

Predation and the evolution of multicellularity

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Abstract

The first step in the major evolutionary transition to multicellularity involves single cells forming a group. Why do cells form a group? Studies have shown that colony formation provides a benefit against predation. However, it is not well known whether this response is generic, or specific to certain prey-predator systems. We sought to assess this by measuring colony formation in nine 'algal-putative predator' combinations; the Chlorophyte algae *Chlorella vulgaris*, *Chlorella sorokiniana* and *Scenedesmus obliquus* as prey, and the flagellate *Ochromonas spp.*, the ciliate *Tetrahymena thermophila* and the crustacean *Daphnia magna* as putative predators. We found that colony formation is a generic response of the algae *C. sorokiniana*, *C. vulgaris* and *S. obliquus* to predation by *Ochromonas spp.*, *T. thermophila* or *D. magna*. Notably, the algae formed colonies regardless of whether the putative predator grazed upon the algae, indicating that predation by itself is not always a prerequisite for colony formation, but this response may have evolved in the algae as a defensive mechanism against potential predation. In six combinations, colonies could not be ingested by the predator, suggesting that colonies have a 'size benefit' against predation. These results outline the importance of predation as a generic factor driving colony formation and help enhance our understanding of the benefits and costs involved in colony formation, the first step in the evolution of multicellularity.

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Chapter 1. General Introduction

1.1. The major evolutionary transitions in individuality

The biological world can be seen as a hierarchy of single units grouping together to form new 'higher level' units (Maynard Smith & Szathmary 1995; Bourke 2011). Genes formed genomes enclosed in cells. Prokaryotic cells symbiotically fused with other prokaryotes and formed eukaryotic cells. Single cells formed multicellular organisms. Social insects with a strictly monogamous lifestyle led to eusocial insect societies. Some species established mutual interdependence with other species, in which case the species involved in the partnership could not reproduce independently any more; in other words the species formed an obligate interspecific mutualism (Maynard Smith & Szathmary 1995; Bourke 2011). Michod & Roze (1997) termed these transitions from previously independent units to new 'higher-level' units, major evolutionary transitions in individuality.

1.2. Steps in the transition to multicellularity

The transition from a unicellular to a multicellular lifestyle has happened at least 25 times throughout evolutionary time (Bourke 2011; Grosberg & Strathmann 2007). In order to understand how this transition occurs, it is useful to divide it into two steps; colony formation and group transformation (Figure 1.1.). The first step, colony formation, can happen by aggregation of cells or by adherence of daughter cells to the parent cell. The second step involves the transformation of the colony into a new 'higher level' individual (Bourke 2011). Cells in this new individual often perform different functions (e.g. division of labour between the somatic and germline cells in

Volvox carteri, Kirk 2005). Furthermore, the new individual can now only reproduce as a whole which means that its cells cannot reproduce independently any more.

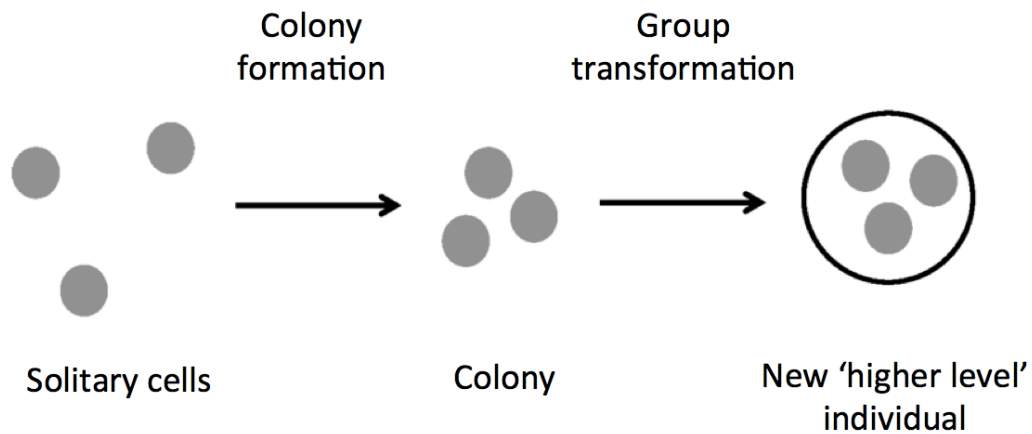


Figure 1.1. Steps in the evolutionary transition to multicellularity. In the first step, single cells come together to form a group. In the second step, the alignment of reproductive interests among the cells, leads to a new 'higher level' individual. Figure adapted from West et al. (2015 in press).

1.3. The problem

The major evolutionary transition from solitary cells to new 'higher level' individuals, poses a problem of cooperation. Putative cheats can arise in the colony, benefiting from the cooperation of others, without paying the cost of cooperation, thus outcompeting cooperators and disrupting the colony (Ghoul et al. 2014). Therefore, how can we explain the formation and transformation of cooperative groups? In other words, what favours and stabilises colony formation and group transformation?

1.4. The solution

Ecological forces can favour cooperation through cells gaining a direct benefit. Additionally, high relatedness amongst cells can favour cooperation, through cells gaining the indirect benefit of their genes, inside their relatives, being passed to the next generation. Specifically, when relatedness (r) weighed by the benefit of the recipient (B) is higher than the cost paid by the actor (C), then a cooperative behaviour can be favoured (Hamilton 1964). This is known as Hamilton's rule ($rB > C$). In the case of clonal individuals ($r=1$), high relatedness resolves the conflict of helping a clonemate or helping ones own offspring, thus facilitating a complete reproductive dependency between members of the group (Queller 2000; Bourke 2011; Boomsma 2007; Boomsma 2009). In other words, cells in the group may become indivisible, therefore forming a new individual. However, clonal cells may not perform a cooperative act if the benefits don't outweigh the costs ($B > C$).

1.5. Benefits of colony formation

Ecological factors have been suggested to drive colony formation (Table 1.1.). Nutrient starvation caused *Myxobacteria* to form branching swarms of cells, which were then able to migrate to nutrient-rich niches (Velicer & Vos 2009; Velicer & Yuen-tsu 2003). Furthermore, Rainey & Rainey (2003) observed that conditions of limited oxygen stimulated *Pseudomonas fluorescens* to form a floating mat of cells at the air-broth interface in liquid medium, where vital oxygen was present. Additionally, cells of *Saccharomyces cerevisiae* benefited from the formation of multicellular clumps, because clumps were able to acquire excreted nutrients more efficiently than single cells (Koschwanez et al. 2013). Also, exposure of *Saccharomyces cerevisiae* to toxins,

such as antimicrobials and ethanol, caused cells to form clusters. In this case, the benefit of forming a cluster was due to the protective shield that tightly adhered cells provided (Smukalla et al. 2008). Boraas et al. (1998) showed that predation by the flagellate *Ochromonas vallescia* triggered colony formation in the algae *Chlorella vulgaris* and that *C. vulgaris* colonies could not be ingested by the predator *O. vallescia*.

Factor promoting colony formation	Species	Reference
Food shortage	<i>Myxococcus xanthus</i>	Velicer & Vos 2009 Velicer & Yuen-tsu 2003
	<i>Dictyostelium discoideum</i>	Bonner 1974
Limited gaseous oxygen	<i>Pseudomonas fluorescens</i>	Rainey & Rainey 2003
Cooperative enzyme secretion	<i>Saccharomyces cerevisiae</i>	Koschwanez et al. 2013
Toxins in the medium	<i>Saccharomyces cerevisiae</i>	Smukalla et al. 2008
Predation	<i>Chlorella vulgaris</i> (prey) <i>Ochromonas vallescia</i> (predator)	Boraas et al. 1998
	<i>Dictyostelium discoideum</i> (prey) <i>Caenorhabditis elegans</i> (predator)	Kessin et al. 1996

Table 1.1. Ecological factors promoting colony formation and examples of species. Table adapted from Bourke (2011).

1.6. Costs of colony formation

The abovementioned examples of colony formation indicate that it is relatively easy for cells to make the first step to colony formation. However, being in a colony can be

costly. Verschoor, Bekmezci and Vijverberg (unpublished) observed that colonies of the alga *Scenedesmus* disintegrated upon removal of the predator *Brachionus*. One of the possible costs is limited access to nutrients mainly to the inner cells of the colony (Boraas et al. 1998 Ploug et al. 1999), which is associated with a low surface to volume ratio (Tollrian & Dodson 1999; Kirk 1994). This hypothesis is supported by the observation that when exposed to low concentrations of mineral nutrients, *C. vulgaris* unicells outcompete colonies (Boraas et al. 1998). Additionally, in photosynthetic organisms such as *Scenedesmus*, colony formation is costly because colonies have higher sinking rates and thus, limited access to light (Reynolds 1984; Lampert et al. 1994). Furthermore, Trainor (1998) and van Rijssel et al. (1997) reported that *Scenedesmus* and *Phaeocystis* cells pay an energetic cost in producing connective material necessary for colony formation.

1.7. Introduction to the algae

The group of algae (from the Latin *alliga*: 'binding') consists of a huge variety of photosynthetic eukaryotic species at different steps in the transition to multicellularity. In the dinoflagellates, the majority of species are unicellular; with only a few species, such as *Gonyaulax monilata*, forming chains of cells (Karleskint et al. 2012). Similarly the euglenoids, the golden-brown algae and the yellow-green algae are predominantly unicellular (Karleskint et al. 2012). However, some species of yellow-green algae, such as *Tribonema*, form small colonies after incomplete cell division (Mauseth 2014). The green algae consist of around 7,000 species. While most green algae are single-celled, such as the species in the genus *Chlorella*, multicellular species also exist (Lee 2008). *Volvox carteri* is an example of a multicellular green alga

containing both somatic cells and gametes (Raven 1998). Two groups that have made the transition to multicellularity are the red and the brown algae (Grosberg & Strathmann 2007). The vast majority of species in these groups, such as the 50 m long brown alga *Macrocystis pyrifera*, are multicellular (Karleskint et al. 2012).

1.8. The Chlorophyte algae

A particularly well studied group within the green algae is the Chlorophyte taxon. This taxon consists of unicellular, facultatively colonial, as well as multicellular species (Figure 1.2.). Examples of unicellular Chlorophyte species are *Chlamydomonas reinhardtii* (Figure 1.2.a) and *C. vulgaris*. Notably, both species form facultatively colonial groups in response to predation (Boraas et al. 1998; Bell 1985; Raven 1998; Fisher et al. 2015 unpublished; Sathe & Durand 2015 in preparation). In other words, the colonies revert to unicells in the absence of the predators. Two other interesting cases are *Gonium pectorale* (Figure 1.2.b) and *Eudorina elegans* (Figure 1.2.c). These species have made the step to colony formation, but not the step to group transformation (Figure 1.1.). Group transformation has happened in the species *Pleodorina californica* (Figure 1.2.d), *Volvox carteri* (Figure 1.2.e) and *Volvox aureus* (Figure 1.2.f), giving rise to new 'higher level' individuals; multicellular organisms. The existence of species in the Chlorophyte taxon ranging from unicells and colonies to multicellular organisms, underlines the usefulness of this taxon for studies on the effect of predation on colony formation.

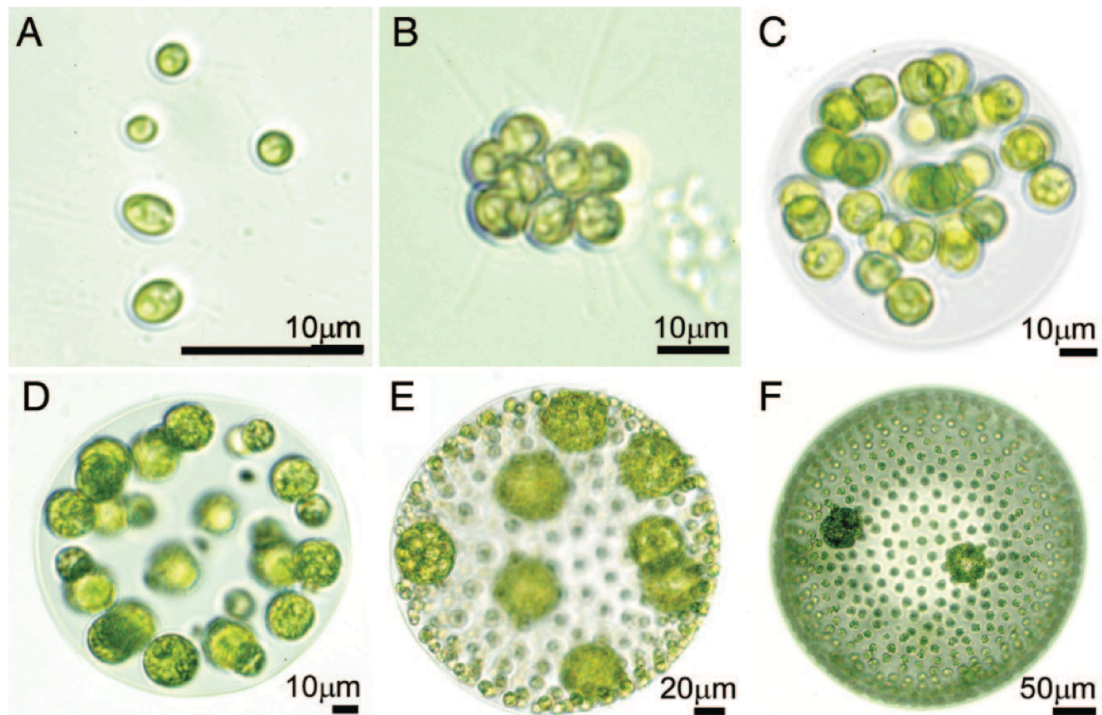


Figure 1.2. Examples of unicellular and multicellular algae in the Chlorophyte taxon. (a) the unicellular species *Chlamydomonas reinhardtii*; (b) a colony of *Gonium pectorale*; (c) a colony of *Eudorina elegans*; (d) the multicellular species *Pleodorina californica*; (e) the multicellular species *Volvox carteri*; (f) the multicellular species *Volvox aureus*. Figure adapted from Michod (2007).

1.9. Aims and structure of thesis

The overall aim of this thesis is to investigate the usefulness of different study systems for long term studies into the social costs and benefits of colony formation. In Chapter 2, I experimentally assess whether colony formation is a general response of algae to predation or whether the response is specific to certain algal species – predator combinations. Firstly, to answer this I use nine predator-prey combinations; the putative predators *Ochromonas spp.*, *Tetrahymena thermophila* and *Daphnia magna*, and the unicellular Chlorophyte algae *Chlorella sorokiniana*, *Chlorella vulgaris* and

Scenedesmus obliquus, and I measure the proportion of cells in colonies. Secondly, I examine whether the mean algal colony size increases in the presence of the putative predators. Thirdly, I test whether *Ochromonas spp.*, *T. thermophila* and *D. magna* are actual predators of the algae by measuring algal density in the presence and absence of predators, and by making observations of the predators' ingesting behaviour.

In Chapter 3, I use the nine 'algal-putative predator' combinations to assess whether cells in a colony have a benefit against predation. Firstly, I test whether a colony is too large to be ingested by a predator, i.e. size benefit. Secondly, I examine whether being in a colony reduces a cell's probability of being attacked by a predator, i.e. dilution benefit. In particular, the hypothesis is that when a cell is in a N-sized colony, the chances of it being attacked are reduced by a factor of $1/N$.

Chapter 2. Colony formation and predation

2.1. Abstract

The major evolutionary transition from unicellular to multicellular life involves single celled organisms forming cooperative multicellular groups. Why do single cells form a group? Defense against predation has been proposed to drive social group formation mainly on the basis of predators being unable to engulf large sized entities. We investigated how widespread predation pressure is as a driving force towards colony formation by using nine different prey-predator combinations; the algae *Chlorella sorokiniana*, *Chlorella vulgaris* and *Scenedesmus obliquus* as prey and *Ochromonas spp.*, *Tetrahymena thermophila* and *Daphnia magna* as predators. Furthermore, we tested the assumption of predation by measuring algal density in the presence of *Ochromonas*, *T. thermophila* and *D. magna*. Our results showed that in all combinations, the algal species formed colonies and in seven cases, *Ochromonas*, *Tetrahymena* and *Daphnia* were clearly predators of the algae. These findings illustrate the generality and importance of predation as a force driving colony formation, the first step towards the evolution of multicellularity.

2.2. Introduction

The tree of life can be described as a hierarchy of major evolutionary transitions in individuality (Table 2.1., Maynard Smith & Szathmary 1995; West et al. 2015 in press). Genes have lost their capacity to replicate independently and can only replicate as part of the whole genome. Similarly, cells in multicellular organisms have lost their ability to

reproduce independently and can only reproduce as part of a whole multicellular organism. However, making a major transition from a previously independently replicating cell, to a new unit that can only reproduce as a whole, is complex. Bourke (2011) and West et al. (2015 in press) have divided this process into two steps; colony formation and group transformation. As a starting point to understand the evolution of multicellularity, we focus on colony formation.

Unit	Cooperative group formation	Major evolutionary transition
gene	compartmentalized genomes	prokaryotic cell
prokaryotic cell	symbiosis of prokaryotes	eukaryotic cell
unicell	colony formation	multicellularity
multicellular organism	origin of societies	eusociality
separate species	origin of interspecific mutualism	Interspecific mutualism

Table 2.1. Cooperative group formation and the major evolutionary transitions in individuality. Table adapted from Bourke (2011).

Why do single cells form a group? Ecological factors have been suggested to drive colony formation. In *Saccharomyces cerevisiae*, environmental stresses have been shown to drive colony formation. For instance, *S. cerevisiae* form clusters as a protection strategy against toxins (Smukalla et al. 2008). Additionally, dispersal has played a major role in driving colony formation, as illustrated in the slime mould *Dictyostelium discoideum* (Bonner 1974). Another example is the advantage of nutrient

storage that groups provide. This is evident in the example of starvation inducing focal individuals to cannibalize groupmates as a means to release stored food (Kerszberg & Wolpert 1998; Raven 1998; Szathmáry & Wolpert 2003). Finally, defense against predation has been proposed mainly on the basis of predators being unable to engulf large sized entities (Stanley 1973; Boraas et al. 1998). Interestingly, the appearance of phagotrophy in evolutionary time possibly selected for larger prey (Boraas et al. 1998; Cavalier-Smith 2002).

The literature on colony formation upon predation pressure is vast, ranging over many taxa (Table 2.2), but these studies have certain limitations. Firstly, the majority of studies assess colony formation by measuring cells per colony and mean particle size, but this measurement does not take into account possible differences in prey growth between the treatment and control. For instance, prey growth may be higher in the treatment, resulting in a higher number of unicells, paired cells, colonies and larger mean colony size. Thus, more colonies and a larger mean colony size in the treatment may be an effect of density and not necessarily predation. In order to assess the effect of predation on colony formation, we controlled for such differences in algal growth by measuring the proportion of cells in colonies. Secondly, these studies do not directly assess whether colony formation is a response of specific algae to specific predators or whether it is a general response to predation.

In this study we attempted to bridge these gaps in the literature by using three freshwater unicellular Chlorophyte algal species: *Chlorella sorokiniana*, *Chlorella vulgaris* and *Scenedesmus obliquus*, and three putative predators: the flagellate

Ochromonas spp., the ciliate *Tetrahymena thermophila* and the crustacean *Daphnia magna* (Figure 2.1). In these nine 'algal-putative predator' combinations we measured the proportion of algal cells in colonies, the mean colony size and the algal density in order to assess colony formation, change in colony size and predation, respectively.

Alga	Predator	Colony formation	Measure of colony formation	Reference
<i>Chlorella vulgaris</i>	<i>Ochromonas vallescia</i>	✓	–	Boraas et al. 1998
<i>Chlorella vulgaris</i>	<i>Tetrahymena thermophila</i>	✓	Proportion of cells in colonies	Fisher et al. unpublished
<i>Scenedesmus obliquus</i> CCAP 276/3A	<i>Daphnia magna</i>	✓	Cells per colony, mean particle volume	Elert & Franck 1999
<i>Scenedesmus obliquus</i> CCAP 276/3B, UTEX 1450 and UTEX 2630	<i>Daphnia magna</i>	✓	Cells per colony, mean particle volume	Lüring 1999; Verschoor et al. 2004
<i>Scenedesmus obliquus</i> MP1	<i>Daphnia magna</i>	✓	Cells per colony, mean particle volume	Lampert et al. 1994; Verschoor et al. 2004
<i>Scenedesmus obliquus</i> NIVA-CHL6	<i>Daphnia magna</i>	✓	Cells per colony, mean particle volume	Van Donk et al. 1999
<i>Scenedesmus obliquus</i>	<i>Brachionus calyciflorus</i>	✓	Cells per colony, mean particle volume	Verschoor et al. 2004
<i>Scenedesmus acutus</i>	<i>Daphnia pulex</i>	✓	Cells per colony, mean particle volume	Lüring & Van Donk 1996
<i>Scenedesmus acutus</i>	<i>Daphnia galeata</i>	✓	Proportion of cells in colonies of different sizes, number of cells per colony	Lüring & Van Donk 1997
<i>Scenedesmus acutus</i>	<i>Brachionus calyciflorus</i>	✓	Cells per colony, mean particle volume	Lüring & Van Donk 1997
<i>Scenedesmus acutus</i>	<i>Bosmina</i>	✓	Cells per colony,	Lüring & Van

	<i>longirostris</i>		mean particle volume	Donk 1997
<i>Scenedesmus acutus</i>	<i>Chydorus sphaericus</i>	x	Cells per colony, mean particle volume	Van Donk et al. 1999
<i>Scenedesmus acutus</i>	<i>Cyclops agilis</i>	x	Cells per colony, mean particle volume	Van Donk et al. 1999
<i>Scenedesmus acutus</i>	<i>Herpetocypris reptans</i>	x	Cells per colony, mean particle volume	Van Donk et al. 1999
<i>Scenedesmus acutus</i>	<i>Cypridopsis vidua</i>	x	Cells per colony, mean particle volume	Van Donk et al. 1999
<i>Scenedesmus subspicatus</i>	<i>Daphnia magna</i>	✓	Cells per colony, mean particle volume	Hessen & Van Donk 1993
<i>Scenedesmus quadricauda</i>	<i>Daphnia magna</i>	x	Cells per colony, mean particle volume	Van Donk et al. 1999
<i>Ankistrodesmus falcatus</i>	<i>Daphnia magna</i>	x	Cells per colony, mean particle volume	Van Donk et al. 1999
<i>Asterionella formosa</i>	<i>Daphnia pulex</i>	x	Cells per colony, mean particle volume	Van Donk et al. 1999
<i>Chlamydomonas reinhardtii</i>	<i>Brachionus calyciflorus</i>	✓	% single cells, mean clump size	Becks et al. 2010
<i>Desmodesmus</i>	<i>Brachionus calyciflorus</i>	✓	Cells per colony, mean particle volume	Verschoor et al. 2004
<i>Micractinium pusillum</i>	<i>Daphnia magna</i>	x	Cells per colony, mean particle volume	Van Donk et al. 1999
<i>Pediastrum duplex</i>	<i>Daphnia magna</i>	x	Cells per colony, mean particle volume	Van Donk et al. 1999
<i>Phaeocystis globosa</i>	<i>Gyrodinium dominans</i>	✓	Colonies ml ⁻¹	Jakobsen & Tang 2002
<i>Raphidocelis subcapitata</i>	<i>Daphnia magna</i>	x	Cells per colony, mean particle volume	Van Donk et al. 1999

Table 2.2. Algal-predator combinations where colony formation was tested in the literature.

Studies on several *S. obliquus* strains are included. Table adapted from Fisher et al. (unpublished), Lürling 2003 and Van Donk et al. 1999. (–) indicates that the test was not performed; ✓ indicates cases where colony formation was observed. x indicates studies where colony formation was not observed.

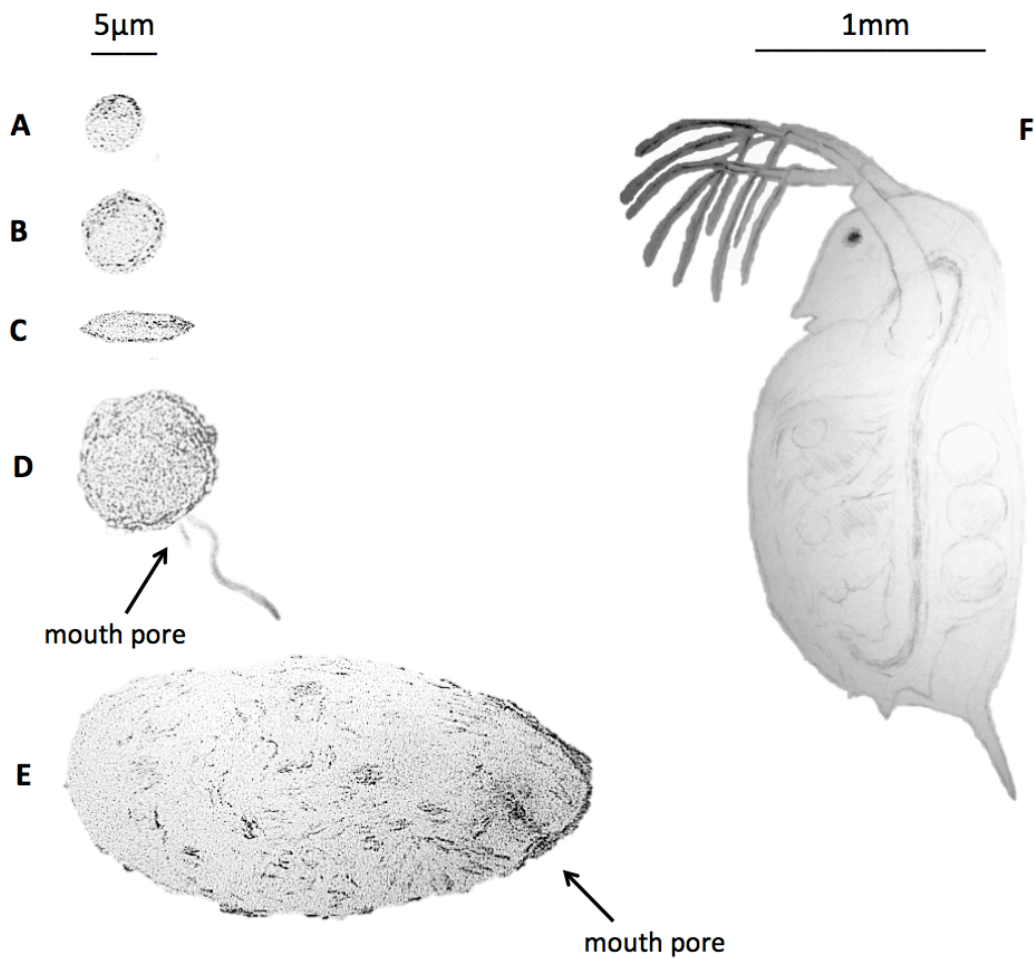


Figure 2.1. Species used in this study. A. *Chlorella sorokiniana*, B. *Chlorella vulgaris*, C. *Scenedesmus obliquus*, D. the flagellate *Ochromonas*, E. the ciliate *Tetrahymena thermophila*, F. the crustacean *Daphnia magna*.

2.3. Materials and Methods

2.3.1. Media

We diluted 20 ml of Bolds Basal medium (from the Culture Collection of Algae and Protozoa; CCAP) in 980 ml of dH₂O and autoclaved the solution at 121°C for sterilization.

We prepared the PPY medium (CCAP) by dissolving 10 g of proteose peptone (Oxoid L85, Unipath Ltd) and 1.25 g of yeast extract (Oxoid L21, Unipath Ltd) in 500 ml of dH₂O using a magnetic-stirrer at 639 rev/min speed, 008 AV heat °C/% AV for approximately 10 min. We transferred 10 ml of the dissolved solution into glass vials and autoclaved at 121°C for sterilization.

2.3.2. Species and growth conditions

Algae. We grew *Chlorella vulgaris* (axenic from CCAP; strain number: 211/11B), *Chlorella sorokiniana* (non-axenic from CCAP; strain number 211/8K) and *Scenedesmus obliquus* (non-axenic from CCAP; strain number 276/3A) cultures in Bolds Basal medium at a 16:8 hour light-dark cycle. We treated samples of 1 ml from these cultures with the antibiotic rifampicin at a concentration of 500 µg ml⁻¹ to inhibit bacterial growth. After 24 h, we diluted the algal cultures 1:300 in Bolds Basal medium and left the cultures to grow in a 1 L-Erlenmeyer flask with shaking at 220rpm and 25°C for at least a week prior to each experiment. **Protists.** We grew *Tetrahymena thermophila* (axenic from CCAP; strain number: 1630/1M) in PPY medium in 20 ml-flat-bottomed flasks at 25°C and a 16:8 hour light-dark cycle. Every week we diluted the culture 1:100 in PPY medium. Similarly, we grew *Ochromonas spp.* (from Corno &

Jürgens 2006) in PPY medium, except that the *Ochromonas spp.* culture was kept in the dark. ***Daphnia magna***. We cultured *Daphnia* (from local fish store) in 1 L-jars with TETRA flake at 25°C and constant air flow to allow for oxygenation.

2.3.3. Experiment 1: testing for colony formation and size change

We tested whether the addition of a putative predator led to the algae being more likely to form colonies and/or increase their colony size. For the *C. sorokiniana* + *Ochromonas spp.* combination, we used 30 50-ml Falcon tubes (Supplementary Material Figure S2.1.) for the control without the putative predator and 30 50-ml Falcon tubes with the addition of the putative predator. In each tube we added 19.6 ml of *C. sorokiniana* to either 0.4 ml of PPY in the control or 0.4 ml of *Ochromonas spp.* in the treatment set. We incubated the tubes at 20°C light-dark 16:8 hour cycle and kept the tube caps loose to allow for oxygenation. We collected samples at four time points after adding the putative predator; 1 h, 24 h, 48 h and 72 h. At each time point, we tilted the falcon tubes five times and transferred 200 µl of each culture into a 96-well plate (Fisher Scientific). We took images using a VisiCam digital camera under an inverted microscope (XDS-3) at X20 magnification; phase contrast: 10. We quantified the proportion of cells in colonies (number of algal cells in colonies:total number of algal cells) and the mean colony size. We define a colony as a group of ≥ 3 cells.

The experimental procedure was similar for the rest of the combinations. The concentrations of algae and putative predators, the total volume used per tube and the number of independent replicates for each combination are described in Table 2.3.

	Concentration of algae (cells ml ⁻¹)	Concentration of putative predators (cells or individuals ml ⁻¹)	Total volume per tube	Number of independent replicates
A) <i>Chlorella sorokiniana</i> + <i>Ochromonas</i> spp.	2 x 10 ⁶	1 x 10 ⁵	20ml	n=30*
B) <i>Chlorella vulgaris</i> + <i>Ochromonas</i> spp.	1 x 10 ⁵	6 x 10 ⁵	20ml	n=9
C) <i>Scenedesmus obliquus</i> + <i>Ochromonas</i> spp.	2 x 10 ⁵	3 x 10 ⁵	5ml	n=9
D) <i>Chlorella sorokiniana</i> + <i>Tetrahymena thermophila</i>	3 x 10 ⁶	1 x 10 ⁴	20ml	n=30*
E) <i>Chlorella vulgaris</i> + <i>Tetrahymena thermophila</i>	2 x 10 ⁶	2 x 10 ⁴	20ml	n=30**
F) <i>Scenedesmus obliquus</i> + <i>Tetrahymena thermophila</i>	2 x 10 ⁵	4 x 10 ⁵	5ml	n=9
G) <i>Chlorella sorokiniana</i> + <i>Daphnia magna</i>	9 x 10 ⁶	1	5ml	n=9
H) <i>Chlorella vulgaris</i> + <i>Daphnia magna</i>	5 x 10 ⁶	1	5ml	n=9
I) <i>Scenedesmus obliquus</i> + <i>Daphnia magna</i>	2 x 10 ⁶	1	5ml	n=9

Table 2.3. Concentrations, total volume per tube and number of independent replicates used

in the Experiment 1. In the combinations of *S. obliquus* + *Ochromonas* spp. (C) and *S. obliquus* + *T. thermophila* (F), we placed 4.04 ml of algae in the tubes with an additional 0.96 ml of PPY in the control set and 0.96 ml of the putative predator in the treatment set. In the combinations with *Daphnia* (G-I), we placed 5 ml of algae in all tubes with an additional five *Daphnia* in each tube of the treatment set. * n_{1h}=3, n_{24h}=9, n_{48h}=9, n_{72h}=9 ** n_{1h}=6, n_{24h}=9, n_{48h}=9, n_{72h}=6

2.3.4. Experiment 2: testing for predation

We tested whether the putative predator had a significant impact on the algal populations. In the combination of *C. sorokiniana* + *Ochromonas* spp., we used nine 50-ml Falcon tubes for the control without the putative predator and nine 50-ml Falcon tubes with the addition of the putative predator. In each tube we placed 4.04 ml of *C. sorokiniana* to either 0.96 ml of PPY in the control or 0.96 ml of *Ochromonas*

spp. in the treatment. We incubated the tubes at 20⁰C light-dark 16:8 hour cycle and kept the tube caps loose to allow for oxygenation. We collected samples at three time points; 0 h: before adding the putative predator, 1 h after adding the putative predator and 24 h after adding the putative predator. At each time point, we tilted the falcon tubes five times and transferred 200 µl of each culture into a 96-well plate. We took images with a VisiCam digital camera under the inverted microscope at X20 magnification; phase contrast: 10. From these images, we counted the total number of algae and converted to log₁₀ cells ml⁻¹.

We followed the same procedure for the rest of the combinations. The concentrations, the total volume used per tube and the number of independent replicates for each combination can be found in Table 2.4.

	Concentration of algae (cells ml ⁻¹)	Concentration of putative predators (cells or individuals ml ⁻¹)	Total volume per tube	Number of independent replicates
A) <i>Chlorella sorokiniana</i> + <i>Ochromonas spp.</i>	3 x 10 ⁵	4 x 10 ⁵	5ml	n=9
B) <i>Chlorella vulgaris</i> + <i>Ochromonas spp.</i>	2 x 10 ⁵	3 x 10 ⁵	5ml	n=9
C) <i>Scenedesmus obliquus</i> + <i>Ochromonas spp.</i>	2 x 10 ⁵	3 x 10 ⁵	5ml	n=9
D) <i>Chlorella sorokiniana</i> + <i>Tetrahymena thermophila</i>	3 x 10 ⁵	4 x 10 ⁵	5ml	n=9
E) <i>Chlorella vulgaris</i> + <i>Tetrahymena thermophila</i>	2 x 10 ⁵	4 x 10 ⁵	5ml	n=9
F) <i>Scenedesmus obliquus</i> + <i>Tetrahymena thermophila</i>	1 x 10 ⁵	4 x 10 ⁵	5ml	n=9
G) <i>Chlorella sorokiniana</i> + <i>Daphnia magna</i>	9 x 10 ⁶	1	5ml	n=9
H) <i>Chlorella vulgaris</i> + <i>Daphnia magna</i>	5 x 10 ⁶	1	5ml	n=9
I) <i>Scenedesmus obliquus</i> + <i>Daphnia magna</i>	2 x 10 ⁶	1	5ml	n=9

Table 2.4. Concentrations, total volume per tube and number of independent replicates used in Experiment 2. In the combinations with *Daphnia magna* (G-I), we added 5 ml of algae in the tubes with an additional five *Daphnia* in each tube of the treatment set.

2.3.4.1. Protist behavioural observations

Throughout the colony induction experiment (Experiment 1), we followed individual *T. thermophila* and *Ochromonas* cells in 96-well plates under the inverted microscope (X20 magnification, phase contrast: 10) to detect any ingesting activity towards unicells. We acquired videos manually with a digital camera (Canon PowerShot A2600).

2.3.5. Statistical analyses

Experiment 1. To compare the proportion of cells in colonies and mean colony size between the ‘without predator’ and ‘with predator’ set, we used the R statistical software. For the proportion of cells in colonies, we used a generalized linear model and quasibinomial, because the residual deviances in the data were much greater than the residual degrees of freedom, indicating overdispersion of the data. Similarly, for the mean colony size we used a generalized linear model and gaussian to account for errors in overdispersion.

Experiment 2. To test whether there were differences in the number of algae between the ‘without predator’ and ‘with predator’ set, we used a generalized linear model fitted with quasipoisson errors.

2.4. Results

2.4.1. Do the algae form colonies?

In all nine combinations, we observed colony formation in the presence of the putative predator (Fig. 2.2.). In most combinations, the proportion of cells in colonies began to increase 24 h after addition of the putative predator (Fig. 2.2. A-H). In the combination *S. obliquus* + *D. magna*, the response appears to be much faster. *Scenedesmus* responded by forming colonies within 1 h after addition of *Daphnia* to the cultures (Fig. 2.2. I).

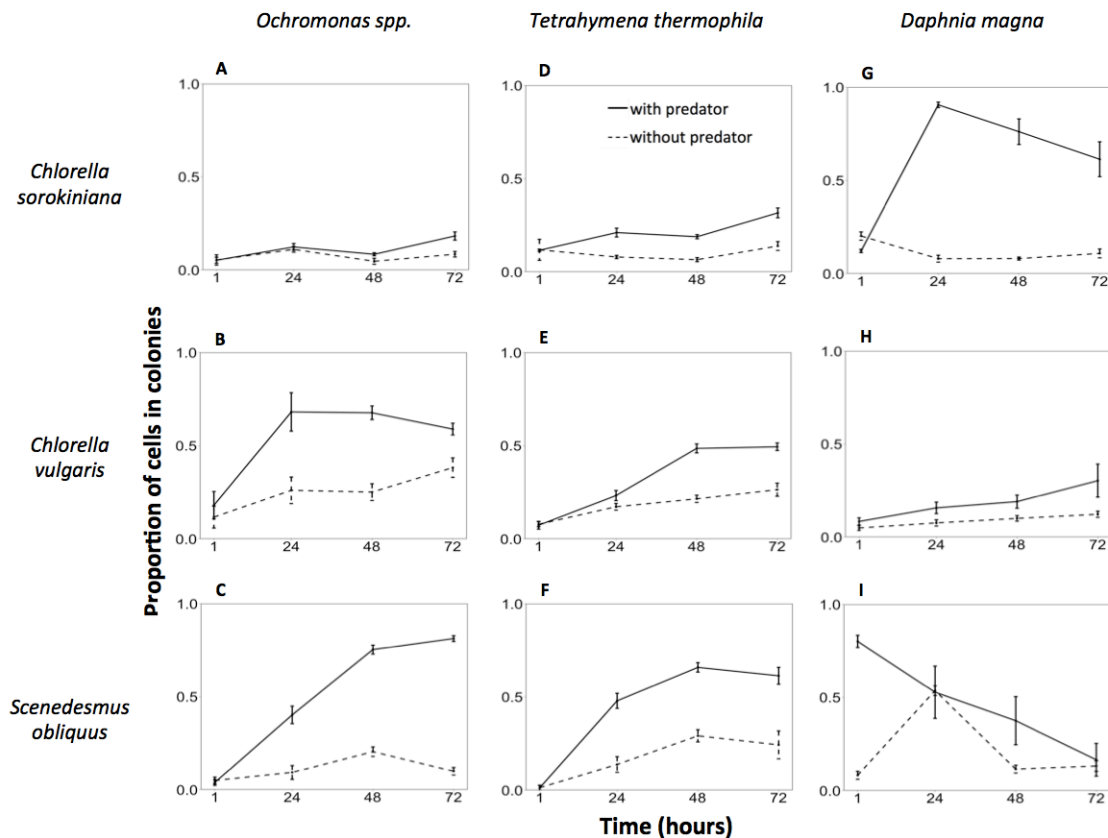


Figure 2.2. Proportion of cells in colonies over 72 h in the presence (solid line) and absence (dashed line) of the putative predator. In all nine combinations, the proportion of cells in colonies is higher in the presence of the putative predator (A. glm, time point 72 h: p_{72} , $F = 15.28$, $df = 16$, $P = 0.00125$; B. glm, p_{72} , $F = 11.14$, $df = 16$, $P = 0.004176$; C. glm, p_{72} , $F =$

220.31, $df = 16$, $P = 8.948 \times 10^{-11}$; **D.** glm, $p72$, $F = 21.63$, $df = 16$, $P = 0.0002663$; **E.** glm, $p72$, $F = 28.71$, $df = 10$, $P = 0.00032$; **F.** glm, $p72$, $F = 10.55$, $df = 16$, $P = 0.00504$; **G.** glm, $p72$, $F = 78.38$, $df = 16$, $P = 1.453 \times 10^{-7}$; **H.** glm, $p72$, $F = 6.38$, $df = 16$, $P = 0.02246$; **I.** glm, $p1$, $F = 202.7$, $df = 16$, $P = 1.667 \times 10^{-10}$). The term 'predator' in the legend refers to a putative predator. Error bars represent standard error of the mean ($\pm se$).

2.4.2. Does colony size change?

Colony size increased in the presence of the putative predator in eight combinations (Fig. 2.3. B-I, Fig. 2.4. B-I). In the combination *C. sorokiniana* + *Ochromonas spp.* (Fig. 2.3. A, Fig. 2.4. A), colony size did not change. Examples of the different colony types seen in the three algal species are illustrated in Figure 2.5.

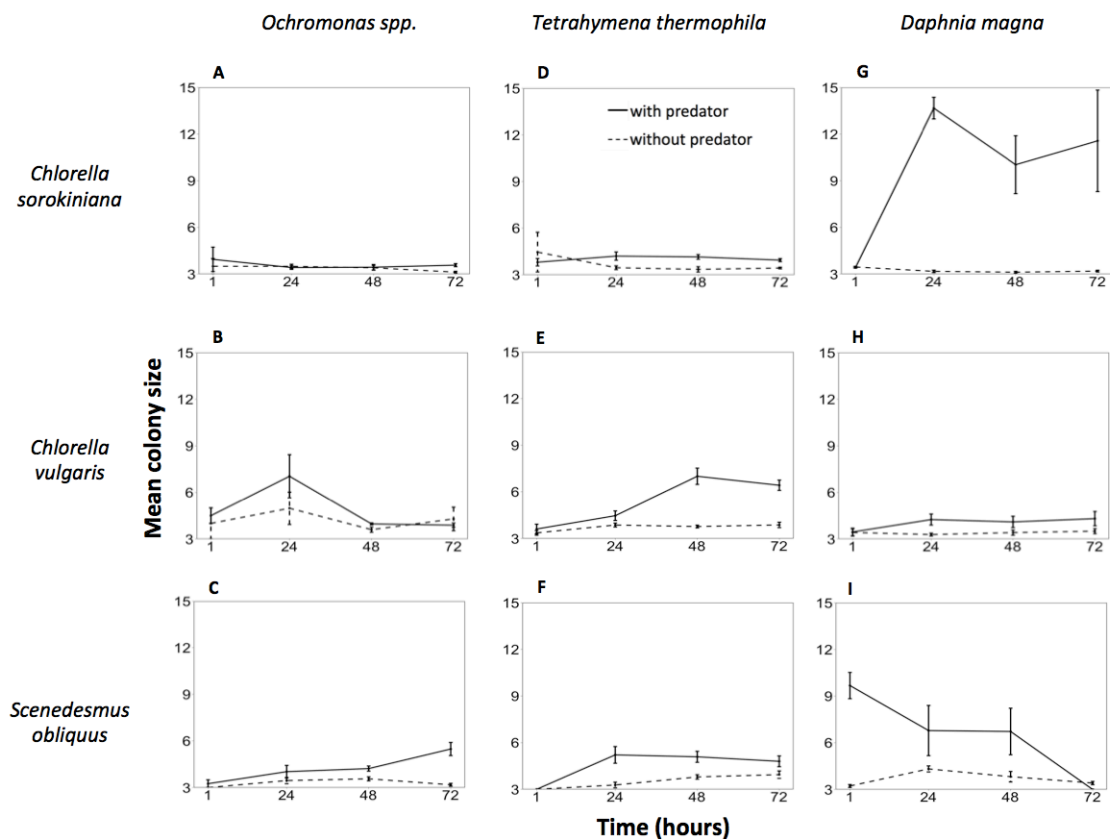


Figure 2.3. Mean colony size over 72 h in the presence (solid line) and absence (dashed line) of the putative predator. In eight combinations, the mean algal colony size was higher in the

presence of the putative predator (**B.** glm, time point 48 h: p_{48} , $F = 4.87$, $df = 15$, $P = 0.0434$; **C.** glm, p_{48} , $F = 9.94$, $df = 16$, $P = 0.006162$; **D.** glm, p_{48} , $F = 12.55$, $df = 16$, $P = 0.002712$; **E.** glm, p_{48} , $F = 37.08$, $df = 16$, $P = 1.566 \times 10^{-5}$; **F.** glm, p_{48} , $F = 11.54$, $df = 16$, $P = 0.003687$; **G.** glm, p_{48} , $F = 13.80$, $df = 16$, $P = 0.001882$; **H.** glm, p_{24} ; $F = 6.93$, $df = 14$, $P = 0.01971$; **I.** glm, p_1 ; $F = 51.089$, $df = 15$, $P = 3.35 \times 10^{-6}$). In the case of *C. sorokiniana* + *Ochromonas spp.*, mean colony size did not differ between the ‘without predator’ and ‘with predator’ set (**A.** glm, p_{48} , $F = 0.03$, $df = 13$, $P = 0.8753$). Error bars are standard error of the mean (\pm se).

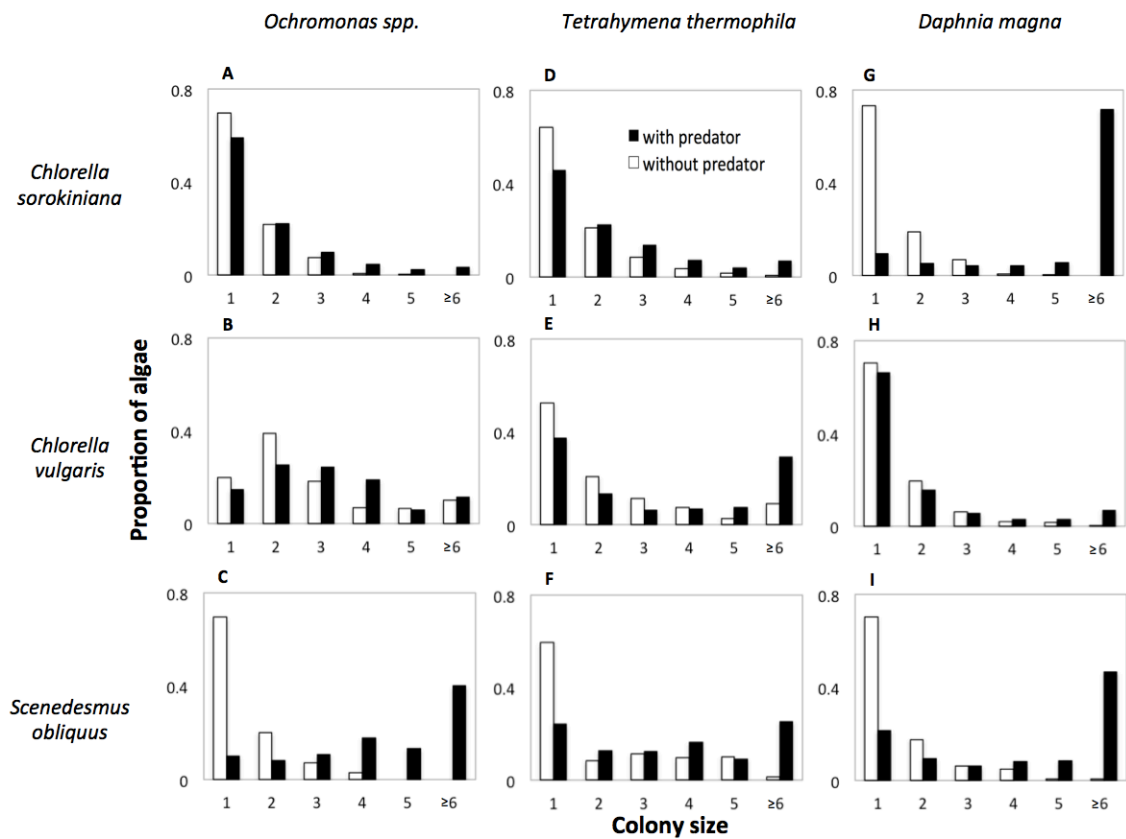


Figure 2.4. Distribution of colony sizes in the presence (shaded bars) and absence (white bars) of the putative predator after 72 h (A-F) and 48 h (G-I). Colony sizes ‘1’ and ‘2’ refer to a unicell and a paired cell, respectively.

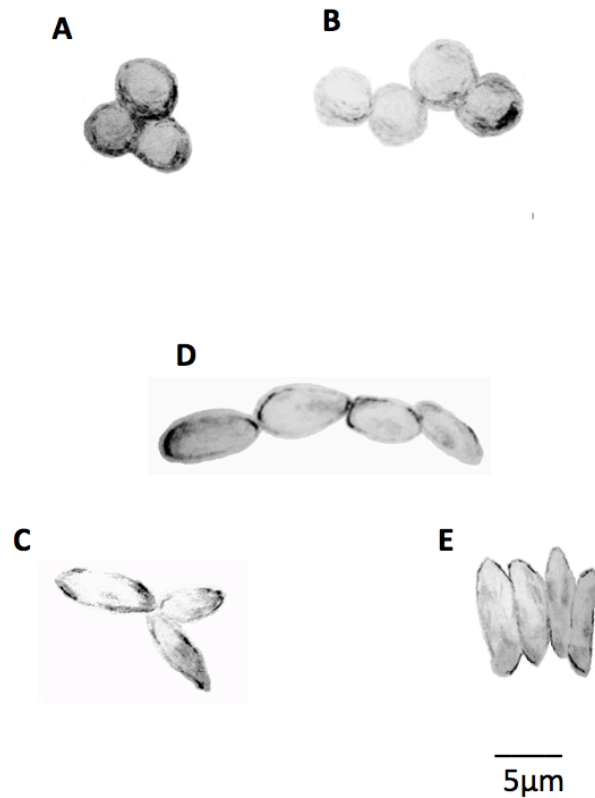


Figure 2.5. Characteristic algal colony types. **A.** 3-celled colony observed in *C. sorokiniana* and *C. vulgaris* cultures, **B.** 4-celled colony observed in *C. sorokiniana* and *C. vulgaris* cultures, **C.** 3-celled colony observed in *S. obliquus* cultures, **D.** and **E.** 4-celled colonies seen in *S. obliquus* cultures.

2.4.3. Do the predators impact algal density?

The number of algae decreased in seven out of nine combinations (Fig. 2.6. A, B, D, F-I) upon addition of the putative predator. This decrease in density supports our assumption that in these seven combinations, the putative predators are eating the algae. Whereas in two combinations, *S. obliquus* + *Ochromonas spp.* (Fig. 2.6. C) and *C. vulgaris* + *T. thermophila* (Fig. 2.6. E), we did not observe a decrease in the number of algae.

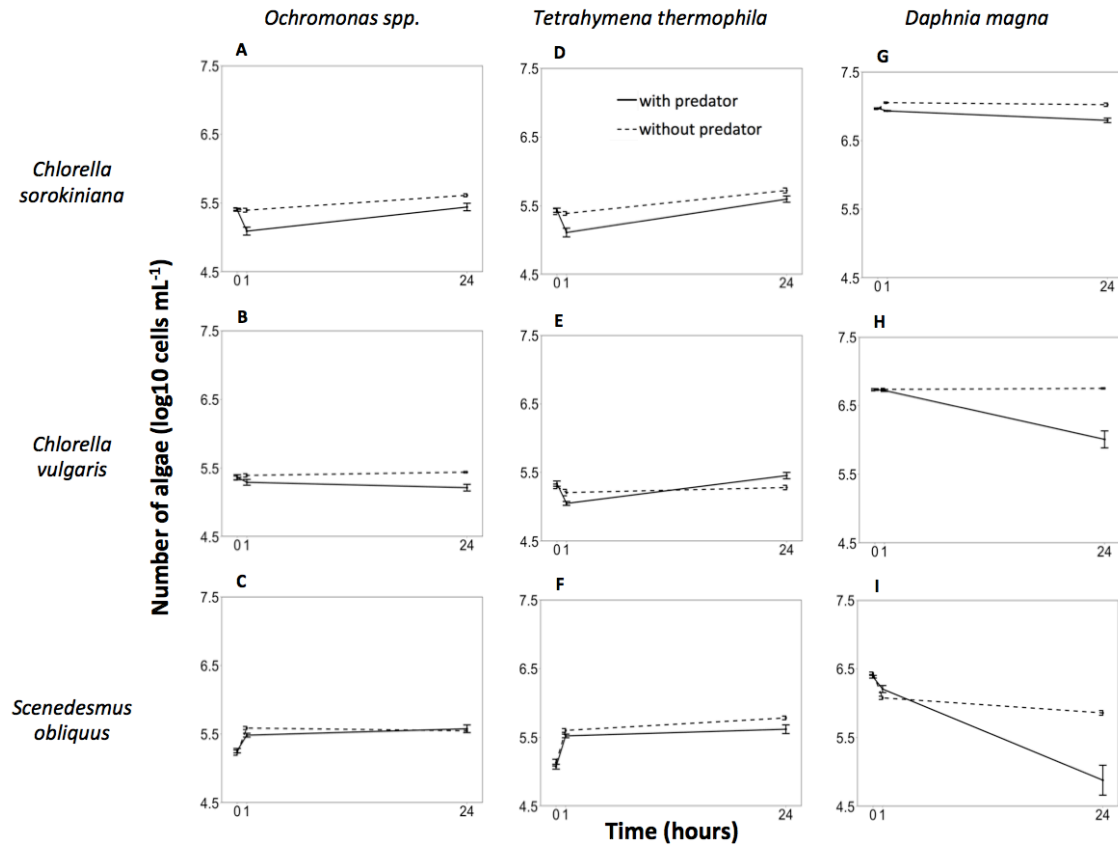


Figure 2.6. Number of algae in the presence (solid line) and absence (dashed line) of the putative predator over 24 h. In seven combinations, the number of algae decreased in the presence of the putative predator (A. glm, time point 24 h: p_{24} , $F = 8.35$, $df = 16$, $P = 0.01068$; B. glm, p_{24} , $F = 21.67$, $df = 16$, $P = 0.0002639$; D. glm, p_1 , $F = 14.51$, $df = 16$, $P = 0.001543$; F. glm, p_{24} , $F = 6.58$, $df = 16$, $P = 0.02078$; G. glm, p_{24} , $F = 32.42$, $df = 16$, $P = 3.324 \times 10^{-5}$; H. glm, p_{24} , $F = 64.59$, $df = 16$, $P = 5.226 \times 10^{-7}$; I. glm, p_{24} , $F = 10.18$, $df = 16$, $P = 0.005685$). While, in the other two combinations, the number of algae did not decrease in the presence of the putative predators (C. glm, p_{24} , $F = 0.72$, $df = 16$, $P = 0.4088$; E. glm, p_{24} , $F = 9.79$, $df = 16$, $P = 0.006477$). Error bars represent standard error of the mean (\pm se).

2.4.4. Further evidence of predation

Observations of the protists capturing (Table 2.5. A, B) and ingesting the algae (Table 2.5. D), as well as the presence of algae inside *T. thermophila* (Table 2.5. F) and *D. magna* (Table 2.5. G-I) further support that in these seven combinations (A, B, D, F-I), *Ochromonas spp.*, *T. thermophila* and *D. magna* are predators.

	<i>Ochromonas spp.</i>	<i>Tetrahymena thermophila</i>	<i>Daphnia magna</i>
<i>Chlorella sorokiniana</i>	<p>A</p> <p>Capture of alga on mouth pore (Movie S2.1.)</p>	<p>D</p> <p>Ingestion of alga and movement across vacuoles (Movies S2.3.-S2.5.)</p>	<p>G</p> <p>Green gut (Figure S2.2.)</p>
<i>Chlorella vulgaris</i>	<p>B</p> <p>Capture of alga on mouth pore (Movie S2.2.)</p>	<p>E</p> <p>—</p>	<p>H</p> <p>Green gut (Figure S2.2.)</p>
<i>Scenedesmus obliquus</i>	<p>C</p> <p>—</p>	<p>F</p> <p>Alga inside vacuole and movement across vacuoles (Movie S2.6.)</p>	<p>I</p> <p>Green gut (Figure S2.2.)</p>

Table 2.5. Direct observations of the protists' feeding behaviour and *Daphnia's* gut. Movies and Figures are provided in the Supplementary material. (—) indicates no observed feeding behaviour towards the alga.

2.5. Discussion

Overall, we observed colony formation upon predation pressure in all nine 'algal-putative predator' combinations tested (Fig. 2.2.). Furthermore, mean colony size increased in eight combinations (Fig. 2.3.). Finally, we found predation, measured as a decrease in algal density, in seven combinations (Fig. 2.6.).

In all nine combinations, we observed colony formation upon predation pressure (Fig. 2.3.). In certain combinations, such as *C. sorokiniana* + *D. magna* and *S. obliquus* + *D. magna*, colonies were visible with the naked eye. When we exposed the culture to the predator *D. magna*, colonies of algae formed in the liquid medium (green points) (Fig. 2.7.). Our findings that predation induced colony formation in all combinations suggest that colony formation is a general response no matter the putative predator used. However, the alga *Scenedesmus acutus* does not form colonies in the presence of the predators *Chydorus sphaericus*, *Cyclops agilis* and *Cypridopsis vidua* (Table 2.2.), indicating that the response to predation is not general in this genus.

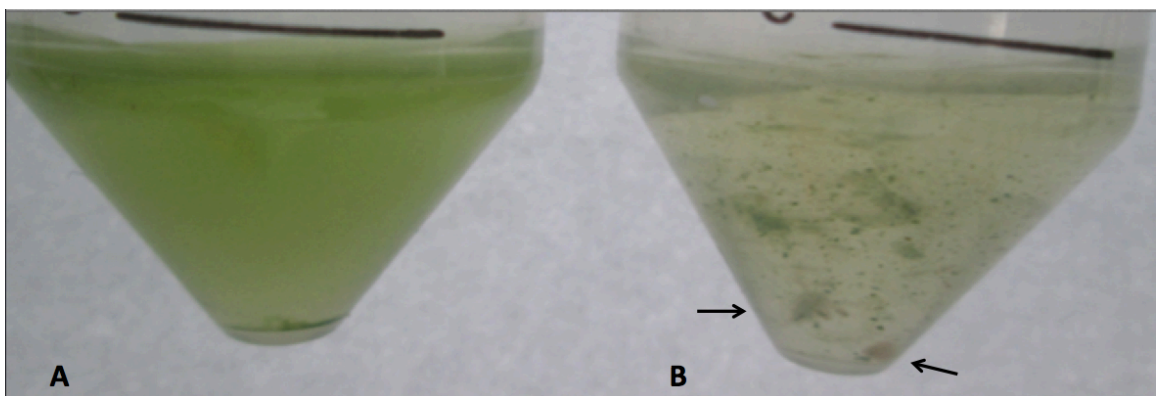


Figure 2.7. Colony formation in *C. sorokiniana* upon predation by *D. magna*. Cultures of *C. sorokiniana*, incubated 72 h in the absence (A) and presence of *D. magna* (B). Colonies of *C. sorokiniana* are visible in the liquid culture (B) as well as two *Daphnia* (arrows).

Previous studies have shown colony formation in three out of these nine combinations. Boraas et al. (1998) found that *C. vulgaris* formed colonies upon predation by *Ochromonas vallescia* (Table 2.2.). This study differs in certain aspects from our study. Specifically, Boraas et al. (1998) used *C. vulgaris* CCAP 211/8A and *O. vallescia* in chemostat cultures, whereas we used *C. vulgaris* CCAP 211/11B and *Ochromonas spp.* in tubes. Regarding the combination *C. vulgaris* + *T. thermophila*, Fisher et al. (unpublished) recently showed that the proportion of *C. vulgaris* cells in colonies increases in the presence of *T. thermophila* (Table 2.2.), but their experiment was performed in 24-well plates. Furthermore, Von Elert & Franck (1999) have shown that *S. obliquus* forms colonies in the presence of *D. magna* (Table 2.2.), but they did not measure the proportion of cells in colonies.

We found that colony size changed in eight combinations (Fig. 2.3. B-I, Fig. 2.4. B-I). In the combination *C. sorokiniana* + *Ochromonas spp.* (Fig. 2.3. A, Fig. 2.4. A), colony size did not change. However, we would expect colony size to increase in all combinations. One observation that may explain this unexpected result is that *C. sorokiniana* only formed large colonies in the presence of the two large predators, i.e. *T. thermophila* and *D. magna* (Fig. 2.3. D, G), but not with the smaller predator *Ochromonas* (Fig. 2.3. A). This indicates that it may be too costly for the algae *C. sorokiniana* to form large colonies (Reynolds 1984; Boraas et al. 1998; Ploug et al. 1999) in the presence of the relatively small predator *Ochromonas* (Fig. 2.3. A).

Finally, we detected a decrease in algal density, i.e. predation, in seven combinations (Fig. 2.6. A, B, D, F-I). We did not observe predation in two combinations; *S. obliquus* + *Ochromonas spp.* (Fig. 2.6. C) and *C. vulgaris* + *T. thermophila* (Fig. 2.6. E). In these two cases, *Ochromonas spp.* and *T. thermophila* were either not grazing on the algae or algal colony formation was so successful (Fig. 2.2. C, E) that the algae could not be grazed upon (Fig. 2.6. C, E). Our behavioural observations (Table 2.5. A, B, D, F-I) supported the findings that in these seven combinations, the predators were eating the algae (Fig. 2.6. A, B, D, F-I). Specifically, *Ochromonas spp.* captured *C. sorokiniana* (Table 2.5. A) and *C. vulgaris* (Table 2.5. B) on its mouth pore. The algae rotated as soon as they reached *Ochromonas's* flagella and then stopped rotating. Although this observation may at first not directly imply ingestion, Boraas et al. (1992) reported that as soon as 50% of the *C. vulgaris* cell is enveloped by *Ochromonas*, the *C. vulgaris* cell stops rotating and then the cell is "drawn into the body of *O. vallescia*". This strongly suggests that our observation may be a preliminary step before ingestion. In the cases of *T. thermophila* with *C. sorokiniana* (Table 2.5. D) and *S. obliquus* (Table 2.5. F), we clearly saw ingestion of the algae and presence of the alga inside *T. thermophila*, respectively. This is the first time such a behaviour of *T. thermophila* towards the algae *C. sorokiniana* and *S. obliquus* has been reported. In all the combinations with *D. magna* (Table 2.5. G-I), we observed a green coloration of *Daphnia's* gut. This has previously been seen in the combinations of *D. magna* + *C. vulgaris* (Ryther 1954) and *D. magna* + *S. obliquus* (Lürling & Verschoor 2003), but not in the case of *D. magna* + *C. sorokiniana*.

2.6. Supplementary Material

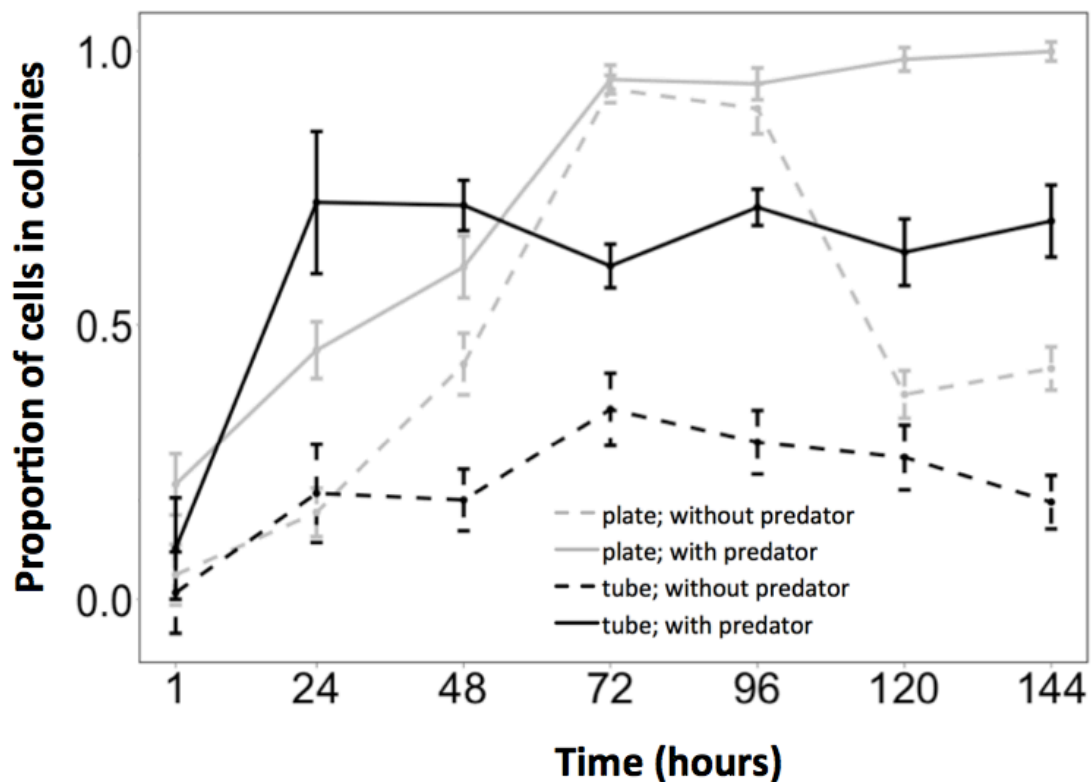


Figure S2.1. Comparison of the proportion of cells in colonies between 50-ml Falcon tubes and 24-well plates. In order to decide whether the 50-ml Falcon tubes or the 24-well plate was the most suitable system to measure the proportion of cells in colonies, we conducted the following experiment. We used nine 50-ml Falcon tubes for the control and nine for the treatment, as well as nine wells on a 24-well plate for the control and nine for the treatment. In each tube we added 19.6 ml of *C. vulgaris* (7×10^4 cells ml^{-1}) to either 0.4 ml of PPY in the control or 0.4 ml of *Ochromonas spp.* (6×10^5 cells ml^{-1}) in the treatment set. We used the same cultures of *C. vulgaris* and *Ochromonas spp.* for the 24-well plate experiment. We added 980 μl of *C. vulgaris* in each well and 20 μl of PPY and *Ochromonas spp.* in the control and treatment, respectively. We incubated the Falcon tubes and the 24-well plate at 20°C light-dark 16:8 hour cycle and kept the tube caps loose to allow for oxygenation. We collected

samples at seven time points after adding the putative predator; 1 h, 24 h, 48 h, 72 h, 96 h, 120 h and 144 h. At each time point, we tilted the falcon tubes five times, to homogenize the solution, and transferred 200 μ l of each culture into a 96-well plate. We acquired microscopy images of these subsamples and of the 24-well plate using a VisiCam digital camera under an inverted microscope (XDS-3) at X20 magnification; phase contrast: 10. We then quantified the proportion of cells in colonies. In both cases the proportion of cells in colonies was higher in the presence of the predator over the 144 h time period (Solid black line – Dashed black line: glm, time point 144 h: p_{144} ; $F = 34.48$, $df = 16$, $P = 2.362 \times 10^{-5}$; Solid grey line – Dashed grey line: glm, p_{144} , $F = 169.09$, $df = 16$, $P = 6.372 \times 10^{-10}$). In the 24-well plate, in the absence of *Ochromonas spp.*, 85% of cells were in colonies within 72 h. For this reason we chose to conduct all our experiments using 50 ml-Falcon tubes. Error bars represent standard error of the mean (\pm se).

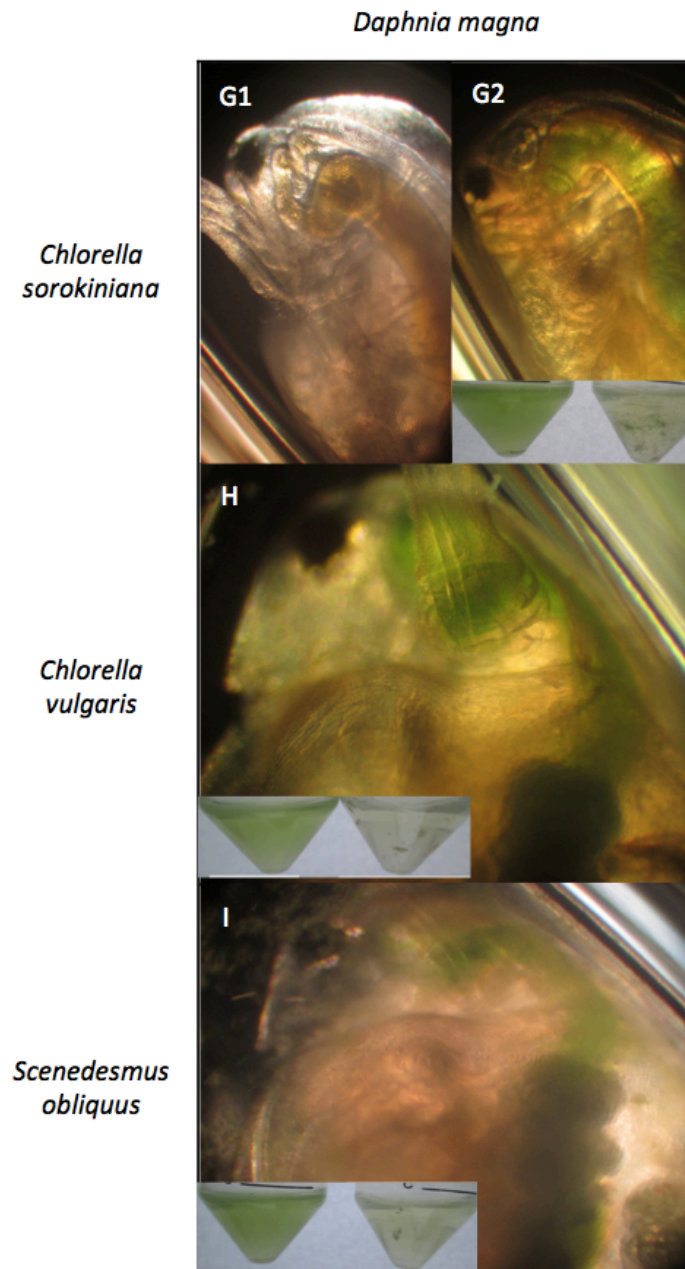


Figure S2.2. Ingestion of algae by *Daphnia*. Gut coloration of *D. magna* after 24 h with no added algae (G1); noticeable green gut 24 h after adding *C. sorokiniana* (G2); green gut 24 h after adding *C. vulgaris* (H); green gut 24 h after adding *S. obliquus* (I). After 72 h, green algal cultures (bottom left image: without *Daphnia*) had become almost transparent due to grazing by *D. magna* (bottom right image: with *Daphnia*).

Movie S2.1. Capture of unicellular *C. sorokiniana* by *Ochromonas*. *C. sorokiniana* rotates for 8 seconds upon contact with *Ochromonas*'s mouth pore and flagella. Then, it stops rotating and remains in contact with *Ochromonas*.

Movie S2.2. Capture of unicellular *C. vulgaris* by *Ochromonas*. *C. vulgaris* rotates upon contact with *Ochromonas*'s mouth pore and flagella. After 20 seconds, it stops rotating and remains in contact with *Ochromonas*.

Movie S2.3. Ingestion of unicellular *C. sorokiniana* by *T. thermophila*. (I)

Movie S2.4. Ingestion of unicellular *C. sorokiniana* by *T. thermophila*. (II)

Movie S2.5. Ingestion of unicellular *C. sorokiniana* by *T. thermophila* and passage from one vacuole to another.

Movie S2.6. Unicellular *S. obliquus* inside *T. thermophila* and passage from one vacuole to another.

Chapter 3. Benefits of colony formation

3.1. Abstract

Predation is one of the forces driving single cells to form colonies, the first step in the evolution of multicellularity. What is the benefit of forming a colony under predation pressure? First, colonies may be too large to be ingested by a predator. This is the 'size benefit' that colonies can provide. Second, being in a colony reduces the probability that a single individual will be attacked. This is termed as the 'dilution benefit'. We tested these two hypotheses using nine predator-prey combinations; *Ochromonas* spp., *T. thermophila* and *Daphnia* as predators, and *C. sorokiniana*, *C. vulgaris* and *S. obliquus* as prey. In six combinations, the predators could not ingest the colonies, indicating that there is a size benefit. In four combinations, *C. vulgaris* + *Ochromonas* spp., *C. sorokiniana* + *T. thermophila*, *C. vulgaris* + *T. thermophila* and *S. obliquus* + *T. thermophila*, the predator spent the same amount of time with either single cells or colonies, supporting the hypothesis that dilution in numbers also provides a benefit to the group. These findings suggest that these four combinations are promising for further studies into the benefits and costs of colony formation.

3.2. Introduction

Unicellular algae rely on light and nutrient uptake from the environment for their survival. Algae inside a colony, have limited exposure to light, due to the higher sinking rate of colonies (Reynolds 1984), as well as limited access to nutrients, due to their position in the colony (Boraas et al. 1998; Ploug et al. 1999). Therefore, we would expect unicells to predominate in a culture. However, in experimental settings where

predators were present (Chapter 2), colonies of algae formed. The question that arises is what are the benefits of forming a colony that outweigh the aforementioned costs.

It has been argued that the formation of colonies in the presence of predators can have two benefits. First, in terms of size, because small predators are unable to ingest large colonies. *Scenedesmus* colonies were too large to be ingested especially by small *Daphnia* (Horn 1981; Lampert 1987; Lürling & Donk 1996; Burns 1968; Lürling et al. 1997). Similarly, the ciliate *Euplotes* sp. could not ingest *Phaeocystis globosa* when *P. globosa* formed colonies (Long et al. 2007). Secondly, there has been suggested to be an advantage of being in a colony in terms of a dilution in numbers. In particular, if different size groups are attacked with the same rate by a predator, it is clearly beneficial for an individual to be in a large group. Such a 'dilution benefit' has been shown in the marine insect *Halobates robustus* upon predation by the fish *Sardinops sagax* (Foster & Treherne 1981; Krause & Ruxton 2002). However, it has not yet been explored to what extent the dilution effect can provide a benefit to microorganisms such as algae being in a colony.

We experimentally tested whether there was a size and/or a dilution benefit of being in a colony and a large colony in our nine algae-predator combinations (A-I). Specifically, we examined the size advantage hypothesis by observing whether the putative predator is capable of ingesting colonies. To experimentally test the dilution effect, which reflects whether the likelihood of predation is approximately the same regardless of whether the prey is singular, in a small or in a large colony, we used the

time spent in the first interaction of the predator with the prey as a proxy for estimating the likelihood of predator attack.

3.3. Materials and Methods

3.3.1. Size benefit: Protist behavioural observations

During the dilution effect experiment (3.3.2.), we followed individual *T. thermophila* and *Ochromonas* cells in each well for 1 min under the inverted microscope (X20 magnification, phase contrast: 10) to detect any ingesting activity towards colonies.

We define a colony as ≥ 3 cells in a group, unless indicated otherwise.

In the combinations with *D. magna*, we were unable to identify whether *D. magna* ingested unicells or colonies. Therefore, we could not examine whether there was a size benefit in forming a colony.

3.3.2. Dilution effect experiment

In this experiment we used 'time spent in contact with the alga' as a proxy of predator attack. The null hypothesis was that the predator spends the same amount of time in contact with an algal cell, regardless of whether the algal cell is singular or in a colony (having controlled for differences in abundance of unicells and colonies). The alternative hypothesis was that the predator spends less time in contact with an algal cell when the algal cell is in a colony. In other words, being in a colony provides a dilution benefit. To test for a benefit in terms of dilution in the combination *C. sorokiniana* + *Ochromonas spp.*, we added 19.6 ml of *C. sorokiniana* and 0.4 ml of *Ochromonas spp.* in 30 50-ml Falcon tubes. We incubated the tubes at 20°C light-dark 16:8 hour cycle and kept the tube caps loose to allow oxygenation. After 72 h, when

the proportion of cells in colonies was ~50%, we tilted all 30 of the tubes five times and transferred samples of 200 μ l on a 96-well plate. We then observed an individual protist from each well under an inverted microscope (at X20 magnification; phase contrast: 10) and tracked whether the first interaction we observed was with an algal unicell or colony, the size of the latter and the time length of the interaction. As interactions we define only those occurring for ≥ 2 s (e.g. see Movie S3.1. time point '0:13-0:19', an example of a '6-second' interaction of *T. thermophila* with a *C. sorokiniana* unicell). In order to find the time spent per individual algal cell, we divided the time length of each interaction with colony size.

We used the same experimental apparatus in the rest of the combinations. The different concentrations, total volume per tube and number of replicates used, are mentioned in Table 3.1.

We did not perform the dilution effect experiment with *D. magna*, because the large-scale difference of the *D. magna* and the algae did not allow us to accurately determine whether an individual was interacting with a unicell or a colony.

	Concentration of algae (cells ml ⁻¹)	Concentration of putative predators (cells or individuals ml ⁻¹)	Total volume per tube	Number of independent replicates
A) <i>Chlorella sorokiniana</i> + <i>Ochromonas</i> spp.	2 x 10 ⁶	1 x 10 ⁵	20ml	n=30
B) <i>Chlorella vulgaris</i> + <i>Ochromonas</i> spp.	1 x 10 ⁶	2 x 10 ⁵	20ml	n=30
C) <i>Scenedesmus obliquus</i> + <i>Ochromonas</i> spp.	4 x 10 ⁵	7 x 10 ⁴	20ml	n=30
D) <i>Chlorella sorokiniana</i> + <i>Tetrahymena thermophila</i>	3 x 10 ⁶	1 x 10 ⁴	20ml	n=30
E) <i>Chlorella vulgaris</i> + <i>Tetrahymena thermophila</i>	2 x 10 ⁶	2 x 10 ⁴	20ml	n=30
F) <i>Scenedesmus obliquus</i> + <i>Tetrahymena thermophila</i>	7 x 10 ⁵	6 x 10 ³	20ml	n=30

Table 3.1. Concentrations, total volume per tube and replicates used in the ‘dilution effect experiment’.

3.3.3. Statistical analyses

In order to predict the response variable ‘Time spent per alga relative to algal frequency’ from the explanatory variable ‘Colony size’, we used a linear model (R package). We fitted regression lines to the data when the relation was positive or negative. Coefficients were expressed as adjusted r^2 values to reduce the effect of variation in sampling.

3.4. Results

3.4.1. Size benefit

In six combinations, *Ochromonas* spp. + *C. sorokiniana* / *C. vulgaris* / *S. obliquus* and *T. thermophila* + *C. sorokiniana* / *C. vulgaris* / *S. obliquus*, we did not observe ingestion of algal colonies by either *Ochromonas* or *T. thermophila*. Specifically, we made 30 independent ‘1 min observations’ of the protists in the presence of the algae (approx.

50% of algal cells in colonies – 50% unicells) and did not observe ingestion of colonies in none of the 30 observations.

In the other three combinations, *Daphnia* + *C. sorokiniana* / *C. vulgaris* / *S. obliquus*, we could not detect whether *Daphnia* was ingesting colonies, due to the large-scale difference between *Daphnia* and the algae.

3.4.2. Dilution benefit of being in a colony

In this experiment our hypothesis was that the predator spends less time with the prey when the prey is in a colony. However, in all four combinations (Fig. 3.1. B, D-F) the predator spent more time in contact with an algal cell when the algal cell was in a colony. This observation indicates that being in a colony does not provide a benefit in terms of dilution.

Similarly, being in a larger colony does not provide a dilution benefit to the alga in these 'alga-putative predator' combinations (Fig. 3.2. B, D-F). The putative predator spent the same amount of time with the alga regardless of whether the alga was in a small or a large colony.

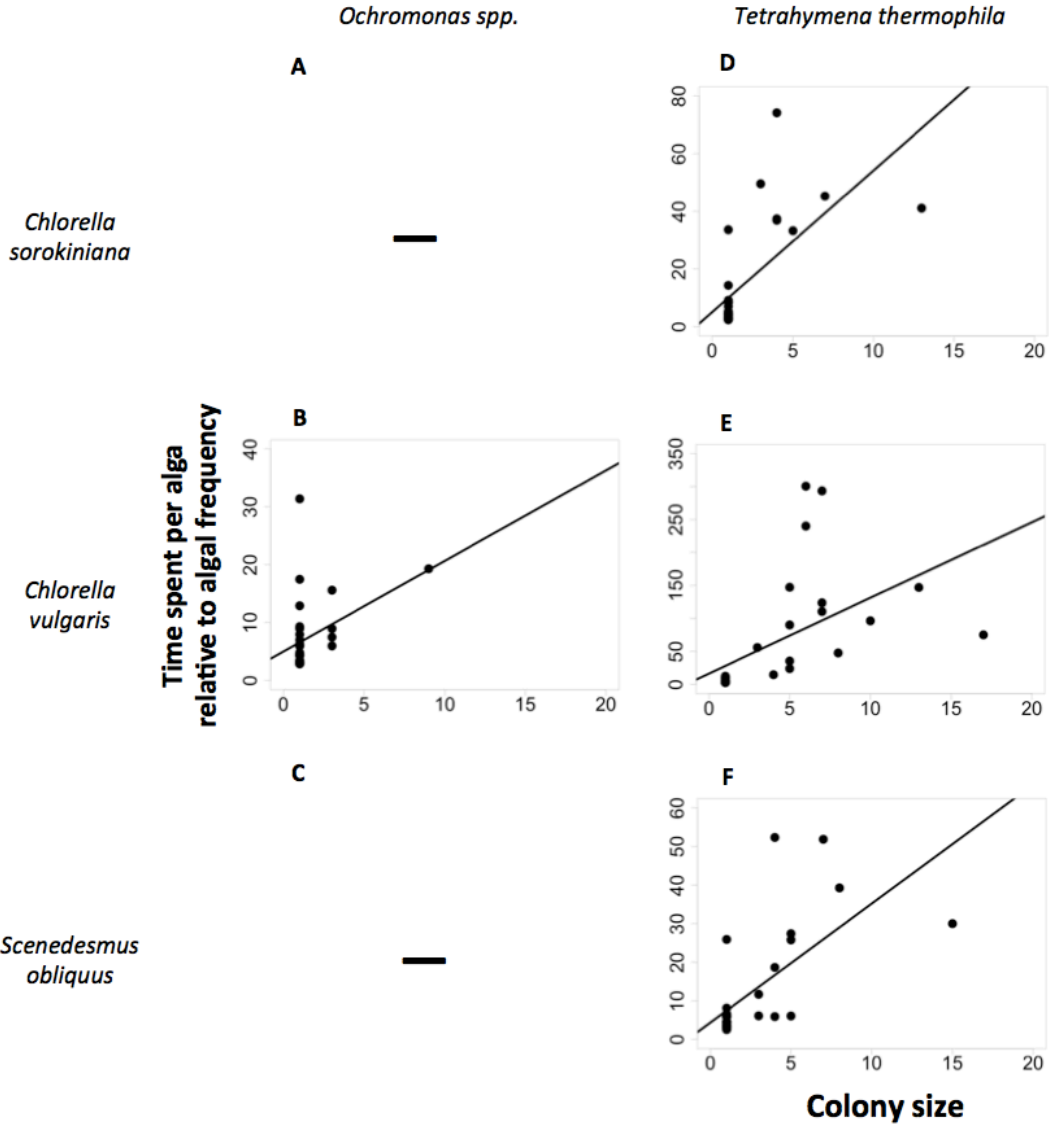


Figure 3.1. Testing for a dilution benefit of being in a colony. Our hypothesis was that the predator spends less time interacting with an algal cell when the alga is in a colony. In none of the combinations, did the putative predator spend less time interacting with an algal cell when the alga was in a colony. In fact, the putative predator spent more time in contact with the algal cell when the algal cell was in a colony (**B**. lm, adjusted $r^2 = 0.1189$, $F = 4.914$, $df = 28$, $P = 0.0349$; **D**. lm, adjusted $r^2 = 0.4242$, $F = 22.37$, $df = 28$, $P = 5.81 \times 10^{-5}$; **E**. lm, adjusted $r^2 = 0.2597$, $F = 10.82$, $df = 27$, $P = 0.00279$; **F**. lm, adjusted $r^2 = 0.4089$, $F = 21.06$, $df = 28$, $P = 8.52 \times 10^{-5}$). Therefore, our results do not support the hypothesis that there is a benefit in terms of dilution. Colony size on the x axis includes unicells and paired cells. (–) For combinations **A** and

C, we could not make robust conclusions regarding the dilution effect, due to a small number of data points.

3.4.3. Dilution benefit of being in a large colony

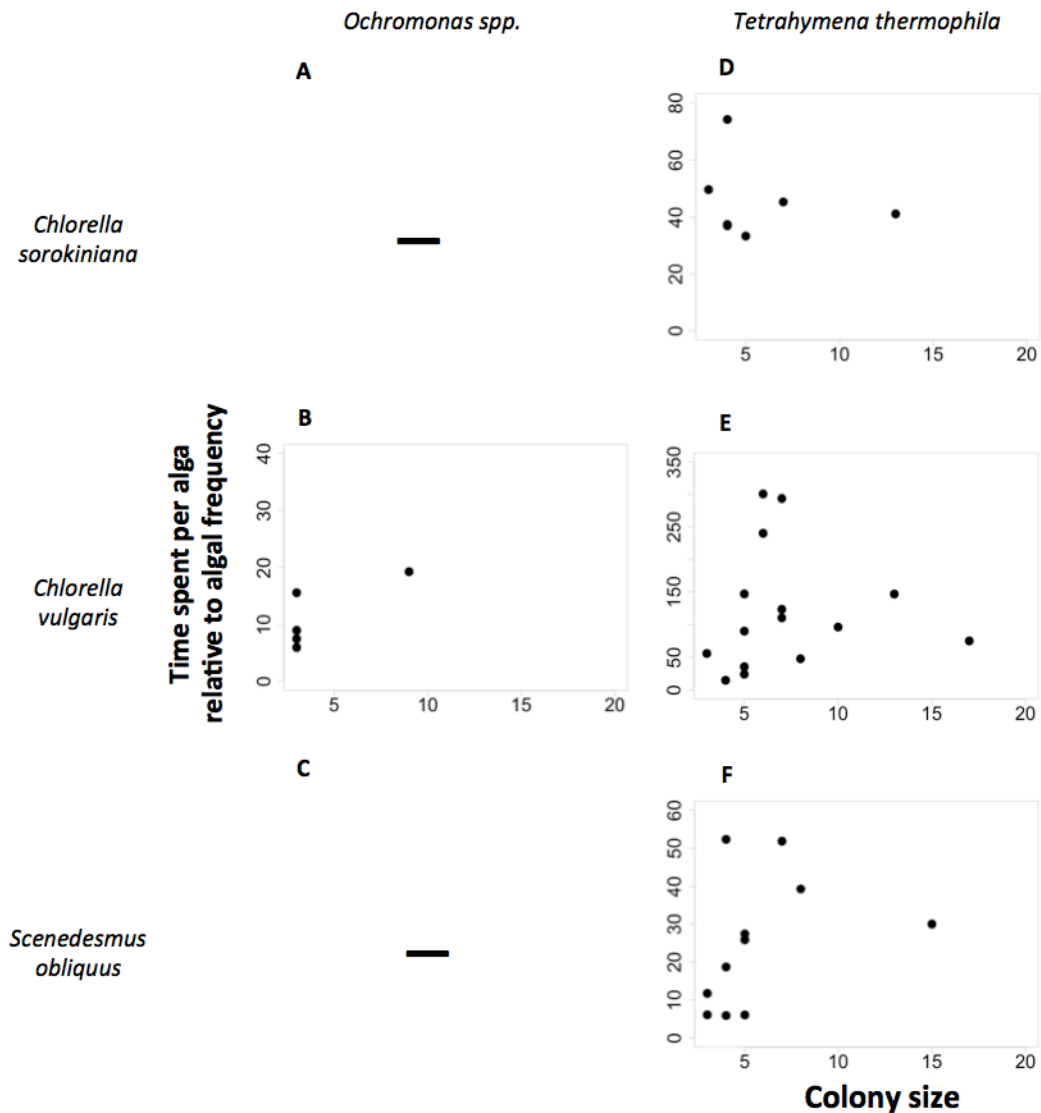


Figure 3.2. Testing for a dilution benefit of being in a large colony. Subpopulation of data points from Fig. 3.1. with colony size ≥ 3 . In none of the combinations does the putative predator spend less time with an alga when the alga is in a colony (B. lm, $F = 4.266$, $df = 3$, $P = 0.131$; D. lm, $F = 0.194$, $df = 5$, $P = 0.678$; E. lm, $F = 0.025$, $df = 13$, $P = 0.878$; F. lm, $F = 1.263$, $df = 9$, $P = 0.29$). Being in a large colony does not dilute the risk of being attacked by a putative predator. (–) Indicates combinations where we observed only one (A) and three (C) interactions with colonies.

3.5. Discussion

In all six combinations examined, the putative predators were not able to ingest colonies. This finding suggests that being in a colony provides a size benefit. However, in four of the combinations tested, *C. vulgaris* + *Ochromonas* spp. (Fig. 3.1. B), *C. sorokiniana* + *T. thermophila* (Fig. 3.1. D), *C. vulgaris* + *T. thermophila* (Fig. 3.1. E) and *S. obliquus* + *T. thermophila* (Fig. 3.1. F), the putative predator spent more time with the algal cell when the algal cell was in a colony. Therefore, being in a colony did not reduce a cell's risk of being attacked by a putative predator. In other words, we did not find a dilution benefit. Also, in these four combinations we did not observe a dilution benefit of being in a large colony (Fig. 3.2. B, D-F).

Regarding the 'size benefit', we were not able to address whether there was a 'size benefit' in colony formation in the cases of *C. sorokiniana* + *D. magna*, *C. vulgaris* + *D. magna* and *S. obliquus* + *D. magna*, because of large differences in scale between the *Daphnia* and the algae. Regarding the 'dilution effect' experiment, we observed that the protists interacted for a longer period of time with an alga when the alga was in a colony, in four cases (Fig. 3.1. B, D-F). By looking at the data points in the case of *C. vulgaris* + *Ochromonas* spp. (Fig. 3.1. B) and *C. sorokiniana* + *T. thermophila* (Fig. 3.1. D), as well as in the cases of *C. sorokiniana* + *Ochromonas* spp. (Fig. 3.1. A) and *S. obliquus* + *Ochromonas* spp. (Fig. 3.1. C), it is clear that the data are not evenly dispersed across the x axis. Most of the data points are 'protist-unicellular alga' interactions.

To improve this study, we need to find the benefits of colony formation in the combinations of *Daphnia* using a different experimental approach. For instance, we could expose the *Daphnia* to either unicellular or colonial algae and then quantify the consumption rate. If we observed that colonies are consumed at a lower rate than unicells, this would support the hypothesis of a ‘size benefit’ of colony formation in the combinations *C. sorokiniana* + *D. magna*, *C. vulgaris* + *D. magna* and *S. obliquus* + *D. magna*. Also, in order to form a robust conclusion on whether there is a ‘dilution benefit’ of being in a colony, we need to address the problem of dispersal in our data (Fig. 3.1. A-D, Fig. 3.2. A-D). This may be solved by using a culture of algae ideally consisting of 50% unicells and 50% cells in colonies.

How do our results relate to previous studies? It is the first time that a size benefit of colonies upon predation pressure has been shown in these six “algal-putative predator” combinations (A-F). It is worth mentioning that a size benefit was observed in a study with *C. vulgaris* + *Ochromonas vallescia* (Boraas et al. 1998). Even though the exact strain of *C. vulgaris* and species of *Ochromonas* used was not the same as in our study, they produced similar results. Specifically, they showed that the predator *O. vallescia* was unable to ingest fully-grown *C. vulgaris* colonies of 15 µm in size, but could ingest unicells and smaller colonies (Boraas et al. 1998). Also, this is the first time that a dilution effect approach has been used to assess the benefit of being in an algal colony.

3.6. Supplementary Material

Movie S3.1. Example of a ‘6-second interaction’ of *T. thermophila* with a single cell of *C. sorokiniana*.

Chapter 4. General Discussion

4.1. Summary

In Chapter 2, we found colony formation in all nine 'algal-putative predator' combinations (Table 4.1.). In all combinations, except *C. sorokiniana* + *Ochromonas spp.*, we observed a change in colony size upon exposure to the putative predators (Table 4.1). Furthermore, in seven combinations we detected a decrease in algal density, as well as ingestion of the algae, capture of the algae on the mouth pore and presence of the algae inside *Tetrahymena* and *Daphnia* (Table 4.1). This strongly suggests that in these combinations, the predators eat the algae.

In Chapter 3, we used the same nine combinations to examine the benefits of colony formation; the 'size benefit' and the 'dilution benefit'. In six combinations (Table 4.1.), the putative predator was unable to ingest colonies of algae, indicating that there is a 'size benefit' in forming a colony. In the combinations with *Daphnia*, the large size of *Daphnia* relatively to the algae did not allow us to draw conclusions regarding ingestion of colonies by the *Daphnia* (Table 4.1.). However, in four combinations the putative predator spent more time with an algal cell when the cell was in a colony, thus indicating that colony formation does not provide a 'dilution benefit' (Table 4.1). Also, in these four combinations the predator spent the same amount of time with the algal cell when the cell was in a large colony and a small colony, suggesting that being in a large colony does not provide a 'dilution benefit' (Table 4.1).

	Chapter 2				Chapter 3		
	Colony formation		Predation		Benefits		
	Proportion of cells in colonies	Mean colony size	Decrease in algal density	Direct observations of predation	Size benefit	Dilution benefit of being in a colony	Dilution benefit of being in a large colony
A) <i>Chlorella sorokiniana</i> + <i>Ochromonas</i> spp.	✓	X	✓	✓	✓	–	–
B) <i>Chlorella vulgaris</i> + <i>Ochromonas</i> spp.	✓	✓	✓	✓	✓	X	X
C) <i>Scenedesmus obliquus</i> + <i>Ochromonas</i> spp.	✓	✓	X	X	✓	–	–
D) <i>Chlorella sorokiniana</i> + <i>Tetrahymena thermophila</i>	✓	✓	✓	✓	✓	X	X
E) <i>Chlorella vulgaris</i> + <i>Tetrahymena thermophila</i>	✓	✓	X	X	✓	X	X
F) <i>Scenedesmus obliquus</i> + <i>Tetrahymena thermophila</i>	✓	✓	✓	✓	✓	X	X
G) <i>Chlorella sorokiniana</i> + <i>Daphnia magna</i>	✓	✓	✓	✓	–	–	–
H) <i>Chlorella vulgaris</i> + <i>Daphnia magna</i>	✓	✓	✓	✓	–	–	–
I) <i>Scenedesmus obliquus</i> + <i>Daphnia magna</i>	✓	✓	✓	✓	–	–	–

Table 4.1. Summary table of our results. (–) represents cases not tested due to a small number of data points (A and C, ‘Dilution benefit’ columns) and difficulty in detecting interactions of *D. magna* with either single unicells or colonies (G-I, ‘Benefits’ columns).

4.2. Is colony formation a general response of algae to predation?

In all nine ‘algal-putative predator’ combinations tested, we observed colony formation. Previous studies have shown colony formation in three of these combinations, although not with the same strains or experimental setting (Boraas et al. 1998; Fisher et al. unpublished; Von Elert & Franck 1999). This suggests that colony formation is a general response of the algae *C. sorokiniana*, *C. vulgaris* and *S. obliquus* to predation by either *Ochromonas* spp., *T. thermophila* or *D. magna*. Although the response seems to be generic in these algae, we cannot make the same conclusion about the whole genus of *Chlorella* or *Scenedesmus*, as *Scenedesmus acutus* does not respond by forming colonies when exposed to different species of zooplankton (Table 2.2.).

4.3. Is actual predation always a prerequisite for colony formation?

Actual predation is not always a prerequisite. In two cases, *S. obliquus* + *Ochromonas* spp. and *C. vulgaris* + *T. thermophila*, we observed colony formation, but not a decrease in the number of algae (Table 4.1.). This may be due to either successful colony formation (Fig. 2.2. C, E) preventing ingestion of the algae by the small predator (Fig. 2.6. C, E) or due to the predators actually not eating the algae. Interestingly, studies on rotifers and the copepod *Eudiaptomus gracilis* show that colony formation is induced in the prey *Scenedesmus*, even though the predators do not eat *Scenedesmus* (Wiltshire et al. 1999). These observations can be explained by taking into consideration the natural environment of these algae, where multiple size-selective predators are present (Tollrian & Dodson 1999; Tollrian & Harvell 1999). In the case of *S. obliquus* + *Ochromonas* spp. and *C. vulgaris* + *T. thermophila*, predation drives colony formation (Table 4.1.), however cells may not have a protective benefit against that particular predator, but might benefit from potential predation.

4.4. Does colony formation provide a benefit?

In six cases, we were able to detect a 'size benefit', but not a 'dilution benefit' of being in a colony (Table 4.1.). This finding suggests that cells may gain a size benefit from being in a colony, even though the per cell risk of attack, measured as time length of 'predator-individual alga' interaction, is not reduced. In the combinations with *Daphnia* we could not measure whether there was a 'size benefit' or a 'dilution benefit' (Table 4.1.). Interestingly, in these combinations colony formation may not provide a 'size benefit', but a benefit of nutrient supply during gut passage. Specifically, *Daphnia* induced the non-gelatinous unicellular *Sphaerocystis schroeteri* to

form gelatinous colonies, and these colonies passed through *Daphnia's* gut, where they gained nutrients from the remains of edible algae and *Daphnia's* metabolites. The algae then emerged intact from *Daphnia's* gut, due to their protective gelatinous sheath (Kampe et al. 2007; Porter 1976).

4.5. Future directions

Further studies on the benefits as well as the costs of colony formation, will help us understand the role of predation in driving colony formation. For example, it will be useful to find the benefits of colony formation in the combinations *C. sorokiniana* + *D. magna*, *C. vulgaris* + *D. magna* and *S. obliquus* + *D. magna*, by exposing the *Daphnia* to either unicells or colonies of algae, and then quantifying colony survival upon passage from *Daphnia's* gut. Also, in order to further assess whether there is a 'dilution benefit' of being in a colony, we need to address the problem of dispersal in our data (Fig. 3.1. A-D, Fig. 3.2. A-D). This may be solved by using an algal culture ideally consisting of 50% unicells and 50% cells in colonies.

In addition, it is necessary to quantify the costs related to colony formation. For example, in the case of *C. sorokiniana* + *Ochromonas spp.*, the costs related to forming a colony may explain the observation that mean colony size did not increase (Table 4.1.). A possible experiment to find the costs, will be to compare the growth rate of cells in colonies and the growth rate of unicells. However, costs in terms of growth rate have proven to be experimentally difficult to detect (Lürding 2001; Hessen & Van Donk 1993; Lampert et al. 1994).

Overall, our observations of colony formation in algae upon predation pressure and measurements of the benefits regarding colony formation, help lead towards a better understanding of the benefits and costs involved in colony formation, the first step in the evolution of multicellularity.

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