg L^{-1}). Two different *Daphnia* waters were used. One was obtained by incubating 35 *D. magna* for 20 h in 100 ml WC* medium with *S. obliquus* as food; the other by incubating 35 *D. magna* in 100-ml filtered Lake Constance water with *S. obliquus* as food. Both *Daphnia* waters were filtered through a 0.2-μm membrane filter (OE66, Schleicher and Schuell, rinsed with 100 ml ultra-pure water before use) before addition to *S. obliquus* cultures. An additional treatment was comprised of inocula of *S. obliquus* in fresh WC* to which 1 μl of 10,000× concentrated *Daphnia* factor was added. The concentration was performed by passing 1 L of *Daphnia* water through a C18-SPE followed by desorption with 10 ml of methanol, which was subsequently evaporated to dryness and resuspended in 100 μl of methanol. The biotest was run in triplicate for 2 d in a climate-controlled cabinet at 20 °C on a rotating shaking device (100 rpm) in continuous light with an intensity of 120 μmol quanta m^{-2} s^{-1}. At the start and at the end of the experiment the algal size distributions and densities were determined routinely in the range of 3–30 μm ESD (equivalent spherical diameter; 150-μm capillary) using a CASY-1 (Schärfe-system) particle counter. Lampert et al. (1994) have shown that the mean particle volumes were highly correlated with the mean number of cells per colony. Thus, for statistical comparison of colony size mean particle volumes were used. The mean particle volumes determined from the particle counter were statistically compared using one-way ANOVA followed by a Tukey's test.

The fractionation experiments—The fact that urea is a polar molecule without lipophilic moieties suggested that urea would not be retained by lipophilic solid phase extraction (C18-SPE). This was tested by applying 50 ml of a solution of urea (2 mg L^{-1}, 1% methanol) to C18-SPE (500 mg, Analytichem) and by subsequent elution of the sorbent with methanol (for more details about the SPE *see* Von Elert and Franck 1999). Subsequently the methanol eluate was evaporated to dryness and resuspended in 15 ml of ultrapure water. The run-through was evaporated to dryness in order to remove the 1% of methanol and resuspended in 50 ml of ultrapure water. Both run-through and methanol eluate were assayed for urea (Merck urea test, results corrected for ammonia content).

In an additional experiment, control medium and *Daphnia* water were applied separately to C18-SPE according to Von Elert and Franck (1999). Both the run-through and the methanolic eluate, of control medium and *Daphnia* water, were assayed in a biotest in WC* as described above for the LI experiment. The same treatments were assayed in CHU12 using *S. obliquus* adapted to CHU12 in a supplemental biotest in 50-ml cellulose-plug stoppered Erlenmeyer flasks containing 25-ml CHU12 medium. Aliquots of 20 μl of the methanolic eluate from the control medium and the *Daphnia* water were added to separate flasks, whereas 5 ml of both types of run-through was added to discrete flasks containing the test algae in 20 ml medium. *S. obliquus* in 20 ml fresh CHU12 medium to which 5 ml *Daphnia* water was added served as positive controls. The inocula were obtained from *S. obliquus* cultures that had been adapted to CHU12 medium for 2 weeks. The initial algal density was 3.7×10^4 particles ml^{-1} and the test was run in triplicate for 2 d in a climate-controlled cabinet at 20 °C on a rotating shaking device (80 rpm) in continuous light with an intensity of 175 μmol quanta m^{-2} s^{-1}. Final algal density and size distribution were determined in the range from 3 to 25 μm ESD using a Coulter Multisizer II (100-μm capillary).

Results of CL experiment—In the bioassay run in CHU12 medium the mean particle volumes showed a rapid increase in the *Daphnia* water treatments compared to the controls, but not in the urea treatments (Fig. 1). One-way ANOVA indicated a significant difference among the various incubations after 48 h ($F_{6,14} = 742.6$; $P < 0.001$) and Tukey's test revealed that only the *Daphnia* water treatments were significantly different from the controls. Microscopic analysis after 2 d confirmed that only the *Daphnia* water treatments induced an increase in colony size with an average of 4.58 (± 0.07) cells per colony, compared to the controls and all urea treatments, which had on average 1.34 (± 0.04) cells per colony. The number of cells per colony in the *Daphnia*

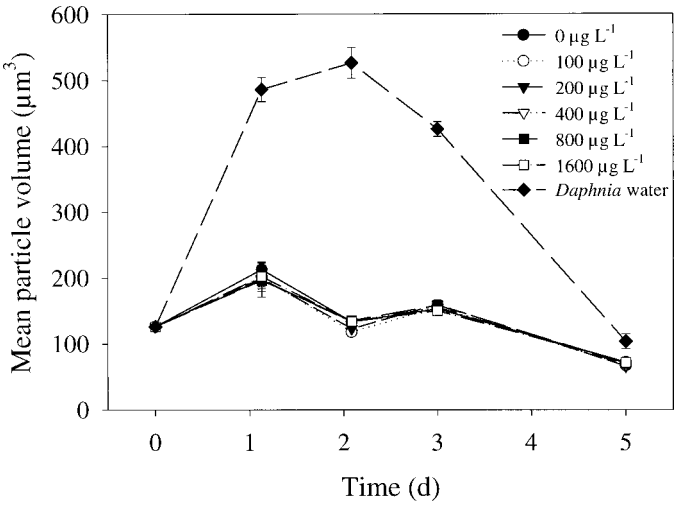

Fig. 1. Effect of *Daphnia* water and different concentrations of urea (0–1600 μg L^{-1}) on the course of the mean particle volume (μm^3) in *Scenedesmus obliquus* cultured for 5 d in CHU12 medium. Error bars represent 1 SD ($n = 3$).

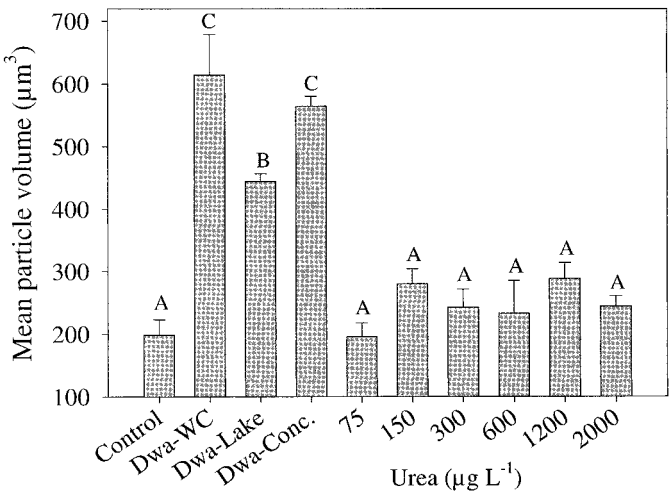

Fig. 2. Effect of *Daphnia* water [in WC* medium (Dwa-WC) and in filtered lake water (Dwa-Lake)], of 1 μl of 10,000× concentrated *Daphnia* factor (Dwa-Conc.) and of different concentrations of urea (75–2000 μg L^{-1}) on the mean particle volume (μm^3) in *Scenedesmus obliquus* cultured for 2 d in WC* medium. Error bars represent 1 SD ($n = 3$). Similar symbols (A, B, C) indicate homogeneous groups that are not significantly different at the 95% level (Tukey's test).

water treatments was significantly different from the controls ($F_{6,14} = 362.9$; $P < 0.001$).

Hence, in this experiment carried out in CHU12 medium urea had no morphogenetic effect on *S. obliquus.* After 5 d, the mean particle volumes of the *Scenedesmus* populations in the *Daphnia* water treatments were again similar to the control populations (Fig. 1).

Results of the LI experiment—One-way ANOVA indicated a significant difference among the various incubations in WC* after 48 h ($F_{9,20} = 66.1$; $P < 0.001$). Both *Daphnia* waters (in filtered lake water and in WC*) were active, as was the 1-μl concentrated *Daphnia* factor (Fig. 2). However, urea added in the range from 75 to 2000 μg L^{-1} was not active. Tukey's test following the ANOVA revealed three homogeneous groups: (1) the *Daphnia* incubation in WC* and the 1-μl concentrate, (2) the *Daphnia* incubation in lake water, and (3) the controls and urea treatments. Hence, also in this experiment urea had no morphogenetic effect.

Fractionation experiments—The addition of the urea solution to C18-SPE revealed that all urea was found in the run-through, whereas no urea was detected in the methanol eluate. This indicates that urea is not retained by C18-SPE, which was expected since urea is a polar molecule without lipophilic moieties.

In both biotests, which were run with the methanolic eluate and with the run-through of C18-SPE with control medium and with *Daphnia* water (Fig. 3), one-way ANOVA indicated significant differences in the mean particle volumes among treatments: WC experiment $F_{3,8} = 4.44$; $P = 0.041$; CHU experiment $F_{4,10} = 615.4$; $P < 0.001$). In both cases, Tukey's test disclosed that the mean particle volumes of *S. obliquus* exposed to the methanol eluate of *Daphnia* water applied to C18-SPE was significantly larger than in the other treatments, indicating that all of the colony-inducing activity is more lipophilic than urea (Fig. 3). No hints for a synergistic effect were found, as the activity of the urea-free fraction of *Daphnia* water did not differ from that of total *Daphnia* water.

Discussion—In both experiments urea had no morphogenetic effect on *S. obliquus.* In *Daphnia* water, as pointed out by Wiltshire and Lampert (1999), urea could act in synergy with other compounds, such as the more lipophilic olefinic carboxylic acid. However, our results revealed no activity in the run-through (containing urea) whereas the metanolic eluate (without urea) was as active as the *Daphnia* water. Hence there is no evidence for a synergy of urea and the lipophilic compounds. Similar results were obtained when the algal assays were run in CHU medium, as in the experiments by Wiltshire and Lampert (1999). These results indicate that all of the activity is more lipophilic than urea, which does not support the hypothesis that urea significantly contributes to the morphogenetic activity released by *Daphnia.* This is corroborated by the negative results from several experiments with urea as a possible colony-inducer in Bristol's medium, WC medium, CHU12, and medium 7 (Lampert et al. 1994; Lürling 1999). *Daphnia* water, however, was active in all of these cases, which confirms that the morphogenetic effect of urea is much more determined by particular growth conditions of *Scenedesmus* than is the infochemical effect of *Daphnia* water. Undoubtedly, urea may have a morphogenetic effect on *Scenedesmus* under certain conditions, but it seems highly unlikely that a general unspecific excretory substance such as urea alone is an infochemical.

The recent study by Wiltshire and Lampert (1999) showed a morphogenetic response to urea in the same strain of *Scenedesmus.* However, the absolute cell size differences induced with urea in that study were rather low: a maximum

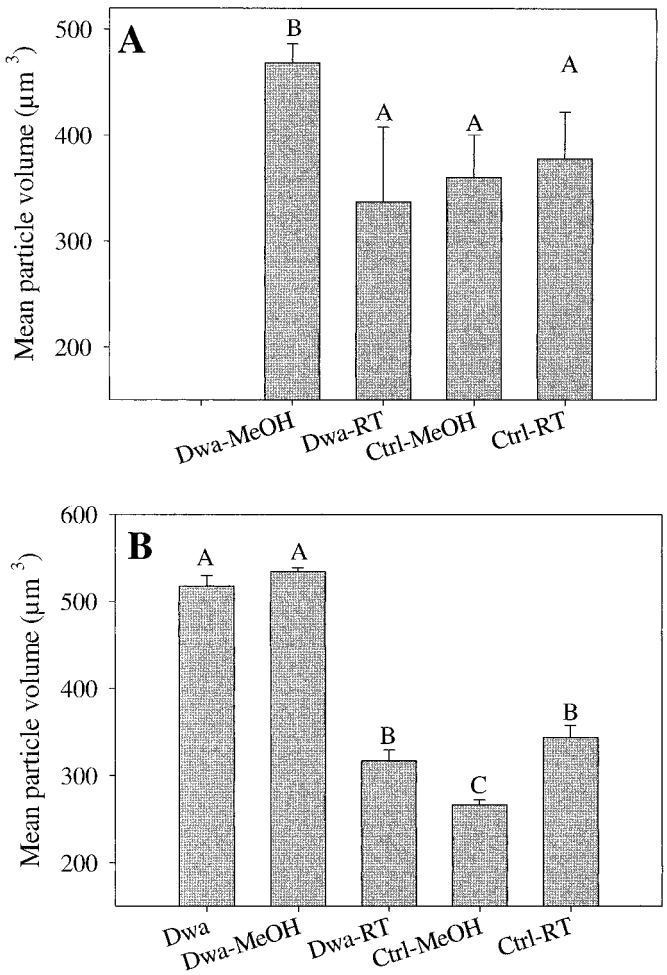

Fig. 3. Mean particle volumes (μm^3) of *Scenedesmus obliquus* exposed in two standard biotests; (A) in WC* medium and (B) in CHU12, to run-through (RT) and methanol eluate of C18-solid-phase extraction (MeOH) of control water (RT/MeOH-Ctrl) and *Daphnia*-incubation water (RT/MeOH-Dwa). Error bars represent 1 SD (n = 3). Similar symbols (A, B, C) indicate homogeneous groups that are not significantly different at the 95% level (Tukey's test).

increase from 1.5 cells per colony to 2.5 cells per colony. In contrast, in *Daphnia* water treatments almost 50% of the population occurred as eight-celled colonies, resulting in 5.8 cells per colony on average (Lampert et al. 1994). It is evident that the effect of *Daphnia* water is stronger than the effect of urea in their assays. Moreover, when animals from the same cohort were incubated in filtered lake water the resulting colony-inducing activity appeared less than when animals were incubated in WC (*see* LI experiment), although the lake water putatively contained urea according to Wiltshire and Lampert (1999).

It is well known from the literature that *Scenedesmus* may respond morphologically to a variety of chemicals, including several nutrients (e.g., Shubert and Trainor 1974; Siver and Trainor 1981; Holtmann and Hegewald 1986). The fact that many of these morphogenetically active compounds are not linked to the presence of grazers, as are other abiotic environmental factors, such as pH (Trainor and Roskosky 1967), temperature (Trainor 1992), and aeration (Hegewald and Schnepf 1991), indicates that colony formation per se in *Scenedesmus* is an unspecific response that must not be regarded as a defense against grazers in all cases.

Recently, Kaler et al. (2000) reported an interaction between the *Daphnia* factor and an unknown nutrient and concluded that *Daphnia* water functions as a carbon and salt cocktail with effects on colony formation in *Scenedesmus.* However, several aspects in their paper make us doubt this. First, the colonies were counted in an electronic particle counter, which is incapable of distinguishing the number of cells per colony. In the highly plastic *Scenedesmus* the number of cells per colony varies, but also there are differences in cell size, especially under different nutrient conditions (Trainor 1998). Kaler et al. (2000) also fractionated *Daphnia* water by hydrophilic gel chromatography. Three resulting UV-spectra-based fractions were added to *Scenedesmus* cultures; however, an essential control with clean medium on the gel and similarly fractionated was lacking. Kaler et al. concluded that the *Daphnia* water contained at least two growth modulators. Unfortunately, they did not report the colony-inducing activity of the different fractions. Analogous to the urea hypothesis, the contribution of nutrients and the interaction with the lipophilic infochemical should be analyzed by C18-SPE of *Daphnia* water, where nutrients are separated from the colony-inducing infochemical.

In the CL experiment, the mean particle volume of the populations in the *Daphnia* water treatments decreased to that of the control after 5 d (Fig. 1). It appears that after *Daphnia* water was added at the start of the experiment, the gradually reduced inductive strength was due to inactivation by absorption, incorporation, or degradation of the inducing chemicals in algal cells and bacteria. The cultures were not completely free of bacteria, but bacterial biomass was very low (based on microscopic analysis and Coulter measurements); nonetheless, bacteria may be important in the turnover of the colony-inducing chemicals (Lürling and Van Donk 1997). Because of the reduced concentration of these chemicals and increased algal population size, gradually fewer cells were exposed to the inducing chemicals and eventually colony formation became negligible and disappeared (Lürling 1998). A certain rate of turnover will limit the persistence of the colony-inducing chemicals and will thus strengthen the reliability of the cue, as it ensures that the concentration of the infochemical reflects the actual risk of predation.

Scenedesmus colonies have higher sinking rates than unicells (Conway and Trainor 1972) that also apply to induced-colonial *Scenedesmus* (Lürling and Van Donk 2000). At first interpreted as a cost, inasmuch as sinking out of the euphotic zone could imply lower growth, sinking could, however, also be interpreted as an escape in time. *Scenedesmus* is capable of surviving prolonged periods of darkness (Dehning and Tilzer 1989), where colonies disintegrate and unicells may serve as inocula for subsequent blooms (Dehning and Tilzer 1989; Egan and Trainor 1989). Enhanced sinking out of the euphotic zone could contribute to the clear-water phase, reducing the food availability to large-bodied zooplankters. In parallel, several flagellated algae have been shown to adjust

their recruitment to the water column in response to the presence of large zooplankters (Hansson 1996). Thus, in the case of very large *Daphnia* harvesting *Scenedesmus*, sinking could provide a refuge from complete decimation by the grazers.

In conclusion, it seems adaptive for *Scenedesmus* to respond morphologically to the presence of grazers to reduce mortality through grazing. Literature data suggest that the grazer-induced colony formation is linked to specific chemicals associated with the algae-grazer interaction and general excretory products, such as urea. However, our results show that *Daphnia* water and lipophilic extracts invariably induce colonies in *Scenedesmus*, while urea-induced colony formation may, if at all, be valid only under certain very limited conditions. The lipophilic infochemical originates from the interaction of algae and grazers, supporting the hypothesis that induced colony formation has evolved as a defense against grazing.

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