THE SMELL OF WATER

Grazer-induced colony formation in *Scenedesmus*

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Miquel Lürling

Proefschrift

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Daphnia pulex

Het snijvlak
Onbarmhartig scherprechter.
Niet op
maar onder,
in een ander blauw
roeit onwetend het leger
met miljoenen
driftig voort.

Adriaen Willemsz - 1999

STELLINGEN

1. De door grazers veroorzaakte kolonievorming in de groenalg *Scenedesmus* is een geïnduceerd afweermechanisme.
   Dit proefschrift

2. Grazergeïnduceerde kolonievorming is mogelijk in alle *Scenedesmus* soorten, mits de celdeling niet geblokkeerd is.
   Dit proefschrift

3. De kolonie-inducerende stof in '*Daphnia*-water' is niet, zoals recentelijk gesuggereerd is door Wiltshire en Lampert, het door *Daphnia* uitgescheiden ureum, maar een tot nu toe onopgehelderd carbonzuur.
   Wiltshire, K.H. & Lampert, W. ASLO-Meeting, 4 Feb. 1999
   Von Elert, E. & Franck, A. (1999) Journal of Plankton Research 21: 789-804
   Dit proefschrift

4. De mening van Scheffer (1998) dat zelfs ernstige graasverliezen gecompenseerd kunnen worden door snelle reproductie van de alg, suggereert ten onrechte dat dit een goede strategie zou zijn om aan de zoöplanktongraasdruk te ontkomen.
   Scheffer, M. (1998) Ecology of shallow lakes, Chapman & Hall, page 186

5. Alle *Scenedesmus* soorten hebben het vermogen om ook ééncellig te zijn.
   Trainor, F.R. (1998). Nova Hedwigia Beiheft 117

6. De communicatie tussen aquatische organismen is vele malen beter dan die tussen onderzoekers.

7. Het watervlooiencircus is een onbetrouwbare methode om de aanwezigheid van bestrijdingsmiddelen in het oppervlaktewater aan te tonen.
   De Geus-van der Eijk, J.G. (1997) Rapport Eijkpunt 'De watervlooientoets, een oriënterend onderzoek naar de bruikbaarheid in akkerbouwgebieden.'

8. Dat watervlooien minder goed groeien wanneer ze gevoerd worden met algen die gekweekt zijn bij een lage fosforconcentratie, wordt ten onrechte toegeschreven aan een directe minerale fosforlimitatie.
Sterner, R.W. (1993) Ecology 74: 2351-2360

9. In de algentaxonomie, en zeker in de *Scenedesmus* taxonomie, is het gebruik van moleculaire technieken een absolute noodzaak.
*Kessler, E. (1991) Botanica Acta **104**: 169-171*

10. De levendige aandelenhandel impliceert dat de *Homo economicus* fictie is.

11. Water is, bij 20°C en een druk van één atmosfeer, geen kleur- en geurloze vloeistof.

12. Geen *Daphnia*, maar *Pulex aquatilis*.

13. In den beginne was er ... chemische communicatie!

14. Een consument produceert geen afval, hij houdt het over.

15. De Amerikaanse droom is voor velen een nachtmerrie.

16. Binnen een opleiding Aquatische Ecologie zou als onderwijselement een duikcursus opgenomen moeten worden.

Stellingen behorende bij het proefschrift:

The smell of water
Grazer-induced colony formation in Scenedesmus

Miquel Lürling
Wageningen, 12 mei 1999.

VOORWOORD

Wat er ontbrak was een zee, maar opgroeien in de polder had ook zijn voordelen. Zo hoefde je slechts door een dun laagje klei om in het grondwater te geraken. En er was genoeg oppervlaktewater wat mijn ouders tot wanhoop dreef. Ze riepen allerlei watermonsters in het leven om me verre van de sloten, grindgaten en plassen te houden. Maar ja, na de bekende salamanders, kikkervisjes, stekelbaarsjes, bloedzuigers en wat al niet meer aan grotere water beesten te hebben gevangen wilde het met die watermonsters niet zo lukken tot grote frustratie van de kleine onderzoeker. Misschien moest ik het water beter bekijken en gelukkig verkreeg ik voor mijn twaalfde verjaardag mijn eerste microscoop. Water uit moeders bloemenvaas tezamen met hooi uit het konijnenhok een week incuberen en warempel een hele mysterieuze wereld ging voor me open. De sloten, vijvers, beken in de omtrek werden veelvuldig bemonsterd. Het veldwerk stopte abrupt met de ontdekking dat water met gemoute gerst en gist tot goddelijk genot kon leiden. De daarop volgende jaren stonden in het teken van vele expedities naar rokerige laboratoria waar tot diep in de nacht werd geëxperimenteerd. Het woord watermonster kreeg ondertussen een andere betekenis. Duizenden monsters werden uit de Breukeleveense Plas gehaald om een indruk te krijgen van de invloed van wind en vis op het lichtklimaat onder water. Leuke vervolgprojecten volgden bij het Centrum voor Limnologie en het RIKZ te Middelburg, en nu nog een promotieplek. Die kwam er, alleen met algen en watervlooien. "Wat weet ik nu van algen en watervlooien?" Vijf jaar later stel ik me die vraag nog steeds, nu echter in de wetenschap dat ik bij lange na niet de enige ben. De toekomst is rooskleurig, er valt nog zoveel te onderzoeken en te ontdekken!

"Wat kom jij hier doen?" was de eerste kennismaking als 'OiO' met de toenmalige vakgroep WKAO. Een bureau werd geconfisqueerd bij een andere vakgroep, een computer opgegraven op het kerkhof en een stoel verkregen van de nabijgelegen kerk. Met de 'onderzoeksfaciliteiten' was het al niet veel beter gesteld, maar daarin lag ook een grote uitdaging. Levendige ruilhandel, creatief bestellen en een goede ondersteuning zorgden ervoor dat in een korte tijd het "Wagenings laboratorium voor plankton onderzoek" een begrip werd. Ondanks dat allerlei randvoorwaarden verbeterd konden en kunnen worden, werd er een topprestatie geleverd, de verwerving van internationale bekendheid. Wageningen werd in het plankton-onderzoek op de wereldkaart geplaatst, eventjes was het HET plankton-infochemicaliën bolwerk. Recentelijk las ik nog: " The laboratory at Wageningen is again adding to our fund of knowledge concerning predator induced alterations in *Scenedesmus*". Hier hebben heel wat personen in meer of mindere mate hun steentje aan bijgedragen. Een woord van dank is dan ook het minste: Allereerst mijn promotor: Beste Wim, we hadden niet zo vaak overleg, maar die keren dat we dat hadden gaven me telkens een zeer goed gevoel. Met name het gemak waarmee jij de zaken overzag was voor mij erg geruststellend. Bedankt voor alles. Beste Ellen, als mijn co-promotor, dagelijks begeleider en ontdekker van de chemische informatieoverdracht tussen watervlooien en algen, was je nauw bij mijn onderzoek betrokken. In de eerste fase kan dan ook gerust over ons onderzoek gesproken worden. Je kijk op het OiO-schap als een tweede puberteit bleek nog niet zo'n verkeerde

inschatting. Ik wil je met name bedanken voor het al vroegtijdig stimuleren van congresbezoeken, het schrijven van artikelen en de vrijheid die me werd gegund om bijvoorbeeld een tijdje in het buitenland te vertoeven. Ich möchte gerne Herr Professor Winfried Lampert vielen Dank sagen für die Möglichkeit einige Monaten in Seinem Labor zu arbeiten. Durch die weltklassische Laborausrustung des Max-Planck-Instituts und die gute Atmosphere war es ein Supererfahrung. An diese Stelle möchte ich auch die Plöner Wissenschaftler Klaus Plath, Claus-Peter Stelzer, Kristin Beck, Petra Limburg, en natuurlijk Maarten Boersma and Karen Wiltshire vielen Dank sagen für die schöne und lehrsame Zeit.
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Daarnaast hebben ook vele studenten in meer of mindere mate een bijdrage geleverd aan de tot standkoming van dit proefschrift. Het besnuffelen van de randgebieden verschafte me veel nieuwe inzichten en ook de 'foutjes' waren vaak erg verhelderend: Johanna Minnaard, Bart Bardoel, Monique Zwiers, Jacco Maissan, Rob Exalto, Roy Geerts, Jeroen Knoef, Caroline Moermond, Remko Roosenboom, Frank Roozen en Esther van der Grinten hartstikke bedankt!
Binnen de LUW heb ik een aantal keren gebruik kunnen maken van de Xe-PAM bij de vakgroep Plantenfysiologie, waarvoor ik Jan Snel hartelijk wil bedanken. Bij Entomologie kon gebruik gemaakt worden van een microbalans en was Marcel Dicke een belangrijke informatiebron over de rol van infochemicaliën in de terrestrische wereld en de Y-buis olfactometrie, mijn dank daarvoor.
Daarnaast zijn er nog vele in den lande en daarbuiten die op uiteenlopende wijze een bijdrage hebben geleverd aan de tot standkoming van dit proefschrift: Bedankt allemaal!
Natuurlijk kan ik mijn familie en vrienden niet vergeten in een dankrede. Ze hebben er in ieder geval voor gezorgd dat er af en toe wat afleiding was en dat de vakidioterie me niet volledig in haar greep kreeg. Inge, jouw liefde, steun en betrokkenheid zijn van onschatbare waarde geweest de afgelopen jaren. Je was er altijd om op terug te vallen als het eventjes wat minder ging. Pap, mam, kei bedankt voor de steun en de interesse. Ontspanning was er ook in de sport, naast het al genoemde hardlopen met de fanatiekelingen, was er de vakgroepsdeelname aan de Veluweloop, het roeien op Koninginnedag, de volleybaltoernooitjes en natuurlijk het Waterteam dat hoge ogen scoorde in de bedrijvencompetitie zaalvoetbal: John, Michiel, Jeroen, Hrasko, Morten, Frits, Marco, Maurice bedankt voor de nodige afwisseling.

CONTENTS

CHAPTER 1

Introduction

"The Precambrian trend from simple producer communities to producer-herbivore and producer-herbivore-carnivore communities can be viewed as a long-term natural cropping experiment resembling those performed by living ecologists"

- S.M. Stanley 1973

1.1 ONE CELL...

All organisms on earth are believed to originate from one cell born over 3.5 billion years ago in a soup of chemicals (Vidal, 1984; Doolittle *et al.*, 1996). Comparison of enzyme amino acid sequences suggests that the common ancestor of pro- and eukaryotes lived about 1.9 billion years ago (Doolittle *et al.*, 1996). The first eukaryote probably originated from fusion of an Archaebacterium with a Gram-negative bacterium (Gupta & Singh, 1994). The precondition for this merging was a dramatic increase in atmospheric O_2 produced by cyanobacteria after the oceanic Fe^{2+}-pool had been exhausted (Schopf *et al.*, 1983). The increased O_2 level was toxic to most anaerobic bacteria favoring only those bacteria that had engulfed respiring bacteria (Vellai *et al.*, 1998). These 'Ur-karyotes' are thought to have captured prokaryotic cells that were not digested but incorporated resulting in organelles, such as mitochondria and flagellae giving rise to heterotrophic flagellates (Vossbrinck *et al.*, 1987; Lake, 1988; Margulis, 1993; Gupta & Singh, 1994; Van den Hoek *et al.*, 1995). The heterotrophic flagellates are considered the ancestors of all fungi, protozoa, metazoa and eukaryotic algae on earth, whereas higher plants evolved from the green algae some 400-500 million years ago (Valentine, 1978). For the evolution of algae the phagotrophic flagellates had to transform into photoautotrophic flagellates. Based on the theory of endosymbiontic origin of organelles, a Precambrian phagotrophic flagellate is believed to have taken up a cyanobacterium in a food vacuole that was not digested, but transformed and incorporated as a chloroplast (Pérasso *et al.*, 1989; Van den Hoek *et al.*, 1995). A prerequisite for predators seemed the development of a nucleus or at least a fluid protoplasm as no phagotrophic prokaryotes are known (Margulis, 1993; Boraas *et al.*, 1998). Because prokaryotes have stiff protoplasm they are unable to form pseudopodia and vacuoles meaning that they cannot feed phagotrophically (Van den Hoek *et al.*, 1995).

Regardless whether life on earth originated at the surface in abundant solar energy or at subsurface hydrothermal systems (Brandes *et al.*, 1998), because of small size and short generation times single-celled organisms could colonize and exploit the earth, as long as liquid water was available. The planktonic mode of life may go back billions of years as indicated by unicellular prokaryotic microfossils (Vidal, 1984). Prokaryotic life remained almost exclusively unicellular for at least the first four-fifth of the history of life on earth (Valentine, 1978), because under conditions without predation, communities would have been structured by competitive interactions, favoring small, free-living cells with the most efficient surface-to-volume ratio (Boraas *et al.*, 1998).

Despite the success of unicellular organisms, multicellular organisms evolved. Multicellularity must have been advantageous as it is widespread (Hallam, 1990) and occurred independently in several unicellular lineages (Bonner, 1988). The selective advantage of exploitation of resources that no unicellular organisms could obtain is believed to have led to

the evolution of multicellular organisms (Valentine, 1978; Brown & Maurer, 1986). Thus, the evolution of multicellularity may be considered a key innovation opening resources from which the organisms were previously barred, a new adaptive zone (Simpson, 1953).

Most research on the emergence of multicellular organisms has been focused on evolution of multicellular animals, the metazoa. The oldest metazoa, the sponges (Porifera) appeared approximately 800 million years ago (Müller, 1997). The emergence of metazoa has been explained by two theories (Willmer, 1990; Ruvinsky, 1997):

1. The colonial theory, based on aggregation of potentially free-living cells of a species in a colony where subsequent specialization resulted in such an interdependency that they had to remain together.
2. The syncytial theory, based on the subdivision of one large single cell and suggesting that multinucleate protists developed cell membranes to become multicellular.

It has been suggested that the Cambrian explosion of metazoa became possible after the evolution of modularization of distinct protein domains allowed the formation of mosiac proteins by exon-shuffling. This exon-shuffling is common to all metazoa, but apparently absent in plants (Müller, 1997). Another basic element in the onset of multicellularity has been suggested to be the sexual process emerging from unicellular eukaryotes (Ruvinsky, 1990; 1997). In the first evolutionary steps sex had nothing to do with reproduction, because cell fusion of opposite 'sexual' types resulted in a dihaploid cell and subsequent division in two meant that the number of cells had not been changed (Ruvinsky, 1997). Even today reproduction in most algae does appear uncoupled from the sexual process or cell conjugation (Van den Hoek *et al.*, 1995). The evolution of meiosis and subsequent adhesion and cooperation of mitotically reproduced cells (cf. colonial theory) may have led to multicellular metazoa (Ruvinsky, 1997).

Among eight groups of eukaryotes that had evolved from the Ur-karyote into multicellular forms are three photoautotrophic phyla: Heterokontophyta, Rhodophyta and Chlorophyta (Van den Hoek *et al.*, 1995). Algal phyla such as the Chlorophyta date back to the end of the Precambrian some 600 million years ago (Vidal, 1984; Van den Hoek *et al.*, 1995). The first unicellular eukaryotic algae appeared about 1800 million years before present (Schopf, 1994). In fact, the eukaryotic cell itself started as a multicellular organism, because of symbiosis with prokaryotic cells, resulting in one cell with integrated and specialized organelles (Gould, 1997). Multicellular photoautotrophs had already evolved at the end of the Precambrian, and some multicellular algae evolved more than a billion years ago (Gould, 1999). These multicellular algae were probably near-shore benthic seaweeds (Han & Runnegar, 1992; Butterfield, 1997). Although late Proterozoic (700 million years ago) colonial cyanobacteria and acritarchs (photosynthetic eukaryotes) have been found (Vidal, 1984), the proterozoic plankton remained profoundly undifferentiated (unicellular), presumably determined by the physico/chemical environment such as nutrient availability,

until an early Cambrian introduction of filter-feeding zooplankton (Butterfield, 1994; Logan *et al.*, 1995).

Despite advantages of sexual populations such as lower mutation load (Kondrashov, 1993) or spreading of molecular symbionts (Hickey, 1993), multicellular photoautotrophs, and especially planktonic species, may have suffered severe competition from unicellular species and might have been out-competed. During most of the Proterozoic aeon (2500 – 540 million years ago) the autotrophic system was assumed to be saturated, self-limiting and biologically monotonous, with low morphological diversity (Stanley, 1973). The presence of numerous unicellular bacteria, cyanobacteria, algae and protozoa today suggests clear benefits of unicellularity. Nevertheless, multicellularity arose explosively in a relatively short period (Stanley, 1973; Valentine, 1978; Vidal, 1984; Gould, 1997). This leads to the hypothesis that phagotrophic predation by heterotrophic flagellates on the photoautotrophic organisms may have exerted a strong selection pressure on reduction of mortality that has given rise to the formation of multicellular organisms (Stanley, 1973; Boraas *et al.*, 1998). The morphological response could have selected for a counter-adaptation in the flagellate and resulted in an "arms-race" that has been proposed as a driving force in the evolution of interacting species (Van Valen, 1973; Dawkins & Krebs, 1979) and that continues indefinitely (Schaffer & Rosenzweig, 1978). Hence, larger photoautotrophs were favored by natural selection since they could not easily be incorporated by phagocytosis and gave rise to the evolution of multicellular life on earth (Stanley, 1973).

Recently, experimental evidence has been gathered that does seem to support this hypothesis. Phagocytosis by a mixotrophic flagellate resulted in a rapid shift from a unicellular algal culture to one dominated by colonies that were virtually immune to predation (Boraas *et al.*, 1998). The phenomenon observed by Boraas and coworkers is probably clonal replacement, since they reported a "*rare multicelled mutant*" in their unicellular cultures. Moreover, they reported that the unicells were superior competitors and that colonies bred true (Boraas *et al.*, 1998).

Whether multicelled algae arose before metazoans, or metazoans, as a result of exon-shuffling (Müller, 1997), sexuality (Ruvinsky, 1997) or a loss of cell wall (Vellai *et al.*, 1998) slightly before metaphyta, it is evident that the evolution of grazing fundamentally had altered the community structure by relieving the resource limitation (Stanley, 1973). The metazoan grazers may have played a crucial role:

"*The exploitation of plankton by metazoan filter-feeders would have fuelled the explosion of Cambrian metazoan evolution. The coincident radiation of planktonic acritarchs becomes explicable as an adaptive response to micro-grazing activity*" (Butterfield, 1994).

1.2 FRESHWATER PLANKTON

In today's freshwater systems rather high species diversity (Hutchinson, 1961) and variation in growth form of algal taxa may be observed (Van Donk *et al.*, 1999). In these variable environments the algae experience rapid nutrient and light changes, temperature fluctuations and variations in abundance of grazers (Sommer *et al.*, 1986).

Both algae and their pelagic predators belong to the plankton, a term which is derived from the Ancient Greek "*πλαγκτοσ*" meaning *wandering*, and refers to all aquatic organisms that drift with water movements (Harris, 1986; Allaby, 1994). In general, plankton organisms have no or weak locomotory powers. All planktonic organisms are faced with the problem that they have to remain in the water column and photoautotrophic organisms in the euphotic zone, but that they have limited capacities to do so. In the continuous struggle for life, competitive interactions are considered a major driving force in determining the biological diversity (MacArthur, 1960; Sommer *et al.*, 1986). Natural selection is presumed to result in the best adapted, optimal phenotypes (Cody, 1974). Since small-sized algae with a large surface-to-volume ratio may grow more rapidly than larger ones (Turpin & Harrison, 1980; Smith & Kalff, 1982), in an aquatic environment selection pressure exists for small organisms that have the most efficient uptake of dissolved nutrients and lowest sinking losses (Reynolds, 1984).

Another strong selective factor is predation by an entire assemblage of protozoan and metazoan grazers (Lehman, 1988). In these systems, the algae-consumer relation is of major importance because it is the first step in the pelagic food chain. There is a broad consensus that freshwater zooplankton feed mainly on nanoplankton, i.e. algae between 2-30 μm in length (Sterner, 1989). The nanoplanktonic algae are often dominant genera in early spring (Sommer *et al.*, 1986), but "*the fate of these algae is to be grazed*" (Reynolds *et al.*, 1982). The high grazing rate on edible algae could favor larger algae that have a refuge from grazing (Sommer *et al.*, 1986; Sterner, 1989). The variability in grazing pressure could, therefore, favor different algal assemblages during different seasons, especially if there is a trade-off between edibility and competition (Sterner, 1989). Most probably the release of nutrients by grazers is beneficial to the larger species (Lehman, 1980; Sterner, 1986) and therefore grazers may also influence the competition for nutrients among algae (Elser *et al.*, 1988).

In general, grazing and sedimentation appear the major algal loss processes operating (Reynolds *et al.* 1982). Assuming that multicellularity has evolved as a defense against predation, one could imagine an adaptive trade-off between defensive multicellularity and competitively advantageous unicellularity. This seems to be confirmed by the study of Boraas *et al.* (1998), who found unicellular *Chlorella* competitively superior to colonial ones. The formation of colonies was interpreted as a defense, because colonies experienced lower mortality through grazing than unicells did (Boraas *et al.*, 1998). Defensive mechanisms may involve some costs (e.g. Dodson, 1984; 1989; Riessen, 1984; Harvell, 1986; Havel & Dodson, 1987; Walls & Ketola, 1989; Petterson & Brönmark, 1997) and it has been suggested that

when the costs of permanent defenses are high, cheeper inducible defenses will be favored (Rhoades, 1979; Harvell & Tollrian, 1999). Although costs may be difficult to detect (Spitze, 1992; Lampert *et al.*, 1994; Tollrian, 1995), the allocation of resources to predator-induced defensive structures implies fewer resources for growth and reproduction (Stearns, 1992). Especially since in the pelagic predation may vary heavily both on temporal and spatial scales (Havel, 1987), the evolution of temporary defenses should be favored compared to fixed defenses (Schlichting, 1989; Clark & Harvell, 1992). Since algae in the pelagic are small relative to their predatory enemies, they will probably not survive an encounter with a grazer (Van Donk *et al.*, 1999). Especially since their "fate" is to be grazed (Reynolds *et al.*, 1982), grazing will exert a strong selection pressure for traits that reduce mortality. A possible trait is that algae may use dissolved chemicals to detect the grazer before they encounter each other in order to elicit a defensive strategy, such as the formation of colonies (Hessen & Van Donk, 1993).

1.3 CHEMICAL INFORMATION

From the 'Ur-soup' 3.5 billion years ago to today's water-bodies, all aquatic organisms have lived and live in an ocean of chemicals. Since "*A living organism is constantly exchanging substances with the environment*" (Maynard-Smith, 1997), chemical substances may play an important role in interactions among organisms. The chemicals are either directly advantageous (nutrients) or disadvantageous (toxins) or may elicit a physiological or behavioral response (information). Analogous to terrestrial systems chemical cues in the water may be used to find prey (e.g. Van Gool & Ringelberg, 1996), to avoid or resist predation (e.g. Havel, 1987), to warn conspecifics (e.g. Pijanowska, 1997), to influence competition (e.g. Gross *et al.*, 1996) or to attract mates (e.g. Snell & Morris, 1993). These information conveying chemicals may be considered metabolic products that leak to the environment and fortuitously convey information (Liley, 1982), and are referred to as infochemicals according to the terminology described by Dicke & Sabelis (1988):

"An infochemical is a chemical that, in the natural context, conveys information between two organisms, evoking in the receiving organism a behavioral or physiological response that is adaptively favorable to one or both organisms".

Infochemicals may be divided into two major groups: Pheromones, intra-specific infochemicals, and allelochemicals, inter-specific infochemicals (Fig. 1.1). Both groups can be further subdivided (cf. Dicke & Sabelis, 1988; Vet & Dicke, 1992).

Theoretically, infochemicals may originate from every chemical process and may be involved in every interaction, simply because all organisms produce "odors" and thus potentially information. However, the suitability of an odor as infochemical depends on its detectability and reliability (Vet & Dicke, 1992; Steinberg *et al.*, 1993).

INFOCHEMICAL	
PHEROMONE: infochemical that is pertinent to the biology of organism #1 and that evokes in a receiving organism #2 of the ***same*** species a response that is favorable to:	**ALLELOCHEMICAL:** infochemical that is pertinent to the biology of organism #1 and that evokes in a receiving organism #2 of a ***different*** species a response that is favorable to:
Organism #1: (+,-) **pheromone** Organism #2: (-,+) **pheromone** Both #1 & #2: (+,+) **pheromone**	Organism #1: **allomone** Organism #2: **kairomone** Both #1 & #2: **synomone**

Figure 1.1: Infochemical terminology cf. Dicke & Sabelis (1988).

Many aquatic organisms use kairomones and/or alarm pheromones to assess their risk of predation (Wudkevich *et al.*, 1997). Consumer-induced defenses are common among freshwater and marine organisms:

In the marine environment, organisms such as seaweeds, sponges and soft corals produce both secondary metabolites and spicules that serve as defenses against consumers (e.g. Harvell & Fenical, 1989; Hay *et al.*, 1994). Several marine organisms may use cues from their predators in deploying their defenses. For example, bryozoans may produce spines (Yoshioka, 1982; Harvell, 1984), snails thicker shells (Palmer, 1985) and barnacles a 'bent' morph, with the rim of its aperture oriented perpendicular rather than parallel to its base (Lively, 1986), in response to cues from their predators. The American lobster has been shown to increase shelter use when exposed to predator-mediated infochemicals (Wahle, 1992). A grazing-mediated chemical defense in the unicellular marine alga *Emiliania huxleyi* has been reoprted (Wolfe *et al.*, 1997). In freshwater systems consumer-induced defenses may be found in fish, amphibians, amphipods, gastropods, zooplankton, protozoa and phytoplankton.

Defenses among fish

Prey fish may be informed by chemical cues from hunting predators (Von Frisch, 1941) or injured conspecifics (Pfeiffer, 1974). As a response the prey fish may decrease their activity (e.g. Mathis & Smith, 1993), make rapid movements (e.g. Reed, 1969), show hiding or schooling behavior (e.g. Magurran, 1989) or avoid dangerous habitats (e.g. Keefe, 1993). The predator (pike *Esox lucius*) avoidance of prey fish (bleak *Alburnus alburnus*) may be influenced by abiotic and biotic variables such as light, the availability of food and the presence of alarm substances (Jachner, 1995; 1996; 1997). The fright response in fathead minnows (*Pimephales promelas*) including increased shelter use, dashing and freezing, was induced when the minnows were exposed to chemical stimuli from the predatory northern pike (*Esox lucius*), but not when exposed to chemical cues from nonpiscivorous peacock

gudgeons (*Tateurndina ocellicauda*) (Mathis *et al.*, 1993). Sticklebacks (*Culaea inconstans*), finescale dace (*Chrosomus neogaeus*) and fathead minnows avoided areas marked with skin extract of sticklebacks indicating that the alarm substances may act both intra- and interspecific (Chivers & Smith, 1994). However, in juvenile rainbow trout (*Oncorhynchus mykiss*) anti-predator responses were only observed in response to skin extract of conspecifics, not to skin extract from swordtails (*Xiphophorus helleri*) (Brown & Smith, 1997). A similar observation was made for three-spined sticklebacks (*Gasterosteus aculeatus*) that exhibited significant increases in anti-predator behavior when presented with alarm substances from skin extract of conspecifics and four-spined stickleback (*Apeltes quadracus*), but not to swordtail skin extract (Brown & Godin, 1997).

Besides behavioral responses, predator-induced morphological changes, i.e. a change in body shape towards a deeper body, in crucian carp (*Carassius carassius*) have been reported (Brönmark & Miner, 1992). In crucian carp, chemicals from injured conspecifcs did not elicit an induced defense, but chemicals related to the piscivorous diet of the predator did so (Brönmark & Petterson, 1994). In the absence of a predator, at high densities shallow-bodied crucian carp gained twice as much body mass as predator-induced deep-bodied fish. Hence, in the absence of predators the inducible defense resulted in fitness costs (Petterson & Brönmark, 1997).

Predator-induced defenses in amphibians, amphipods and gastropods

The use of chemical cues to detect predatory fish is widespread among amphibians (Kats *et al.*, 1988). Larval amphibians may increase shelter use (Kats *et al.*, 1988) or reduce their activity in response to predator-associated chemicals (Skelly & Werner, 1990) at the expense of reduced growth and development (Skelly, 1992). Streamside salamander larvae (*Ambystoma barbouri*) have been shown to exhibit an adaptive 'sink to the bottom' response to chemical cues from predatory green sunfish (*Lepomis cyanellus*) (Sih & Kats, 1994). Tadpoles of gray treefrog (*Hyla chrysoscelis*) are relatively inactive and possess smaller, less colored tailfins when exposed to chemicals from predatory dragonfly larvae (*Anax junius*). The predator-induced phenotype appeared less vulnerable to predation, but suffered greater mortality from other causes (McCollum & Vanbuskirk, 1996).

The gastropod *Gammarus lacustris* decreased activity when exposed to predator kairomones from pike and larval dragonfly. Moreover, *G. lacustris* possesses an alarm pheromone as a similar response has been observed when exposed to crushed conspecifics (Wudkevich *et al.*, 1997). Several freshwater snails have been reported to crawl out of the water in response to predator-associated chemicals (Covich *et al.*, 1994).

Predator-induced defenses in zooplankton

Predators that release defense-inducing infochemicals show a broad taxonomic distribution. Zooplanktivorous fish such as roach (*Rutilus rutilus*) and perch (*Perca fluviatilis*), dipterans of the genus *Chaoborus*, the hemipterans *Notonecta* and *Anisops*, and the copepod *Epischura* have been reported to induce defense mechanisms in cladocerans (for review Havel, 1987). However, the cue might also originate from injured conspecifics (Pijanowska, 1997). The most examined cladocerans are members of the genus *Daphnia*. Predator-induced defenses include morphological, behavioral or life history changes. Helmet growth (e.g. Hebert & Grewe, 1985; Hanazato, 1991; Tollrian, 1994), formation of neck-spines (e.g. Dodson, 1989; Hanazato, 1990; Tollrian, 1994), crest formation (Grant & Bayly, 1981), tail spine elongation and carapace broadening (Havel, 1985) are possible morphological features to avoid predation. Enhanced diel vertical migration (Ringelberg, 1991; DeMeester, 1993; Loose *et al.*, 1993), swarming and somersaulting (Larsson & Dodson, 1993; Pijanowska, 1994) are also induced by predator infochemicals. The life history of *Daphnia* is also influenced by unidentified infochemicals released from fish. Animals mature earlier at smaller size, clutches are larger and neonates smaller in presence of fish infochemicals (Stibor, 1992; Macháček, 1993; Reede, 1995).

In rotifers, defenses against predation may be induced by chemicals released from carnivorous rotifers like *Asplanchna*, copepod predators like *Mesocyclops, Tropocyclops*, *Epischura*, and herbivorous competitors like *Daphnia* (Stemberger & Gilbert, 1987). Predator released chemical cues have been shown to induce spine formation and increase body size in several rotifer species (e.g. Gilbert, 1966; Gilbert & Stemberger, 1984; Stemberger & Gilbert, 1984; 1987).

Induced-defenses among protozoa

Ciliates of the genus *Euplotes* alter their morphology as response to proteineous substances released from a variety of predators, such as the ciliate *Lembadion*, the rhizopod *Amoeba proteus* or the turbellarian *Stenostomum* (Kuhlmann & Heckmann, 1985; Kusch, 1993; Kusch & Kuhlmann, 1994). The combination of large lateral wings, increased cell size and an additional dorsal crest appear highly effective in reducing predation (Kuhlmann & Heckmann, 1994), but involve energetic costs (Kusch & Kuhlmann, 1994; Wiackowski & Szkarlat, 1996).

Induced-defenses among phytoplankton

Although infochemically induced reactions have been reported frequently, especially in fish and zooplankton, very little is known about the role of infochemicals in the grazer-phytoplankton interaction. The rapid response of the beat rate of the appendages of daphnids by cyanobacterial extracellular products may be an example of chemical information transfer

(Forsyth *et al.*, 1992; Haney *et al.*, 1995). Whether this is a defense strategy governed by the cyanobacteria or by *Daphnia* is unclear. An inducible morphological defense against a ciliate grazer (*Pseudomicrothorax dubius*) has been reported in two strains of the cyanobacterium *Phormidium* (Fialkowska & Pajdak-Stós, 1997). Several flagellate algae seem able to use grazer associated chemicals to express consumer-avoidance behavior (Hansson, 1996). Non-flagellate green algae of the genus *Scenedesmus* may alter their morphology in order to reduce their vulnerability against grazing (Hessen & Van Donk, 1993; Lampert *et al.*, 1994).

Infochemicals released by competitors

All of the above mentioned examples describe the effects of infochemicals excreted by predators (or by predator activity) that reduce the mortality of the prey species. However, also within a certain trophic level infochemicals may be acting. Several studies have reported effects in zooplankton by infochemicals released from potential competitors. Feeding in *Daphnia* is reduced by infochemicals released from congeners (Matveev, 1993) and conspecifics (Helgen, 1987). Feeding in the copepod *Diaptomus* is reduced by a high-molecular weight chemical released from its competitor and predator *Epischura* (Folt & Goldman, 1981). Effects on fecundity have been reported in competing rotifers of the genus *Brachionus* (Halbach, 1969) and in cladocerans (e.g. Seitz, 1984; Hobæk & Larsson, 1990; Goser & Ratte, 1994; Burns, 1995; Cleuvers *et al.*, 1997). Chemicals released from *Daphnia* induced a lower fecundity and decreased population growth rate in the rotifer *Keratella* (Conde-Porcuna, 1998). Pheromones may be involved in mate recognition in rotifers (Rico-Martinez *et al.*, 1996).

Infochemicals from aquatic plants

Analogous to terrestrial systems aquatic plants may use chemicals to defend themselves against grazing or to gain a better competitive position. The palatability of foliage to herbivores was reduced after artificial damage, even in undamaged leaves (Jeffries, 1990). Szecpanska (1978) has reviewed allelopathy between aquatic macrofytes. Growth inhibition in cyanobacteria by chemicals released from *Ceratophyllum* has been reported (Kogan *et al.*, 1972). Also *Chara* inhibits algal growth (Gibbs, 1973; Wium-Andersen, 1982; 1987), but Forsberg *et al.* (1990) found no allelopatic effects. The reduced algal growth in the presence of *Stratiotes* appeared due to nutrient competition rather than allelopathy (Brammer, 1979). It has been demonstrated that polyphenols released from *Myriophyllum* were responsible for observed algal inhibition (Gross *et al.*, 1996). The interaction between macrofytes and zooplankton is merely providing a refuge for zooplankton and may in turn be beneficial for macrofytes because of enhanced algal mortality (Timms & Moss, 1984; Scheffer *et al.* 1993). On the other hand, *Daphnia* has been reported to swim away from *Elodea, Nitella* and *Myriophyllum* (Pennak, 1973; Lauridsen & Lodge, 1996). *Daphnia* has been shown to

respond to (planktonic) algal associated chemicals by preference for water with odor from edible food (Van Gool & Ringelberg, 1996).

A sea of infochemicals

From all reports on chemically mediated information transfer in aquatic systems one could get the impression that a water-body is a chaotic continuum of chemical information. Indeed, aquatic organisms live in an ocean of chemicals and probably in a sea of infochemicals. Numerous of those infochemicals may act simultaneously on one or more organisms of the same species or the same infochemical may affect numerous species. Most infochemicals are probably not excreted on purpose to convey information, in contrast with hormones in our body fluid. However, the amount and chemical diversity of human hormones, i.e. small molecules derived from amino acids, proteins, steroids and ecosanoids, clearly demonstrate that an enormous diversity of infochemicals may be present simultaneously (Stryer, 1988). In the marine environment, information molecules apparently possess a common structure consisting of low-molecular-weight peptides or peptide-containing molecules with a basic amino acid residue at the carboxyl terminus (Browne *et al.*, 1998; Decho *et al.*, 1998). In freshwater systems, however, the chemical information substances may be both proteins and small organic molecules (e.g. Parejko & Dodson, 1990; Tollrian & Von Elert, 1994; Peters-Regehr *et al.*, 1997).

Compared to other means of information transfer, for example by acoustic and visual cues, infochemical transmission in water is relatively undirected and slow. It depends on diffusion and is affected by water movement. However, the latter may not necessarily affect the organism's response to the chemical cue. It has been shown for oyster larvae that they do settle in response to a waterborne chemical cue in both still and flowing water (Turner *et al.*, 1994). Advantages are possible long-range and long-term transmissions, independence of light and insensitivity to obstacles (Liley, 1982). Thus, water appears an appropriate medium for chemical information transfer.

1.4 *SCENEDESMUS*

In pelagic freshwater systems, the position of the organisms in the different trophic levels corresponds with the time of interest in chemical information transfer. Since Karl von Frisch (1941) demonstrated the existence of infochemicals in fish interactions, the following decades a wealth on information has been gathered on infochemicals and chemical communication among fish (Liley, 1982). The last 10-15 years considerable attention has been focussed on variable predator-induced defenses in zooplankton (Havel, 1987), but, although Harvell (1984) suggested pronounced evolution of induced defenses in clonal taxa, such as algae (Havel, 1987), still very little is known about induced defenses in algal taxa.

Morphological variability seems widespread among phytoplankton, including cyanobacteria, diatoms and chlorophytes (Trainor *et al.*, 1971), but research on causal factors focussed on the physico-chemical environment (e.g. Mur, 1971). The biological environment and especially the grazers were believed to feed on specific size classes of algae, thereby regenerating nutrients for larger grazing-protected cells (Lehman, 1980; Sommer *et al.*, 1986; Sterner, 1989). However, it was known that the presence of grazers such as *Daphnia* could alter the morphology of phytoplankton. *Aphanizomenon* showed a shift from flakes in the presence of *Daphnia* to smaller flakes and single filaments in its absence (Lynch, 1980; Holm *et al.*, 1983). Two chlorophytes, *Chlamydomonas* and *Scenedesmus*, showed colony formation in the presence of herbivorous zooplankton (Mikheeva & Kruchkova, 1980), but since this was reported in Russian it remained unknown elsewhere. A first attempt to examine grazer-induced colony formation was briefly mentioned by Fulton III & Paerl (1987b), but chemicals released from *Daphnia ambigua* appeared ineffective as colony-inducing agents in *Microcystis*. Also chemicals released from *Daphnia magna* were ineffective in inducing *Microcystis* colonies (Hessen & Van Donk, 1993). However, with *Scenedesmus* the latter investigators were more successful.

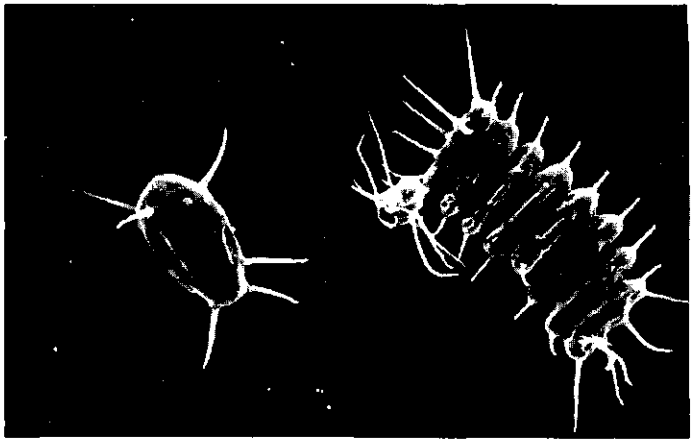


Figure 1.2: *Scenedesmus subspicatus* unicell (left panel) and *Daphnia*-induced eight-celled coenobium (right panel). *From Hessen & Van Donk (1993).*

Hessen & Van Donk (1993) were the first who discovered that chemical substances released from a grazer, *Daphnia*, induced a morphological defense in the green alga *Scenedesmus subspicatus*. Unicellular populations of this alga were rapidly transformed into populations dominated by eight-celled coenobia that were protected from grazers (Fig. 1.2).

In culture, whether actively growing, P-limited or in stationary phase their strain of *S. subspicatus* was unicellular (Van Donk & Hessen, 1993; Hessen & Van Donk, 1993), not unusual for laboratory strains (Trainor, 1998). A year later, the *Daphnia*-induced colony formation was confirmed by Lampert *et al.* (1994), using another *Scenedesmus*, *S. acutus*. A

dramatic increase in the number of eight-celled colonies was observed when the culture was exposed for 48 hours to medium with only 2% (v/v) medium from a *Daphnia* culture (Lampert *et al.*, 1994).

Scenedesmus is one of the commonest genera in freshwater systems with a worldwide distribution (Canter-Lund & Lund, 1995; Trainor, 1998), but morphologically also extremely variable (Trainor, 1991). Although Chodat (1913; 1926) already discussed phenotypic plasticity within the genus, his reports were dismissed as were scant other reports. "*Pickled sampling at one point in time, rough examination, use of continuous cultures and prejudiced scientists who dismissed reports on phenotypic plasticity in Scenedesmus as irrelevant, all contributed that we ended up with approximately 1330 taxa nicely assembled by Hegewald & Silva (1988) in an "annotated catalogue of Scenedesmus and nomenclaturally related genera*" (Trainor, 1998). However, intensive efforts by Trainor and co-workers revealed detailed information on *Scenedesmus* plasticity in several strains. Trainor and co-workers concluded that "*many morphological expressions of Scenedesmus are not all separate taxa, but represent ecomorphs of a limited number of species...the number should be very low, not 1300, not 130, but perhaps closer to 13!*" (Trainor & Egan, 1990a). Moreover, "*morphological stability of Scenedesmus in the field is an illusion*" (Egan & Trainor, 1990). However, opening a textbook one will undoubtedly find *Scenedesmus* presented as a four- or eight-celled colony: its 'typical form'. Unicells are often not 'recognized' as *Scenedesmus*, and have been placed in other algal genera. Swale (1967) reported *Chodatella-quadriseta*-like unicells in an isolate of *Scenedesmus armatus*. Spiny *Scenedesmus* 'disintegrated' in the laboratory in *Chodatella-subsalsa*-like unicells (Fott, 1968). Trainor & Egan (1990b) clearly demonstrated *Lagerheimia hindakii* to be the unicellular stage of *Scenedesmus*. In a recent study, based on 18S rRNA analysis three strains of *Chlorella fusca* had to be placed within the genus *Scenedesmus* (Kessler *et al.*, 1997). Spined unicells may resemble *Chodatella, Franceia* or *Lagerheimia*, whereas non-spiny unicells could resemble *Ankistrodesmus, Chlorella, Oocystis, Raphidium* and *Selenastrum* (Trainor, 1998). In the non-spiny subgenus (Euscenedesmus), during asexual reproduction, coenobia will be formed inside the parent cell wall. The initial cementing does not hold when the cells are released from the parent cell and the coenobium disintegrates into unicells (Nilshammer & Walles, 1974; Trainor, 1998). This appears the usual pathway for unicell formation in *S. acutus* and *S. obliquus* (Trainor, 1998). In the spiny subgenus (Desmodesmus), *Scenedesmus* may produce developmental unicells (true unicells) that simply do not cement together or degenerative unicells as a result of coenobium fragmentation (pseudounicells). Unicells that respond to morphogenetic substances released from grazers are considered true unicells (Trainor, 1998). The sequence of the formation of a *Daphnia*-induced eight-celled coenobium in the non-spiny *S. acutus* is presented in figure 1.3.

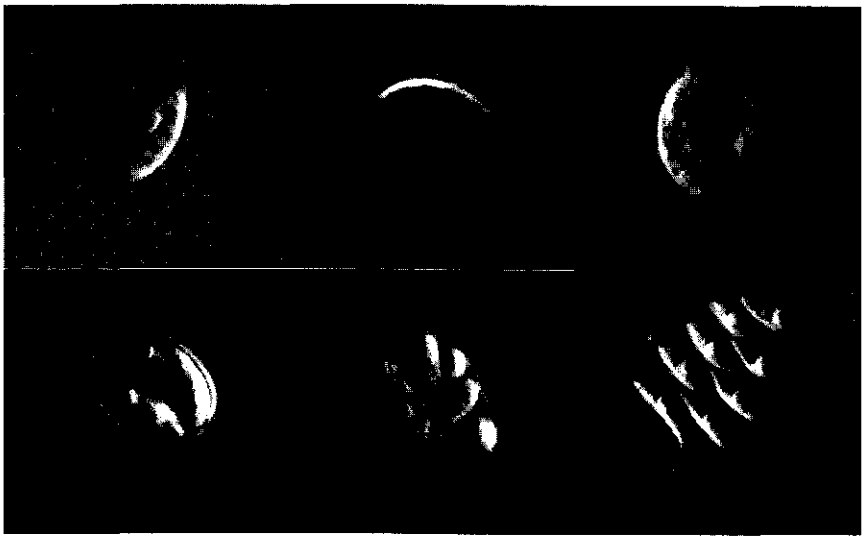


Figure 1.3: Asexual reproduction in *Scenedesmus acutus*: Formation of autospores inside the parental cell resulting in an eight-celled coenobium.

An ordered sequence of ecomorph development has been reported in several spiny *Scenedesmus* (Egan & Trainor, 1990; Trainor, 1992a;b; 1993). Unicells appeared during logarithmic growth, spiny coenobia when cultures aged, finally resulting in short-spined and spineless coenobia (Trainor, 1993; 1995; 1998). In non-spiny *Scenedesmus*, so far no research on the sequence of ecomorph development has been undertaken.

Several factors have been demonstrated to affect the morphological development in *Scenedesmus*. Formation of different ecomorphs will not only depend on photoperiod, nutrients, pH, temperature, cell density, age and growth rate (e.g. Overbeck & Stange-Bursche, 1966; Swale, 1967; Trainor & Roskosky, 1967; Steenbergen, 1975; Trainor & Shubert, 1974; Gavis *et al.*, 1979; Ramos-Cárdenas & de Lara-Isassi, 1985; Holtmann & Hegewald, 1986; Egan & Trainor, 1989; 1990; Trainor, 1992a;b; 1993), but it will also depend on the grazers (Hessen & Van Donk, 1993; Lampert *et al.*, 1994). The latter phenomenon not only confirmed that one taxon produced two distinct morphotypes, but also presents a plausible ecological explanation for the colony formation, i.e. defense against predation. *Daphnia* can easily ingest small *Scenedesmus* coenobia, but not large eight-celled coenobia (Hessen & Van Donk, 1993). Most coenobia will undoubtedly be too large to be grazed by protist grazers, such as the phagotrophic flagellates *Paraphysomonas* (Grover, 1989) and *Ochromonas* (Boraas *et al.*, 1998).

In *Scenedesmus* coenobial cells may be arranged in several forms: linear, costulatoid (in staggered groups of four), alternating, irregular and dactylococcoid (Fig. 1.4).

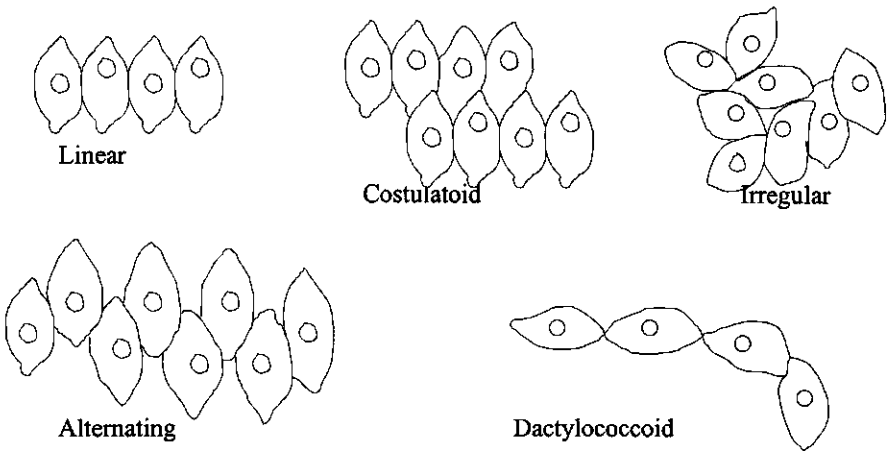


Figure 1.4: Several arrangements of *Scenedesmus* coenobia

1.5 THIS THESIS

This thesis focuses on the morphological variability in the non-spiny *Scenedesmus acutus* Meyen. The examination of the phenotypic plasticity has been carried out with emphasis on the role of grazing-associated chemicals based on a cost-benefit analysis. Although the *Daphnia-Scenedesmus* interaction is used as a model system, several experiments were performed with other algae and zooplankters to ascertain more insight in the variability and taxonomic distributions of the investigated response.

CHAPTER 2 reports on the effects of grazing-associated chemicals on the growth and development in the green alga *Scenedesmus acutus* Meyen. It turns out that the typical eight-celled morph is only abundant in the presence of a '*Daphnia*-factor'.

To examine the effects of various factors on growth and colony formation in *S. acutus* a reliable biotest is of utmost importance. CHAPTER 3 reports on the development of such a biotest. It is shown that the induction of coenobia depends on the amount of algae grazed upon. *Daphnia* needs to feed on digestible food to induce coenobia. Another outcome is that only herbivorous zooplankton elicits a morphological response. Moreover, certain detergents trigger the unicell-colony transformation.

CHAPTER 4 reports on the effect of different nutrient conditions on growth and morphology in *S. acutus*. The response of nutrient-replete and deplete cells to *Daphnia*-infochemicals is examined. It appears that *Daphnia*-induced colony formation may occur over under a broad range of nutrient conditions.

In CHAPTER 5 the effects of various temperatures on growth and morphological development in *S. acutus* are examined. The inducible nature of coenobia formation suggests costs associated with this defensive trait. Analyses of possible costs associated with colony

formation, such as lower growth rates, hampered light harvesting and enhanced sedimentation are presented in CHAPTER 6.

Benefits of coloniality are supposed to be a reduced grazing pressure. The effects of unicellular and colonial *Scenedesmus* on feeding behavior and life history characteristics of herbivorous zooplankton are presented in CHAPTER 7.

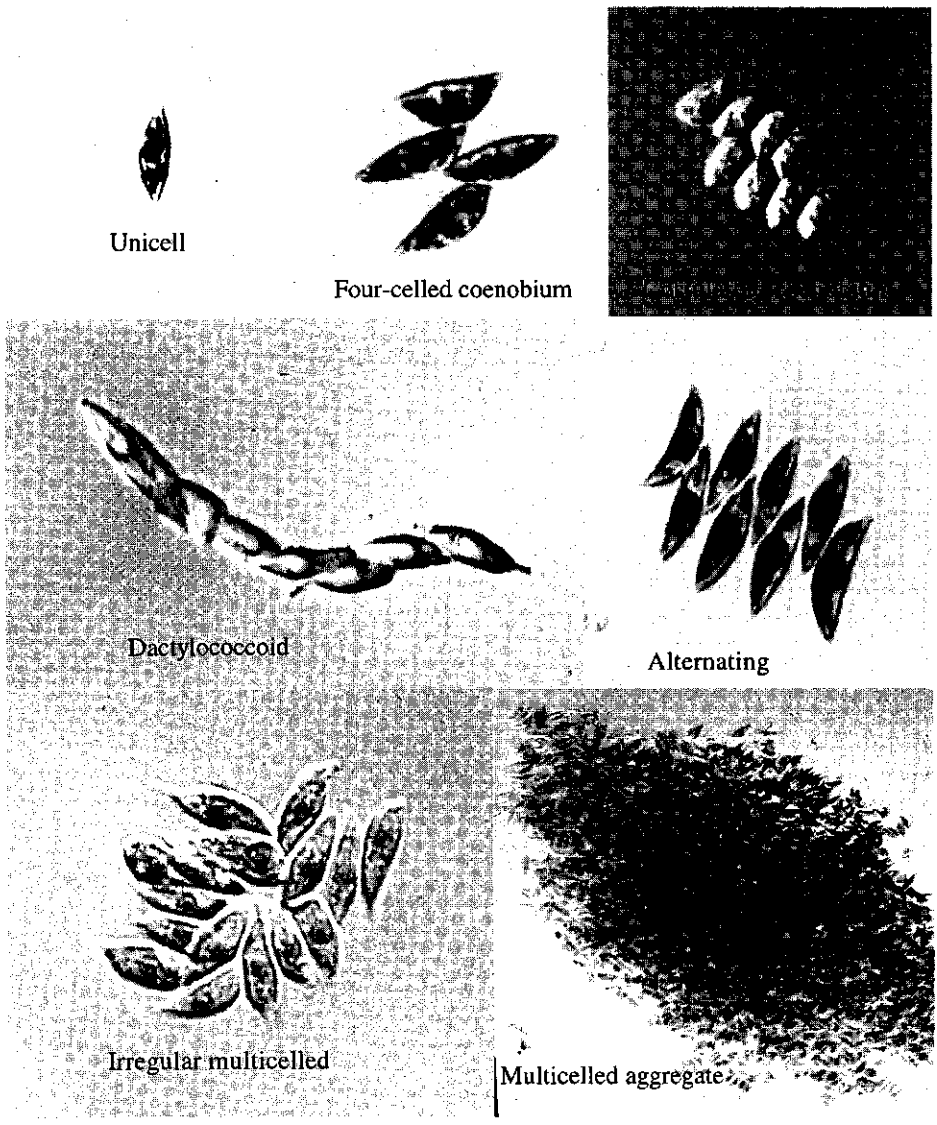
To gain more insight into the taxonomic distribution of consumer-induced colony formation, in CHAPTER 8 the results are presented of biotests with several clones and species of *Scenedesmus*, with some other green algae, and some algae from other taxonomic groups. The *Daphnia*-induced colony formation appears not universal in *Scenedesmus* and is not restricted to the genus. An additional clogging of cells into large aggregates in the presence of live *Daphnia* was observed. No evidence was obtained to support grazer-induced spine formation in *Scenedesmus* or colony formation in *Microcystis*.

CHAPTER 9 discusses the direct effects of grazing-associated chemicals on other zooplankters and reports the response in swimming behavior of *Daphnia* exposed to chemicals released from food and competitors.

Finally, CHAPTER 10 summarizes the results presented in the previous chapters.

INTERMEZZO

Morphological appearance in *Scenedesmus*



CHAPTER 2

Herbivory related chemicals affect growth and morphological development in *Scenedesmus acutus* Meyen

Parts of this chapter have been published in:
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"Some still remain uncomfortable with
the concept that an individual microalgal
species can exhibit ***extensive*** *phenotypic plasticity,*
even to the point of not incorporating
essential facts into their research"

- F.R. Trainor 1998

2.1 INTRODUCTION

Herbivory is one of the main losses among algae (Sterner, 1989) and therefore a strong selection pressure exists on the development of traits that reduce mortality. Pelagic algae may use two strategies to reduce grazing losses, either avoid being ingested or, if ingested, avoid being digested. Many algae are notoriously plastic in morphology, growth and biochemical composition and variable traits have been interpreted as defense mechanisms against grazing.
It is not surprising that zooplankton feeds with differing success on various phytoplankton species, primarily due to parameters like cell size, shape, cell wall structure and the presence of toxins. Changing environmental conditions, favoring different clones of the same species, could lead to replacement of one clone by another one (Wood & Leatham, 1992), possibly with a variation in the defensive trait. Another intraspecific change, phenotypic plasticity, occurs when changed environmental variables alter the defensive trait of cells belonging to the same clone (Schlichting, 1989; West-Eberhard, 1989).

Algal species belonging to the genus *Scenedesmus*, one of the commonest genera of freshwater algae (Canter-Lund & Lund, 1995) in the Netherlands (Mur, 1971), vary in their phenotype. *Scenedesmus* exists as unicells or as two or more celled coenobia (e.g. Uherkovich, 1966; Egan & Trainor, 1990). Formation of unicellular stages or coenobial ecomorphs depends on initial cell density (Egan & Trainor, 1989), nutrients and pH (e.g. Trainor & Roskosky, 1967; Ramos-Cárdenas & de Lara-Isassi, 1985; Holtmann & Hegewald, 1986) and temperature (Trainor, 1992a,b; 1993). An ordered sequence of ecomorph development has been reported in *Scenedesmus armatus* Chodat (Trainor, 1992a), in *S. communis* Hegewald (Trainor, 1992b) and in *S. subspicatus* Chodat (Trainor, 1993). These changes are interpreted as cyclomorphosis (sensu Black and Slobodkin [1987]), driven by nutrients and temperature (Trainor, 1992a,b; 1993).

Chemicals released from grazers also induce morphological changes in *Scenedesmus*. Hessen & Van Donk (1993) discovered that chemicals released from the grazer *Daphnia* triggered the unicell-colony transformation in *Scenedesmus subspicatus* Chodat. In the presence of either live *Daphnia* or filtered water from a *Daphnia* culture, *S. subspicatus* formed numerous eight-celled coenobia and more rigid spines. The expressions of grazer-induced phenotypic plasticity do not seem to be restricted to spiny *Scenedesmus* species. Coenobia formation could also be induced in the non-spiny *Scenedesmus acutus* Meyen (Lampert *et al.*, 1994). Those experiments were short-term experiments in which *Scenedesmus* cells were cultured for only a few days, and described the phenomenon of rapid transition from almost entirely unicellular populations to ones consisting of mostly eight-celled coenobia. The studies were not focused on the development of different *Scenedesmus* ecomorphs, but emphasized mostly the possible effects for the inducing animal, *Daphnia*. The studies examining the developmental morphology of *Scenedesmus*, however, did not take into account the possible effects of grazer (predator)

chemicals. Therefore, in this chapter the effects of chemicals released by *Daphnia* on the growth and morphological development of the non-spiny *Scenedesmus acutus* Meyen were examined.

2.2 METHODS

The green alga used in this study, *Scenedesmus acutus* Meyen, was derived from the Max-Planck-Institute for Limnology (Plön, Germany). The algal cells were cultured in our laboratory in 1.0 liter chemostats on slightly modified WC-medium (Guillard & Lorenzen, 1972). The chemostats were continuously illuminated at an irradiance of 100 $\mu mol \cdot m^{-2} \cdot s^{-1}$ provided by circular fluorescent tubes (Philips TLEM 40 W/33RS) in a temperature-controlled chamber at 20°C and at a dilution rate of 1.2 day^{-1}.

An inoculum of exponentially growing unicellular *S. acutus* was derived from the chemostats and was transferred into 300 ml cellulose-plug stoppered Erlenmeyer flasks containing 150 ml of medium. Each batch culture contained 134 ml autoclaved WC-medium, 1 ml algal inoculum and either 15 ml additional WC-medium filtered through a 0.1 µm membrane filter (controls) or 15 ml membrane-filtered test water (treatments). All filters had been rinsed with 200 ml nanopure water before use. The test water was produced prior to the experiment by allowing 200 adult *Daphnia magna* Straus to feed for 24 h on a 1.0 liter suspension of *S. acutus* (*ca.* 10^5 particles$\cdot ml^{-1}$; i.e. *ca.* 4 mg $C \cdot l^{-1}$) in a mixture of WC- and RT-medium (Tollrian, 1993). The batch cultures were incubated at 20°C on a rotating shaking table (80 rpm) and continuously illuminated from above by fluorescent cool-white tubes (Osram L 36W/21-840) at 100 $\mu mol \cdot m^{-2} \cdot s^{-1}$. Controls and treatments were run in quadruplicates for 35 days. The initial algal density was 7903 ± 28 particles$\cdot ml^{-1}$ (*ca.* 14000 cells$\cdot ml^{-1}$). Algal densities and particle size distributions were determined routinely in the size range 3.0 - 20.0 µm ESD (equivalent spherical diameter) using a Coulter Multisizer II (100 µm capillary). For each replicate the number of cells per colony was determined microscopically by counting at least 120 aggregates (i.e. unicells as well as coenobia) in a subsample preserved in Lugol's fixative. Cell dimensions (length and width) of different ecomorphs were measured using a Leica Quantimet 500 MC image-analyzer coupled with a Nikon light-microscope at 600 x magnification. Both the WC-medium and the test water were analyzed for their total (in)organic C, inorganic-N and inorganic-P content. Total (in)organic carbon was determined using a TOC-analyzer (model 700, OI-Analytical). NH_4^+-N, NO_2^-/NO_3^--N and PO_4^{3-}-P were determined using a SKALAR autoanalyzer.

Growth rates were calculated from increase in biovolumes and from cell multiplication. Total cell numbers were computed by multiplying the number of particles (determined by Coulter Multisizer II) by the number of cells per particle (determined by microscope). Growth curves were fitted by non-linear regression using Genstat 5 program (Genstat, 1993) according to the logistic curve model:

$$y = \frac{c}{(1 + \exp(-b \times (t - a)))}$$

where y = either cell number (ml^{-1}) or biovolume ($\mu m^3 \cdot ml^{-1}$), t = time (d), a = curve bending coefficient, b = intrinsic rate of population increase (d^{-1}) and c = carrying capacity ($cells \cdot ml^{-1}$ or $\mu m^3 \cdot ml^{-1}$). Maximal growth rates (μ_{max}, d^{-1}) were estimated from increase in cell number (N) and biovolume (V) from the first until the third day after inoculation according to $\mu_{max} = [\ln(N_3) - \ln(N_1)] * t^{-1}$. Model parameters ($a$, b and c) and the estimated μ_{max} were statistically compared applying one-tailed t-tests. Estimated μ_{max}, based on both cell multiplication and increase in biovolume, were compared using two-way ANOVA, with the used estimation method and with/without *Daphnia* water as the two fixed effects. Cell dimensions (length and width) were statistically compared by one-way ANOVA (Sokal & Rohlf,1995).

2.3 RESULTS

Growth of *S. acutus* differed between populations cultured in pure medium (controls) or in medium with filtered water (10% v/v) from a *Daphnia* culture (treatments). Estimated parameters of the nonlinear logistic model based on cell numbers differed significantly between populations in the two media-types (Table 2.1). However, the model based on algal volume gives no significant difference in intrinsic rate of population increase (Table 2.1). The latter seems to be the result of increased cell volume in the treatments, making-up for reduced cell division rates. Exponential growth was only observed during the first 3 days (Fig. 2.1).

Table 2.1: Estimated parameters (means ± 1 SE) of logistic growth model with a = curve bending coefficient, b = intrinsic rate of population increase (d^{-1}) and c = carrying capacity ($cells \cdot ml^{-1}$ or $\mu m^3 \cdot ml^{-1}$) and means (± 1 SE) of estimated exponential growth rates (μ_{max}), including t- and P-values of t-tests.

MODEL: $y = c / (1 + \exp(-b \times (t - a)))$					
y_1 = Cell number (ml^{-1})					
	Controls	Treatments	t-values	df	P-values
r^2	0.949	0.983			
a_1	7.802 ± 0.360	7.585 ± 0.257	0.491	34	0.313
b_1	0.603 ± 0.119	0.420 ± 0.038	4.818	34	<0.001
c_1	$9.953\ 10^6 \pm 3.99\ 10^5$	$6.744\ 10^6 \pm 1.64\ 10^5$	7.434	34	<0.001
$\mu_{max,1}$	0.995 ± 0.131	1.242 ± 0.048	1.70	22	0.103
y_2 = Biovolume ($\mu m^3 \cdot ml^{-1}$)					
	Controls	Treatments	t-values	df	P-values
r^2	0.980	0.972			
a_2	9.318 ± 0.381	7.803 ± 0.391	2.775	38	0.004
b_2	0.309 ± 0.027	0.365 ± 0.040	1.156	38	0.127
c_2	$9.35 \cdot 10^8 \pm 3.18 \cdot 10^7$	$7.94 \cdot 10^8 \pm 2.80 \cdot 10^7$	3.328	38	0.001
$\mu_{max,2}$	1.712 ± 0.025	1.752 ± 0.044	0.84	30	0.407

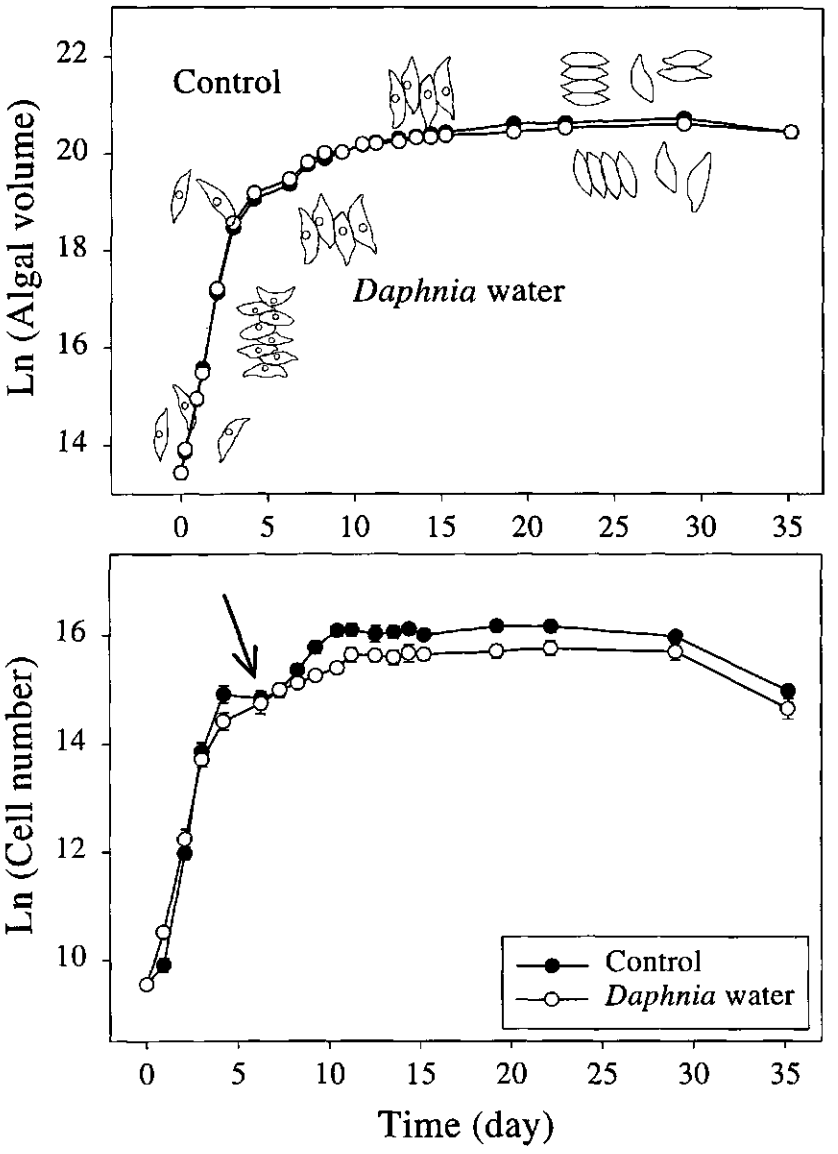


Figure 2.1: Growth (means ± 1 SD; n = 4) of *Scenedesmus acutus* in standard WC-medium (control) and in WC-medium with water (10% v/v) from a *Daphnia* culture (*Daphnia* water), expressed as ln algal volume (upper panel) and as ln cell numbers (lower panel). Also included the expression of dominant morphotypes in time (upper panel).

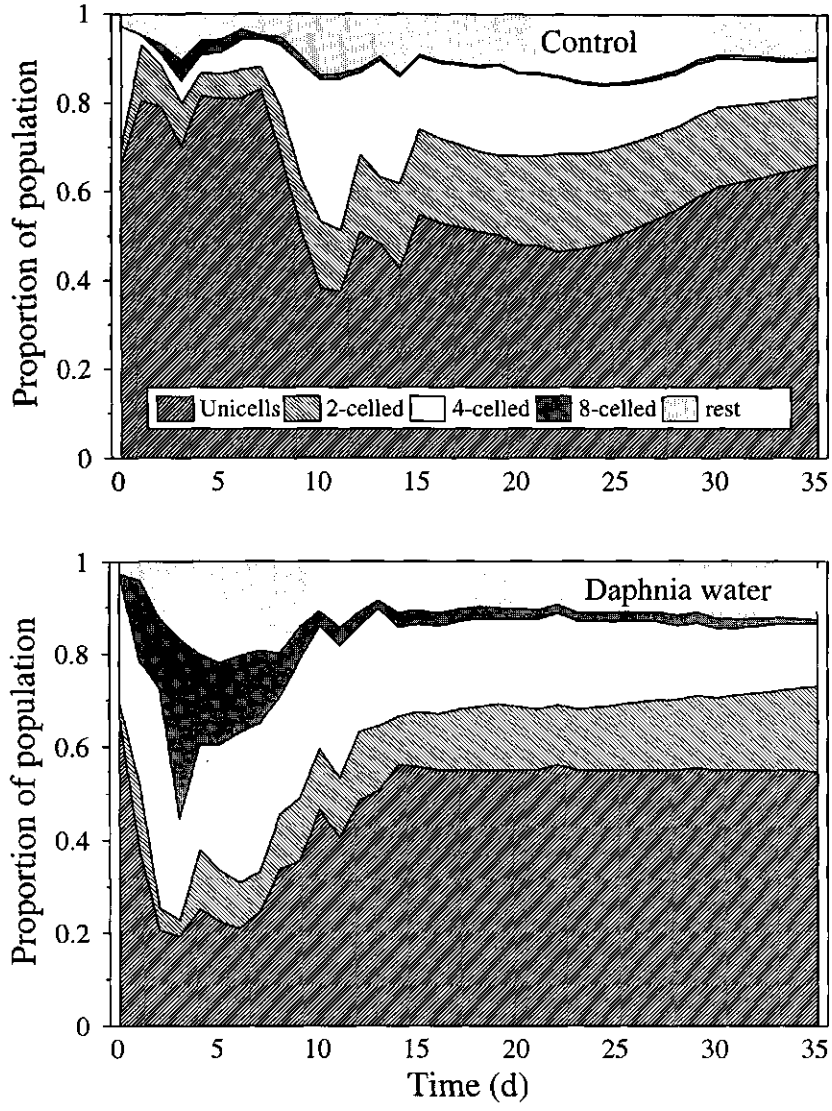


Figure 2.2: Expression of dominant morphotypes (unicells, 2-, 4- and 8-celled coenobia; rest-group includes 3-, 5-, 6-, and 7-celled coenobia) as proportion of *Scenedesmus acutus* populations cultured in standard WC-medium (upper panel) and in WC-medium with water (10% v/v) from a *Daphnia* culture (lower panel).

These exponential growth rates were not significantly different between control populations and treatment populations when based on either cell multiplication or increase in biovolume (Table 2.1). Also the two-way ANOVA indicated no significant differences in estimated μ_{max} between control and treatment populations ($F = 2.27$; $P = 0.139$). The used estimation method, however, had a significant effect on the estimation of μ_{max} ($F = 62.2$; $P < 0.001$); no significant interaction was observed ($F = 3.22$; $P = 0.079$). Although there was a tendency of higher exponential growth in the treatment populations (Table 2.1) gradually growth became lower in the treatment populations (Fig. 2.1) and resulted in significantly different carrying capacities of both groups of populations in both models (Table 2.1).

While growth was unaffected during the first days, *Scenedesmus* morphology was changed drastically in the treatment populations. In the treatment populations, a rapid formation of four-celled coenobia (47% of population on day 2) and eight-celled coenobia (38% on day 3) could be observed followed by a subsequent recovery of unicell abundance (Fig. 2.2). The control populations were dominated by unicells that made up more than 80% of the population. From 7 to 14 days, the dominance of unicells in the control populations gradually decreased to 38% on day 11, while the proportion of four-celled coenobia concomitantly increased to 34% on day 11 (Fig. 2.2).

Meanwhile, after 15 days, as cultures reached carrying capacity, population composition seemed to stabilize and was more or less comparable between control and treatment populations (Fig. 2.2).

The rapid morphological response of *Scenedesmus* in the treatments is also reflected in the mean number of cells per colony and in the mean particle volume (Fig. 2.3). The mean particle volume remained larger in treatment populations compared to the controls throughout the entire experiment. During the first 2 weeks, the treatments contained more cells per colony than the controls; in the following weeks, individual cell size, as reflected in cell volume, also appeared larger in treatments than in control populations (Fig. 2.4).

The differences in cell size also were observed with image analysis. The major morphological differences occurred during the exponential growth phase. Hence, the presented analyses of cell dimensions in Table 2.2 are based on data derived during that period (i.e., days 2 and 3). Cell dimensions of unicells and coenobia differed significantly between control populations and treatment populations (Table 2.2). In the treatment populations, two types of four-and eight-celled coenobia could be observed: relatively small-sized coenobia and large coenobia. The differences were due to both significantly increased cell lengths and widths of coenobial cells in the larger size class (Table 2.2).

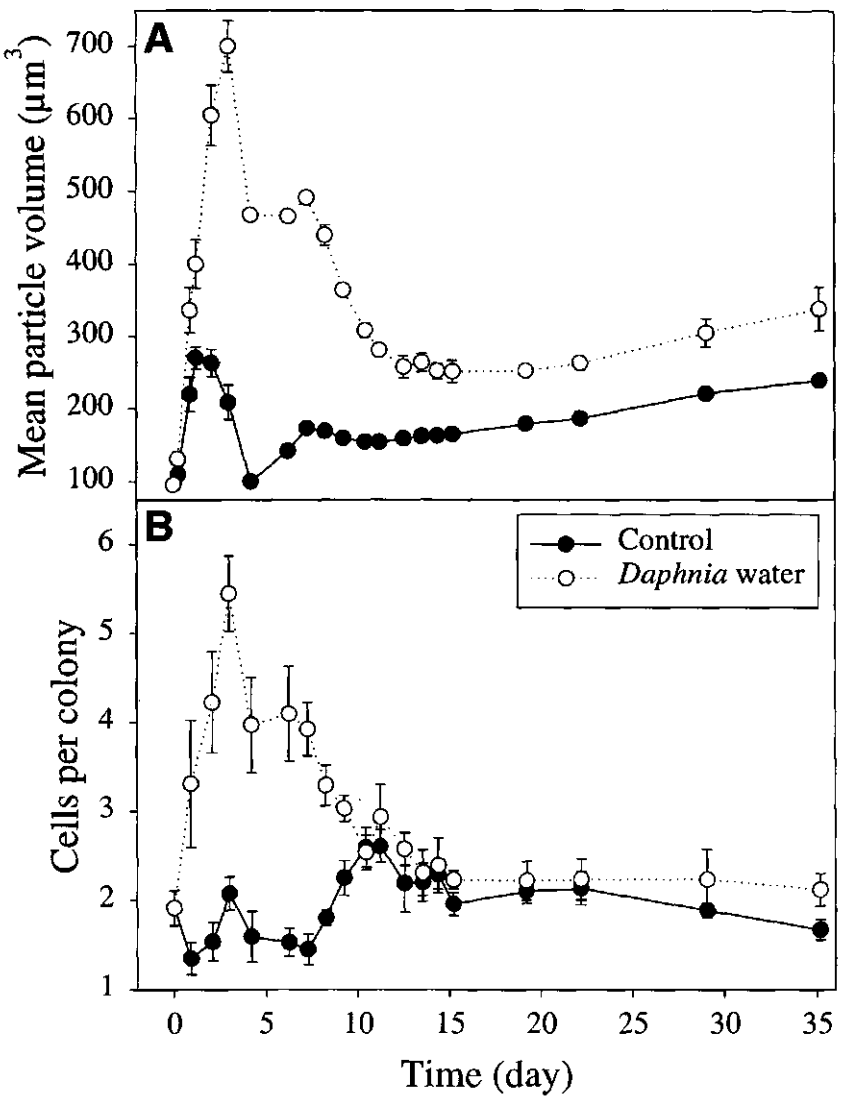
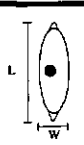
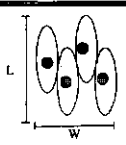
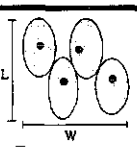
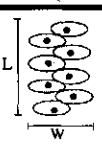
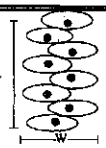


Figure 2.3: Morphological response expressed as means ± 1 SD of mean particle (colony) volume (in μm^3; panel A) and cells per colony (panel B) of *Scenedesmus acutus* populations cultured in standard WC-medium (control) and in WC-medium with water (10% v/v) from a *Daphnia* culture (*Daphnia* water).

Table 2.2: Length and width dimensions (length, L × width, W; µm ± 1 SD) of unicells, 4-celled coenobia (small- and large-types), 8-celled coenobia (small and large) and cells belonging to 8-celled coenobia, including *F*- and *P*-values of one-way ANOVA's. Similar symbols [a,b,c] within a column indicate homogenous groups that are not significantly different at th 95% level (Tukey's test).

Morphotype					
	unicells	small 4-celled	large 4-celled	small 8-celled	large 8-celled

Morphotype		**Dimensions**	
Unicells	***Treatment***	***Length (µm)***	***Width (µm)***
	Control	15.7 (1.9)[a]	5.4 (1.0)[a]
	Daphnia water	16.5 (1.8)[b]	5.6 (1.3)[a]
	One-way ANOVAs:	$F_{1,87} = 6.52$; $P = 0.012$	$F_{1,87} = 3.20$; $P = 0.077$
4-Celled	Control	20.4 (3.6)[a]	14.4 (3.9)[a]
Small	*Daphnia* water	25.9 (3.1)[b]	21.4 (4.6)[b]
Large	*Daphnia* water	30.9 (1.6)[c]	25.5 (4.9)[c]
	One-way ANOVAs:	$F_{2,24} = 20.9$; $P < 0.001$	$F_{2,24} = 12.9$; $P < 0.001$
8-Celled	Control	22.5 (2.4)[a]	19.9 (1.4)[a]
Small	*Daphnia* water	30.7 (5.3)[b]	24.0 (2.4)[b]
Large	*Daphnia* water	56.6 (7.2)[c]	30.3 (4.4)[c]
	One-way ANOVAs:	$F_{2,41} = 122.8$; $P < 0.001$	$F_{2,41} = 32.0$; $P < 0.001$
Cells in 8-celled			
Coenobia	Control	12.0 (0.8)[a]	3.7 (0.4)[a]
Small	*Daphnia* water	15.8 (1.1)[b]	5.3 (1.1)[b]
Large	*Daphnia* water	19.9 (3.4)[c]	9.5 (0.7)[c]
	One-way ANOVAs:	$F_{2,45} = 56.4$; $P < 0.001$	$F_{2,45} = 232.7$; $P < 0.001$

Populations never consisted solely of unicells and two-, four- or eight-celled coenobia, but always contained a fraction of three-, five-, six-, and seven-celled coenobia and even some aggregates with more than eight cells (all indicated as rest-group in Fig. 2.2). Although the majority of the coenobia were isofacial alternating coenobia, costulatoid, linear and tetradesmoid coenobia also were observed. Some of the observed morphotypes are presented in figure 2.5.

The NO_2^-/NO_3^- and PO_4^{3-} concentrations determined in both the control WC-medium and in the test water (WC and RT medium from a *Daphnia* culture) were similar. Only the NH_4^+-N concentration was considerably higher in the test water (0.83 mg·l^{-1}) compared to the WC medium (0.02 mg·l^{-1}). Both total inorganic (TIC) and organic carbon (TOC) were slightly higher

in the test water with TIC concentrations of 16.7 and 19.2 $mg \cdot l^{-1}$ and TOC concentrations of 2.8 and 3.9 $mg \cdot l^{-1}$ for WC and test water, respectively.

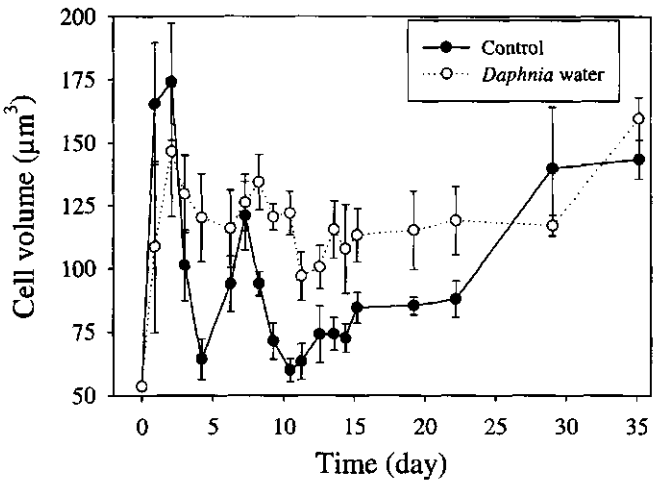


Figure 2.4: Cell volume (μm^3) of unicells and coenobial cells of *Scenedesmus acutus* cultured in standard WC-medium and in WC-medium with water (10%v/v) from a *Daphnia* culture (error bars represent ±1 SD).

2.4 DISCUSSION

Scenedesmus growth and development were strongly influenced by the presence of filtered *Daphnia* water. The formation of eight-celled coenobia in the treatment populations occurred during the exponential growth phase. Although often observed in the field (e.g. Uherkovich, 1966; Krienitz, 1987), eight-celled *S. acutus* coenobia are rarely found in laboratory cultures. In our control populations, only a negligible fraction occurred as eight-celled coenobia. The occurrence in treatment populations of eight-celled coenobia during exponential growth when nutrients were still available in excess, strongly suggests grazing-associated chemicals to be the inducing agents rather than nutrients. In fact, *S. acutus* has been reported to remain mainly unicellular when cultured under varying nutrient conditions (Sterner *et al.*, 1993; Sterner & Smith, 1993; Lürling & Van Donk, 1997a) but to form coenobia within 2 days after exposure to grazing-associated chemicals (Lampert *et al.*, 1994; Van Donk *et al.*, 1999). Although ammonium and organic carbon concentrations were somewhat higher in the test water from the *Daphnia* culture, the addition of 15 ml test water to 135 ml algal suspensions resulted in such a dilution that these differences became negligible. Moreover, ammonium and urea (Lampert *et al.*, 1994; *see* CHAPTER 3 & 4), and organic carbon sources (Nagy-Tóth *et al.*, 1992) had no effect on colony formation in *S. acutus*.

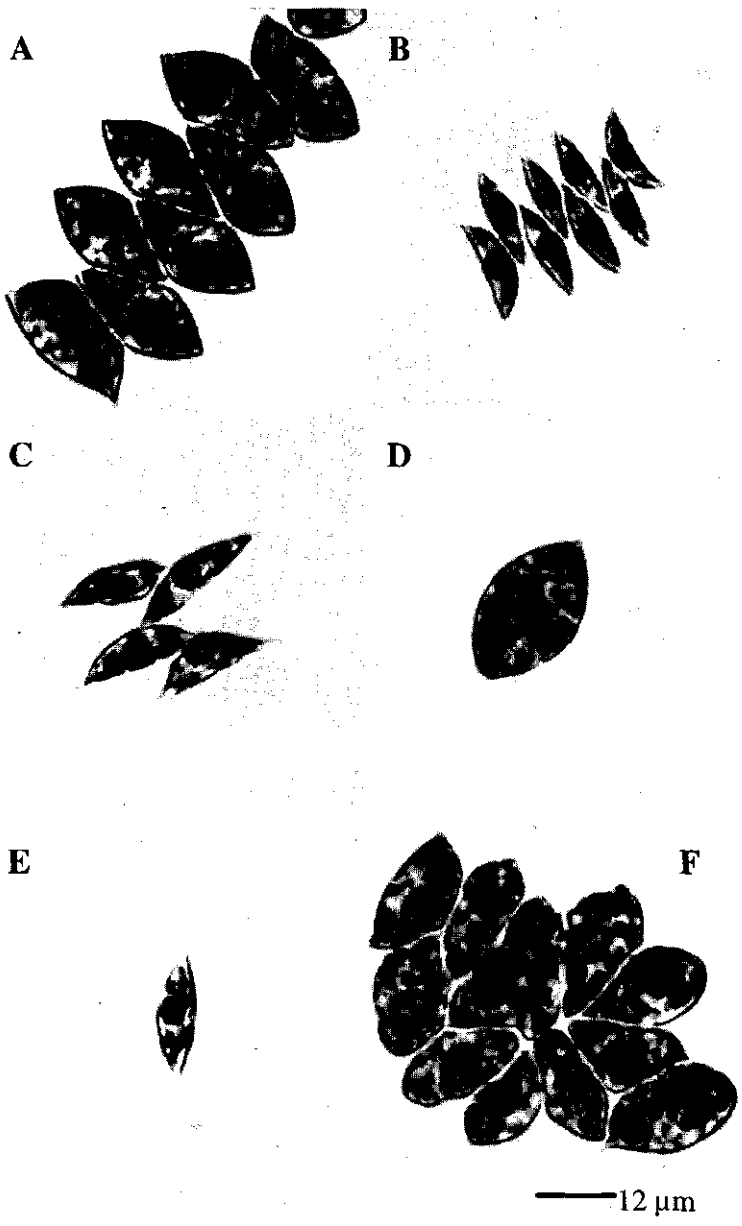


Figure 2.5: *Scenedesmus acutus* morphotypes. A: Large eight-celled coenobium. B: Small eight-celled coenobium. C: four-celled coenobium. D: Large unicell. E: Small Unicell. F: Irregular coenobium.

After 1 week, coenobia gradually disappeared from the treatment populations, and began to resemble the control populations after *ca.* 11-12 days (see Figs. 2.2 and 2.3). Possibly, the gradually reduced inductive strength of the culture medium was due to inactivation by

biodegradation, including bacterial activity as absorption and incorporation of the inducing chemicals in the algal cells. Because of the reduced concentration of these chemicals and increased population size, gradually fewer cells were exposed to the inducing chemicals and eventually coenobia formation became negligible and disappeared.

Besides coenobia formation, cell dimensions also increased in the treated populations. This increase in individual cell size does not seem to be a consequence of the induced colony formation, because both unicells and coenobial cells showed larger dimensions, but rather the result of an additional process. It may be a result of higher TOC levels (*ca.*14% higher) in the treatment medium. Aqueous fuel oil extract increased significantly the cell dimensions and volume of several *Scenedesmus* species (Tukaj & Bohdanowicz, 1995). Nagy-Tóth *et al.* (1992) reported increased cell dimensions of *S. acutus* when organic carbon sources were added to the medium. However, in their studies no formation of eight-celled coenobia was induced. In fact, some studies have reported unicell dominance in an organic carbon rich environment, i.e. sewage oxidation pond, soil extract media or the addition of glycolic acid (e.g. Mattoni *et al.*, 1965; Trainor, 1971; Siver & Trainor, 1981; 1983). By contrast glucose has been reported to favor coenobia formation in the non-spiny *S. obliquus* (Trainor, 1964). The colony-inducing chemical is most likely an organic molecule, too (Lampert *et al.*, 1994). Therefore, no generalized statements on the effect of organic carbon on *Scenedesmus* morphology seem justified.

The expression of four-celled coenobia in control populations resembles the reported cyclomorphosis of other *Scenedesmus* species. After dominance of unicells at the end of log-growth, formation of mostly four-celled coenobia has been reported in the spined species *S. subspicatus* (Trainor, 1993), *S. communis*, and *S. komarekii* (Egan & Trainor, 1990). Analogous to observations by Egan & Trainor (1989) and Trainor (1993), a deflection in the growth curve (indicated with an arrow in Fig. 2.1) could be observed before the control populations changed from unicellular to four-celled dominance. Cell death just before the unicell-colony transformation seems the most probable cause.

After the exponential growth phase, growth rates were reduced in the treatment populations resulting in a significantly lower carrying capacity. This may reflect either nutrient depletion in the cultures or a cell-size effect and hence reduced nutrient uptake. Nutrient-depletion may be a result of the added *Daphnia* water, which was a mixture of WC- and RT-medium. However, no differences in major nutrients between the WC-medium and the *Daphnia* water were measured.

Costs of colony formation were not reflected in growth rates, which has also been observed by Hessen & Van Donk (1993) and Lampert *et al.* (1994). Benefits of remaining unicellular include smaller sinking velocities (cf. Reynolds, 1984) and an advantageous surface-to-volume ratio in terms of nutrient uptake and light harvesting. In contrast, colonies may exceed the size of grazable particles and may experience a reduced grazing pressure (Hessen & Van

Donk, 1993). Small four- and eight-celled coenobia with dimensions of 26 × 21 µm and 31 × 24 µm, respectively, are protected only against small *Daphnia*. However, eight-celled coenobia with dimensions of on average 57 × 30 µm, but up to 65 × 40 µm, will undoubtedly confront even the largest *Daphnia* species with ingestibility problems.

Because of clonal variability (e.g. Mladenov & Furnadzieva, 1995; 1997) in natural water bodies, an altered morphology of a *Scenedesmus* population could be the result of clonal replacement (Wood & Leatham, 1992) or phenotypic plasticity (West-Eberhard, 1989). The observed differences in growth and morphological development of *S. acutus* in the absence or presence of grazing-associated infochemicals clearly demonstrate that the effect of grazing also should be taken into account to explain the plasticity of *Scenedesmus*. Moreover, with increasing evidence on phenotypic plasticity in *Scenedesmus*, the frequently observed coenobia in nature and unicells in lab-cultures may be the result of both selective grazing on unicells and chemically induced coenobia formation rather than clonal replacement.

CHAPTER 3

The biotest: an important tool to investigate coenobia formation in *Scenedesmus*

Parts of this chapter are based on:

Lürling, M. (1998) *Journal of Phycology* **34**: 578 –586

Lürling, M., Van Donk, E. & Beekman, W. *Accepted for publication in Verhandlungen der internationalen Vereinigung für theoretische und angewandte Limnologie*

Lürling, M. & Beekman, W. *Submitted to Limnology & Oceanography*

"We have seen that the members of the same class, independently of their habits of life, resemble each other in the general plan of their oganisation. The whole subject is included under the general name of morphology. This is the most interesting department of natural history, and may be said to be its very soul.

- C.R. Darwin 1859

3.1 INTRODUCTION

In the previous chapter a clear effect of unknown grazing-associated chemicals on growth and morphology of *Scenedesmus acutus* was demonstrated. However, several factors such as initial cell density, age of the culture, pH, nutrients and temperature may affect morphology and colony size (e.g. Trainor & Roskosky, 1967; Ramos-Cárdenas & de Lara-Isassi, 1985; Holtmann & Hegewald, 1986; Egan & Trainor, 1989; Trainor, 1992a, b; 1993). Organic carbon sources have been reported to increase cell dimensions and hence biovolume of several *Scenedesmus* species (Nagy-Tóth *et al.*, 1992; Tukaj & Bohdanowicz, 1995).

Moreover, close examination of the media (Bristol's and medium 7) used in several studies after the *Scenedesmus* plasticity (e.g. Egan & Trainor, 1989a,b,c; Ramos-Cárdenas & de Lara-Isassi, 1985) revealed the absence of an (in)organic carbon source, meaning that the major constituent (*c.* 50% of biomass) of an algal cell had to diffuse into the medium from the air. Hence, the occurrence of carbon limitation under these conditions is not unlikely.

Among the factors involved in the ecomorph expression in *Scenedesmus* are several that are related to the amount of carbon available to the algae. As demonstrated in the previous chapter and in recent literature, the algal predator *Daphnia* releases a chemical substance that triggers the unicell-colony transformation in *Scenedesmus* (Hessen & Van Donk, 1993; Lampert *et al.*, 1994). But *Daphnia*'s are referred to as sloppy feeders. They ingest more food than they use for biomass and as a result many products are released from *Daphnia* (Peters, 1987), including organic carbon (Lampert, 1978), cyclic AMP (Francko & Wetzel, 1982), phosphorus (Peters & Rigler, 1973) and ammonia and amino acids (Gardner & Miller, 1981). The colony-inducing chemical is probably an organic molecule (Lampert *et al.*, 1994). Nagy-Tóth *et al.* (1992) examined the effect of several carbon sources on the morphology of *S. acutus*, but did not report any formation of eight-celled coenobia. They discussed whether the dominance of unicells in the presence of glucose was a peculiarity of their strain or just the result of bubbling. Interestingly, glucose has been reported to induce coenobia in a non-spiny *Scenedesmus* strain (Trainor, 1964). Glucose being the major building block of cellulose could be released from the algal predator *Daphnia*, which is a sloppy feeder that may release 10-17% of the ingested carbon (Lampert, 1978).

To test, for example, for the effects of nutrients, chemicals, or amount of medium with grazer-associated chemicals from zooplankton cultures, a reliable biotest is of uttermost importance. In this chapter, I first report experiments performed to establish a reliable bioassay, followed by biotests in which the influence of biotic and abiotic factors on coenobia formation in *Scenedesmus acutus* Meyen was examined.

3.2 DEVELOPMENT OF THE BIOTEST

3.2.1 General

A biotest should be simple, fast, but reliable. This means that as many factors as possible should be maintained constant. Therefore, to ensure a as constant as possible inoculum of test algae, the green alga *Scenedesmus acutus* Meyen was cultured in 1.0 litre chemostats on slightly modified WC-medium (Guillard & Lorenzen, 1972; Table 3.1). The chemostats were continuously illuminated at an irradiance of 125 $\mu mol \cdot m^{-2} \cdot s^{-1}$ provided by circular fluorescent tubes (Philips TLEM 40 W/33RS). The chemostats were positioned in a temperature-controlled chamber at 20°C and run with a dilution rate of 1.2 day^{-1}. Harvested algae from the chemostat were used as inocula in batch cultures serving as tests.

Table 3.1: Composition of algal growth medium (final concentrations in $mg \cdot l^{-1}$).

Major nutrients	$mg \cdot l^{-1}$	Trace elements	$mg \cdot l^{-1}$
$NaNO_3$	85.01	$Na_2EDTA \cdot 2H_2O$	4.36
$MgSO_4 \cdot 7H_2O$	36.97	$FeCl_3 \cdot 6H_2O$	1.00
$CaCl_2 \cdot 2H_2O$	36.76	$MnCl_2 \cdot 4H_2O$	0.18
$Na_2SiO_3 \cdot 9H_2O$	28.42	$CuSO_4 \cdot 5H_2O$	0.001
H_3BO_3	24.00	$ZnSO_4 \cdot 7H_2O$	0.022
$NaHCO_3$	12.60	$NaMoO_4 \cdot 2H_2O$	0.022
K_2HPO_4	8.71	$CoCl_2 \cdot 6H_2O$	0.012
		Na_3VO_4	0.0018
TES[a]	85.00	H_2SeO_3	0.0016

[a] TES = *N*-Tris(hydroxymethyl)-methyl-2-aminoethane-sulphonic acid ($C_6H_{15}NO_6S$); Sigma T-1375

A second approach would be the use of continuous cultures themselves. A chemostat system in a steady state, with very low short-term noise and long-term drift, could be used as its own control before and after the addition of test-water, in fact a pulse perturbation (Boraas, 1993). An advantage of chemostats is that they are controlled with a precisely defined nutrient environment. However, a disadvantage is that fewer cultures can be maintained or tests performed than with short-term batch cultures. To accommodate quickness and simplicity of the desired biotest, short-term batch culture growth experiments were prefered. At low inoculum size and relatively short experimental incubation periods, cultures will approximate steady state conditions. The set-up was partly based on the biotest as developed by Lampert *et al.* (1994). Nevertheless, chemostat cultures were used to examine the effect of nutrients, and growth rate (*see* §4.3 & 4.4) on morphology of *S. acutus*.

Initially, short-term batch experiments were performed without the colony inducing chemicals. These experiments had the main objective to examine the variability in *Scenedesmus* growth and morphology exhibited under various conditions. Some experimental conditions were chosen *a priori*, such as an incubation temperature of 20°C, the use of a shaking device to prevent sedimentation (80 rpm), continuous illumination from above at an irradiance of 125 $\mu mol \cdot m^{-2} \cdot s^{-1}$ (Osram L 36W/21-840 cool-fluorescent white tubes) and 100 ml Erlenmeyer flasks.

Algal densities and particle size distributions were determined using an electronic partical counter; Coulter Multisizer II (100 µm capillary) or CASY-1 (150 µm capillary). The number of cells per colony was determined microscopically by counting at least 100 aggregates (i.e. unicells as well as coenobia) in algal subsamples preserved in Lugol's fixative. Cell dimensions (length and width) of different aggregates were measured using a Leica Quantimet 500 MC image-analyzer coupled with a Nikon light-microscope at 500 × magnification.

Growth rates were calculated from increase in biovolumes and from cell multiplication. Total cell numbers were computed by multiplying the number of particles (determined by Coulter) by the number of cells per particle (determined by microscope).

Test-water with colony inducing *Daphnia* chemicals was produced prior to the experiments by allowing a few hundred adult *Daphnia* to feed on a suspension of *S. acutus* (*ca.* 4 $mgC \cdot l^{-1}$) in WC-medium. After 24 hours incubation at 20°C in the dark, the *Daphnia* were removed from the vessel and the medium was filtered. In their study, Lampert *et al.* (1994) showed that mean particle volumes were highly correlated with the mean number of cells per colony. Thus, for statistical comparison mean particle volumes could be used. I compared for 78 measurements the mean number of cells per colony with the mean particle volume (MPV), which revealed that both parameters were highly correlated ($r^2 = 0.770$; Fig. 3.1). The regression is:

$$\log(MPV) = 2.241 + 0.730 \times \log(\text{cells colony}^{-1})$$

This is close to the regression obtained by Lampert *et al.* (1994):

$$\log(MPV) = 2.127 + 0.726 \times \log(\text{cells colony}^{-1})$$

In both regressions, the slope is less than 1, which indicates that the individual cell sizes decrease with increasing colony size. However, under certain conditions detection of colony formation solely based on determinations of mean particle volumes may imply unjustified conclusions as cell dimensions could be increased rather than colony formation being induced. On the other hand, colony formation (cell division) may be triggered, but may not necessarily coincide with a proportional increase in mean particle volume, for example as a result of a limiting resource. In these cases microscopic analysis is of importance.

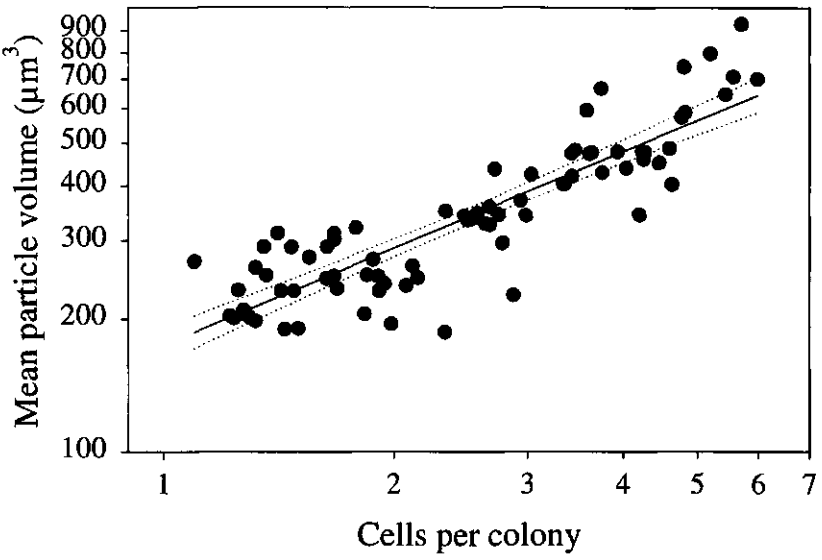


Figure 3.1: Relationship between the mean particle volume (μm^3) and the mean number of cells per particle in *Scenedesmus acutus*. Solid line represents linear regression, dotted lines the 95% confidence intervals.

3.2.2 Plugs?

Following Lampert *et al.* (1994) 100 ml Erlenmeyer flasks were selected as experimental vessels to be used in the biotest. Due to C uptake by the algae and a consequent rise in pH, replenishment of C from the air will occur (Portielje & Lijklema, 1995). Therefore, in a first experiment the effect of different sealing methods applied to experimental vessels on growth and morphology of *S. acutus* was examined to select for appropriate plugs to seal the experimental vessels.

The coverings used were parafilm, silicon rubber plugs, cellulose plugs and cotton wool, while an additional series of Erlenmeyer flasks was not closed. Each Erlenmeyer contained 50 ml of *S. acutus* in WC-medium (without TES-buffer). The initial cell density was 45000 ± 450 particles·ml^{-1} (i.e $4.2 \cdot 10^6$ $\mu m^3 \cdot ml^{-1}$). The different treatments were run in quadruplicates. The pH and temperature of the medium were recorded routinely using a pH 96-meter (WTW). Cell dimensions of *Scenedesmus* were determined of samples taken after 48 hours of incubation. Length and width of the unicells were statistically compared applying one-way ANOVA, followed by a Tukey's test.

Application of different plugs on the experimental Erlenmeyer flasks resulted in different growth and cell morphology (Fig. 3.2). Open flasks and flasks closed with either cotton wool or cellulose-plugs supported the highest growth, while silicon-rubber plug stoppered or parafilm sealed flasks showed a reduced growth. The significantly smaller cell dimensions (Table 3.2), the courses of the measured pH and cell volume (Fig. 3.2) suggest

possible carbon limitation in silicon-rubber plug stoppered and in parafilm sealed flasks occurring already within one day. However, after 48 h also in the other treatments pH-values had increased dramatically. Based on this experiment, cellulose plugging was chosen for closure of experimental Erlenmeyer flasks.

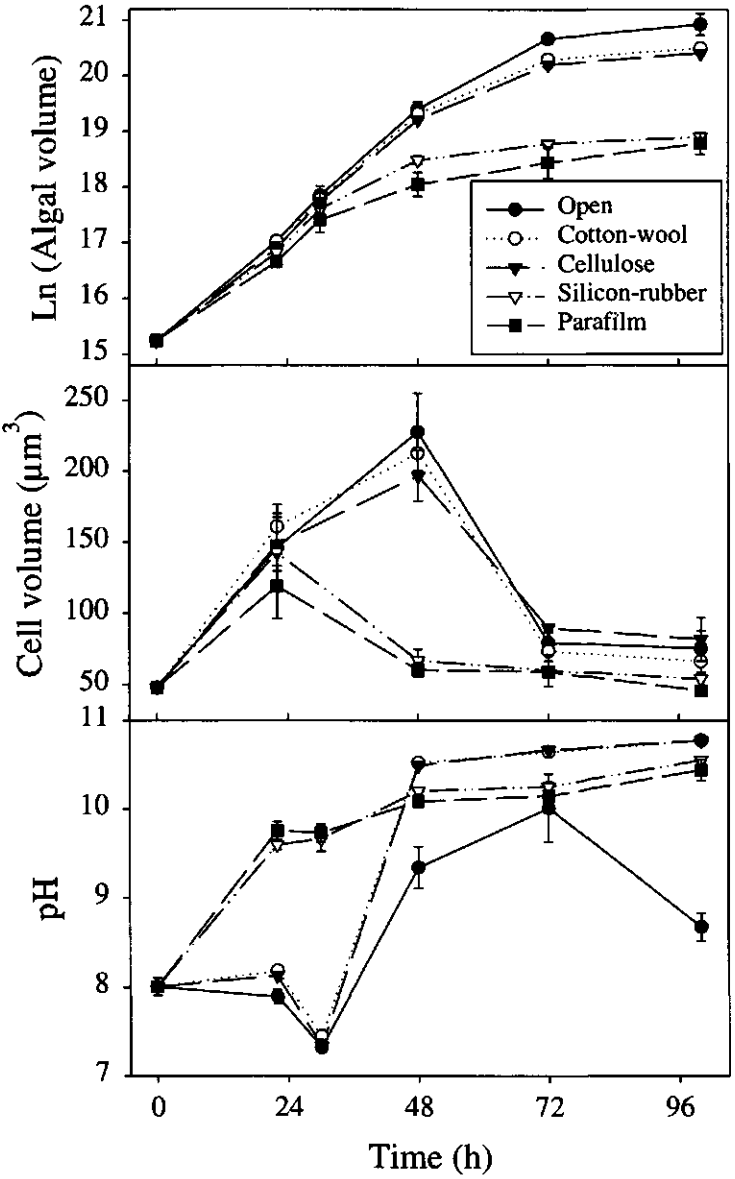


Figure 3.2: *Scenedesmus acutus* growth based on total volume (A), *S. acutus* cell volume (B) and the course of the pH (C) in incubations with different sealings

Table 3.2: Mean length and width dimensions (± 1 SD; n = 4) of *Scenedesmus acutus* cultured for 48 h in batches with different stops including *F*- and *P*-values of one-way ANOVA. Similar symbols [A,B] within a column indicate homogeneous groups (Tukey's test).

Plug-type	Length (µm)	Width (µm)
Open	17.6 (2.4) [A]	7.1 (1.5) [A]
Cotton-wool	17.9 (2.7) [A]	7.1 (1.7) [A]
Cellulose	17.2 (2.9) [A]	6.9 (1.5) [A]
Silicon-rubber	15.6 (2.3) [B]	4.3 (0.8) [B]
Parafilm	14.9 (1.3) [B]	3.7 (0.4) [B]
F- and	$F_{4,196} = 12.1$	$F_{4,196} = 73.6$
P values	$P < 0.001$	$P < 0.001$

3.2.3 Buffer & Carbon

The WC medium contains only 1.8 mg $C \cdot l^{-1}$, but this seemed not to hamper *Scenedesmus* growth, as exponential growth was observed during the first 48 h (*see* Fig. 3.2A). However, in cellulose plug stoppered flasks the pH increased to values above 10 (Fig. 3.2C). Therefore, a buffer (TES = *N*-Tris(hydroxymethyl)-methyl-2-aminoethane-sulphonic acid ($C_6H_{15}NO_6S$); Sigma T-1375) in a final concentration of 85 $mg \cdot l^{-1}$ was added to the medium. Moreover, the amount of carbon in the medium was varied and added in normal amount, 10 times and 25 times the standard amount of $NaHCO_3$. The effect of the buffer and carbon on growth and colony formation in *S. acutus* was examined both in the absence and presence of filtered medium from a *Daphnia* culture.

The addition of the buffer resulted in lower pH values after 48 h than in the previous experiment (Fig. 3.3). Two-way ANOVA indicated no differences in the pH after 48 h that was 9.33 (± 0.30).

Growth rates were not affected by the *Daphnia* water or the carbon content of the medium and were on average 2.079 (± 0.066) d^{-1}. By contrast, two-way ANOVA indicated that the number of cells per colony was significantly affected by *Daphnia* water ($F = 85.7$; $P < 0.001$), but not by the amount of carbon ($F = 0.21$; $P = 0.812$). Although the individual cell volumes in control populations were increased at higher C-levels, with values of 142 (± 29.1), 149.0 (± 18.5) and 171.3 (9.9) μm^3 in standard, 10 times and 25 times carbon, respectively, the differences were not significant.

Since the buffer did not seem to affect growth and morphology in a negative way, but kept the pH at reasonable values, and the addition of more carbon had no significant effect, it was decided to maintain the carbon as in the WC medium and add the buffer. The temperature

in this experiment was higher than the desired 20 ± 1 °C, but constant over the incubation period at 23.3 ± 0.4 °C, and could explain the high volume based growth rates.

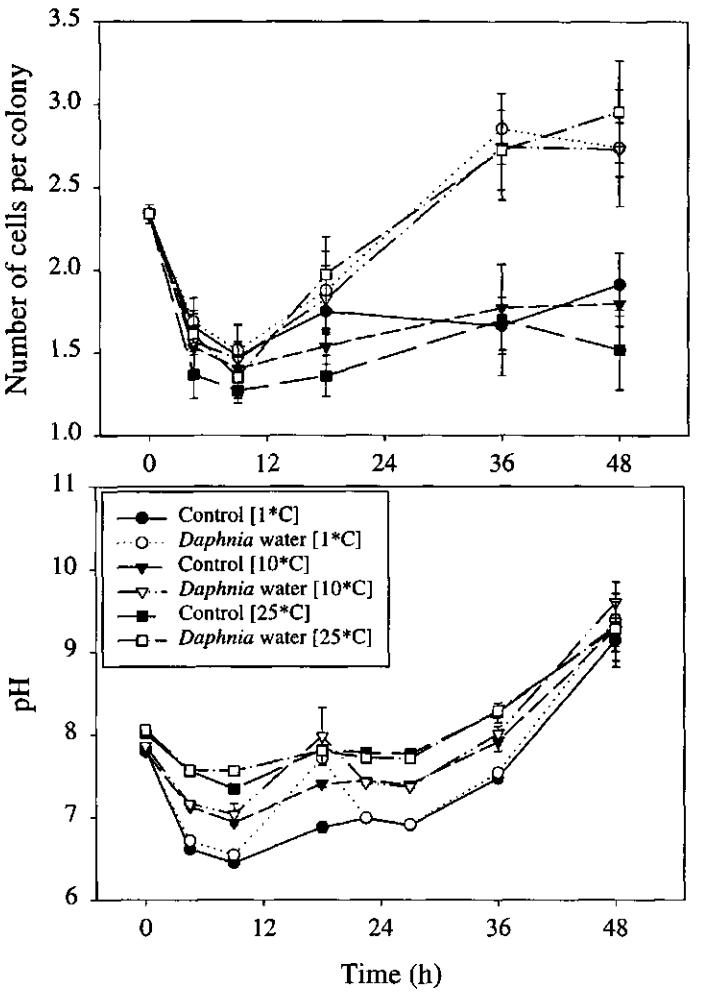


Figure 3.3: Course of pH (lower panel) and number of cells per colony (upper panel) in *S. acutus* cultured in the absence (Control; filled symbols) and presence of water from a *Daphnia* culture (*Daphnia* water; open symbols) in medium with the standard amount of carbon [1*C], 10 times [10*C] and 25 times the standard amount [25*C]. Error bars represent 1 SD (n = 4).

3.2.4 Filters

To separate dissolved zooplankton chemicals from algae, bacteria and debris a filtration step was included. However, filters may release extractable compounds that could influence growth and morphology of the test-algae. Therefore, the effect of filtration was

examined and compared with another separation technique, centrifugation. In a first experiment 0.1 µm membrane-filters (NC10) were rinsed with different amounts of nanopure water, 0, 50, 100, 200 and 500 ml respectively, before 10 ml WC-medium was filtered through it. Five ml filtrate from four filters was added separately to 45 ml *S. acutus* suspensions in 100 ml Erlenmeyer flasks. As control served incubations without test-water (Control) while incubations with 5 ml supernatant of medium from a *Daphnia magna* culture, centrifuged for 5 min. at 4000 rpm, served as a positive control, i.e. incubations in which colony formation was expected (Dm-Unfilt.). Tests were run in quadruplicate. After 48 h, colonies had not only been induced in incubations with *Daphnia* water without filtration (Dm-Unfilt.), but also in incubations with medium filtered through non-rinsed filters (Fig. 3.4). Hence, extractable compounds from cellulose nitrate filters (NC10) induce colonies in *Scenedesmus* as well.

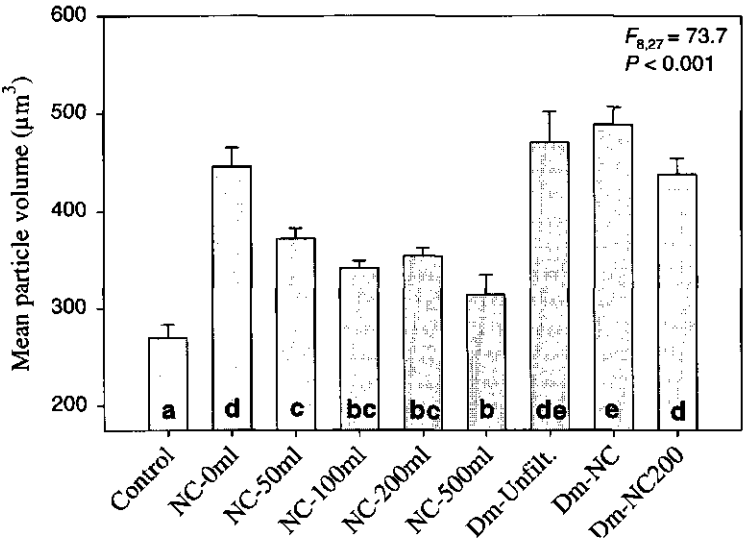


Figure 3.4: Effect of medium filtered through unrinsed and rinsed cellulose-nitrate membrane filters on mean particle volumes in *S. acutus*. Error bars represent 1 SD (n = 4). Similar symbols a,..,e represent homogeneous groups (Tukey test). NC-xml = Represents treatments with 5 ml filtrate from a cellulose nitrate membrane filter (NC10) after the filter had been rinsed with x ml water. DM-Unfilt. = treatments with 5 ml medium from a *Daphnia magna* cultured centrifuged at 4000 rpm (5 min.). DM-NCx = treatments with 5 ml filtered medium from a *Daphnia* culture after the filter had been rinsed with x ml water.

In a second experiment, filtrate from different filter types was tested on their possible effect on the algae. Aliquots of 25 ml WC-medium were filtered through glass-fiber (GF52),

mixed esters of cellulose (ME24) or cellulose nitrate (NC10) filters (all filters from Schleicher & Schuell, Germany). Filtrate (5 ml) was added to 45 ml of *S. acutus* suspensions in fresh WC-medium in 100 ml Erlenmeyer flasks with an initial algal density of 45000 particles·ml^{-1}. As control served incubations without test-water (Control) while incubations with 5 ml supernatant of medium from a *Daphnia magna* culture, centrifuged for 5 min. at 4000 rpm, served as a positive control, i.e. incubations in which colony formation was expected (*Daphnia* water). Based on the previous experiment, a treatment with glucose (1 mg·l^{-1}) was added. Both mixed-esters and cellulose nitrate filters significantly induced colonies in *Scenedesmus* (Fig. 3.5). However, filtrate from GF-filters had no morphogenetic effect. Although Trainor (1964) observed glucose-induced colony formation in *S. obliquus* UTEX 393, in this experiment glucose appeared not to induce colonies in *S. acutus*, which has also been found by Nagy-Tóth *et al.* (1992).

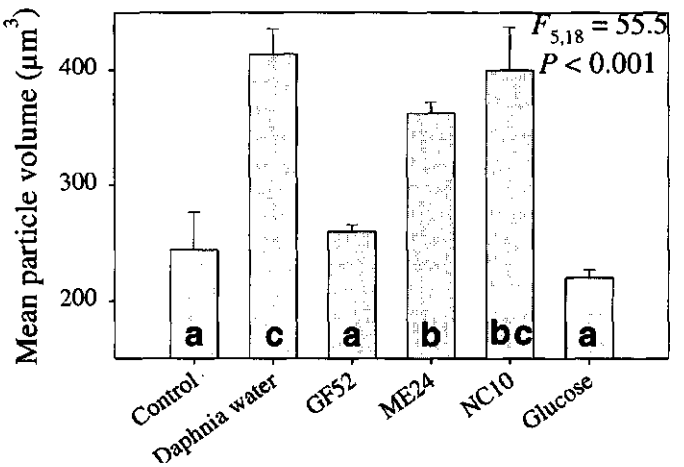


Figure 3.5: Effect of medium run through different filters on mean particle volumes in *S. acutus*. Error bars represent 1 SD (n = 4).

Based on these experiments, glass-fiber filters seemed most suited, although cellulose-nitrate filters may still be used after rinsing or as a positive control to ensure a constant colony induction. Moreover, analysis of the filtrate may reveal the chemical structure of the inducing compounds and thus may lead to a more narrowed search for the "*Daphnia* factor".

3.2.5 Inoculum size

This experiment was designed to examine the effect of initial algal density on *Daphnia*-induced colony formation and growth in *S. acutus*. In previous experiments Lampert *et al.* (1994) used inocula of $1.25 \cdot 10^5$ cells ml^{-1}, while Hessen & Van Donk (1993) have used

$5.4 \cdot 10^5$ cells·ml^{-1}. These relatively heavy inocula could result in nutrient depletion or self-shading and hence affect the growth rate.

Series of three replicate flasks with or without *Daphnia* test-water (10% v/v) received different amounts of test-algae according the range 10^3, 10^4, $2.5 \cdot 10^4$, $5 \cdot 10^4$, 10^5 and $5 \cdot 10^5$ particles·ml^{-1}.

Colony formation appeared negatively correlated with the initial algal density (Fig. 3.6 panel A). The two-way ANOVA indicate a significant effect of inoculum size on the colony formation, a significant effect of the *Daphnia* water and a significant interaction (Table 3.3).

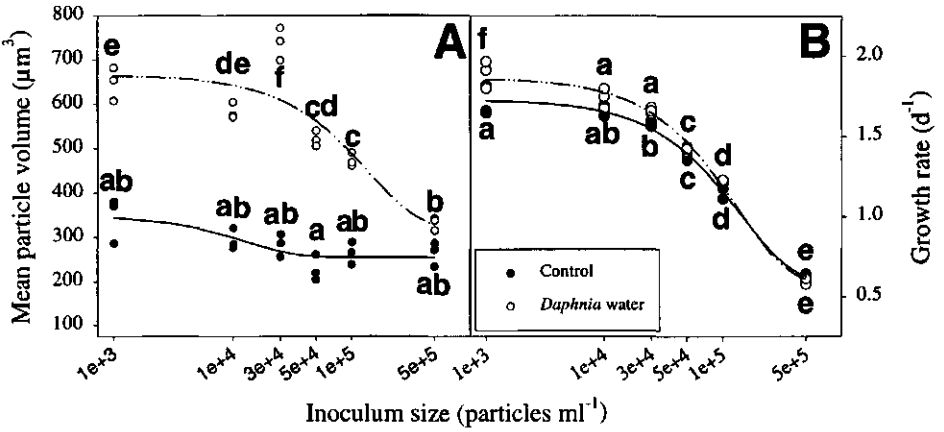


Figure 3.6: Effect on inoculum density on colony formation, expressed as mean particle volume (Panel A) and growth (Panel B) in *S. acutus* in the absence (Control) and presence of water from a *Daphnia* culture (*Daphnia* water). Similar symbols, **a,..,f** indicate homogeneous groups (Tukey's test).

Table 3.3: *F*- and *P*-values of two-way ANOVAs on mean particle volumes (MPV in µm^3) and volume based growth rates (d^{-1}).

	MPV (µm^3)			Growth rate (day^{-1})		
	df	*F*	*P*	df	*F*	*P*
Inoculum-size	5	49.0	<0.001	5	534.3	<0.001
Daphnia water	1	785.9	<0.001	1	22.7	<0.001
Interaction	5	29.0	<0.001	5	3.12	0.026

Growth rates were significantly reduced with increasing inocula sizes (Fig. 3.6; panel B). Moreover, growth rates were significantly higher in treatments with *Daphnia* water compared to controls when initial algal density was below $5 \cdot 10^4$ particles·ml^{-1}, while rates were similar above this density. This experiment could, however, not unravel whether the

lower colony formation at larger inocula sizes was due to reduced growth or due to a relatively reduced availability of inducing chemicals to individual cells. Concluding, an inoculum around $2.5 \cdot 10^4$ cells·ml^{-1} is advisable on the one hand to ensure sufficient algal biomass necessary for microscopical counting, on the other to prevent reduced growth and colony formation.

In an additional experiment 5 ml test-water from a *Daphnia* culture was added to 20 ml algal suspensions with initial concentrations of 0, $2 \cdot 10^3$, $2 \cdot 10^4$, 10^5, $2 \cdot 10^5$, $5 \cdot 10^5$ and 10^6 particles·ml^{-1}. After 24 hours these suspensions were filtered through a 0.1 µm membrane filter and 20 ml was added to *S. acutus* in 30 ml fresh medium to examine the effect on coenobia formation.

Colony formation was significantly affected ($F_{6,14} = 14.0$; $P < 0.001$) by the pretreatment in a way that test-water from high pre-test algal concentrations resulted in significantly reduced colony formation (Fig 3.7). Growth rates were not significantly different ($F_{6,14} = 2.75$; $P = 0.055$), but a tendency to somewhat reduced growth in vessels with test-water from high pre-test algal densities could be observed (Fig 3.7). Thus it seems that the coenobia inducing compounds are inactivated when exposed to heavier inocula.

Whether the colony-inducing chemicals are inactivated by passive adsorption to algal cells or by metabolic activity was examined in another experiment. In a first series, 20 ml test-water from a *Daphnia* culture was added to 30 ml algal suspensions with initial concentrations of 0, $2.5 \cdot 10^4$, 10^5, $5 \cdot 10^5$ and 10^6 particles·ml^{-1}. In a second series, 20 ml test-water was added to 30 ml medium without algae, to 30 ml with 10^6 particles·ml^{-1}, to 30 ml heat-killed algae equivalent to 10^6 particles·ml^{-1}, and to 30 ml containing a 1:1 mixture of heat-killed and live algae corresponding to 10^6 particles·ml^{-1}.

After 24 hours, these suspensions were filtered through a glass-fiber filter and 5 ml was added to *S. acutus* in 45 ml fresh medium to examine the effect on coenobia formation. Colony formation was significantly affected ($F_{6,14} = 16.8$; $P < 0.001$) by the pretreatment in a way that test-water from high pre-test algal concentrations resulted in significantly reduced colony formation (Fig. 3.8). Growth rates were significantly different ($F_{6,14} = 2.90$; $P = 0.047$), but this was due to one treatment ($5 \cdot 10^5$ particles·ml^{-1}) which was significantly different from the control (Fig. 3.8). Heat-killed algae, however, had no effect on the colony-inducing chemicals. Test-water exposed to heat-killed algae remained active, whereas live algae significantly reduced the activity ($F_{4,10} = 17.7$; $P < 0.001$) (Fig. 3.8). Growth rates based on biovolume were not different ($F_{4,10} = 1.85$; $P = 0.196$) with a mean (± 1SD) of 1.646 ± 0.042 (n = 5).

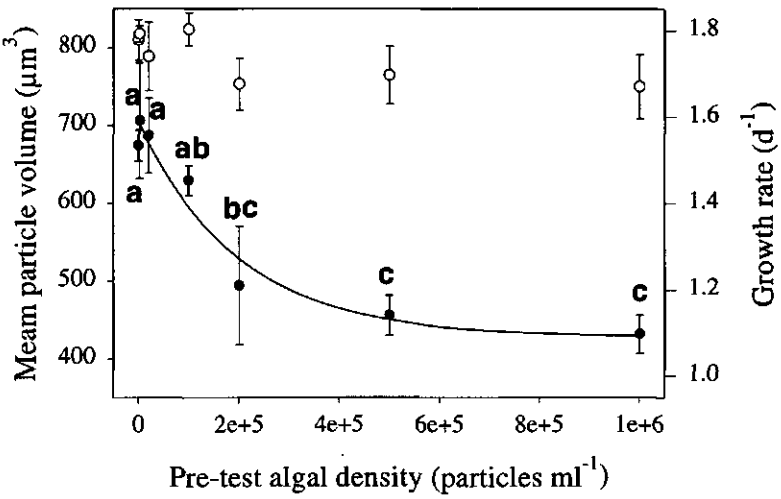


Figure 3.7: Effect on the colony formation in *S. acutus* of *Daphnia* water exposed for 24 h to algal suspensions varying in density, prior to use in a biotest. Error bars represent 1 SD (n = 3). Closed symbols (●) refer to mean particle volumes, open symbols (o) to growth rates. Similar symbols (**a,b,c**) represent homogeneous groups that are not statistically different at the 95% level (Tukey's test).

However, it should be noted that the strain *S. acutus* MPI is not axenic. The inactivation of colony-inducing activity could also be due to bacterial degradation. Therefore, antibiotics were added to *S. acutus* suspensions with *Daphnia* water and the growth and colony formation compared with incubations without antibiotics, but with (positive controls) or without *Daphnia* water (negative controls). Although the inoculum density was ~$4 \cdot 10^4$ particles·ml^{-1}, and 5 ml test water was added, based on the observation that only a proportion of the *S. acutus* population occurred as eight-celled coenobia, it was hypothesized that this proportion would be enhanced in the presence of antibiotics. After 48 h, one-way ANOVA indicated significant differences ($F_{2,6,}$ = 100.8; $P < 0.001$) in mean particle volumes, but Tukey's test revealed that only the negative controls were significantly different. The mean particle volumes (± 1 SD; n = 3) for negative controls, positive controls and treatments with antibiotics were 238.9 (27.0), 671.9 (8.9) and 598.5 (63.2) µm^3, respectively. Growth rates were similar ($F_{2,6}$ = 2.74; P = 0.143) and on average 1.69 (0.05) d^{-1}. This experiment may not completely rule out bacterial degradation, but it rules out modification by bacteria attached to the algae.

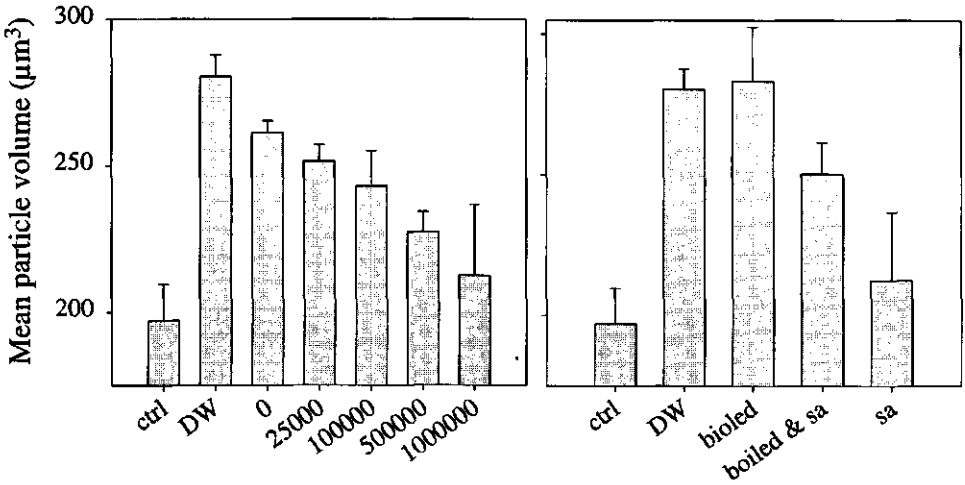


Figure 3.8: The effect of exposure of *Daphnia* water to different amounts of *Scenedesmus acutus* (left panel; 0...10^6 particles·ml^{-1}), and to live (*S.a.*10^6), heat-killed (boiled) or a 1:1 mixture of both (boiled & *S.a.*) on mean particle volume (MPV ± 1 SD; n = 3) and growth (± 1 SD) in *Scenedesmus acutus*. Similar symbols indicate homgeneous groups that are not significantly different at a 95% level (Tukey's test).

3.2.5 Amount of test-water

Different amounts of test-water, i.e. 0, 0.1, 1.0, 2.0, 5.0 and 10 ml, were added to *S. acutus* suspensions in WC-medium. To produce the test-water, prior to the experiment approximately 100 *Daphnia magna* were transferred into 700 ml WC-medium with *S. acutus* as food (10^7 µm^3·ml^{-1}). After 24 h, medium from this culture was filtered through a glass-fibre filter and used as test-water. An additional treatment received one adult *D. magna* (~ 4 mm), the biotest was run in triplicate. After 48 h, colony sizes, expressed as the mean particle volume, were measured. One-way ANOVA indicated significant differences ($F_{6,14} = 46.2$; $P < 0.001$). Three homogeneous groups could be distinguished: 1) "0" and "0.1", 2) "1.0, 2.0", "5.0", "10" ml of test-water, and 3) the "one-live *Daphnia* treatment (Fig. 3.9). The addition of 1.0 ml (2% v/v) already significantly promoted coenobia formation however the response with 5.0 ml *Daphnia* water was ~10% larger. Hence, 5 ml was selected as standard amount of test-water to be added to test-algae in biotests. The volume based growth rates were significantly different among treatments ($F_{6,14} = 199.1$; $P < 0.001$), however Tukey's test revealed that this was due to the treatments with one live *Daphnia* added. Omitting this treatment showed that the addition of different amounts of test-water had no effect on the volume based growth rates in *S. acutus* ($F_{5,12} = 1.49$; $P = 0.265$) with a mean (± 1 SD) of 1.479 (± 0.038) d^{-1}.

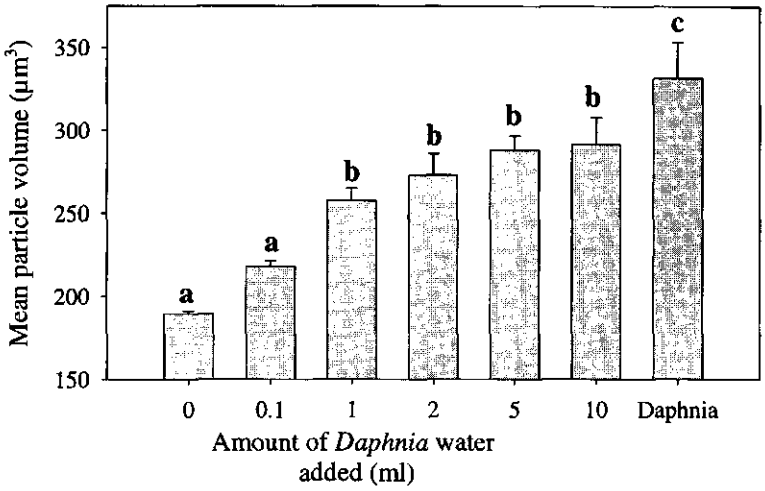


Figure 3.9: Effect of different amounts of filtered water from a *Daphnia* culture (0.1 ... 10 ml) and of one live *Daphnia* added (Daphnia) on the mean particle volume (µm³) of *Scenedesmus acutus*. Error bars indicate 1 SD (n = 3). Similar symbols a,b,c represent homogeneous groups.

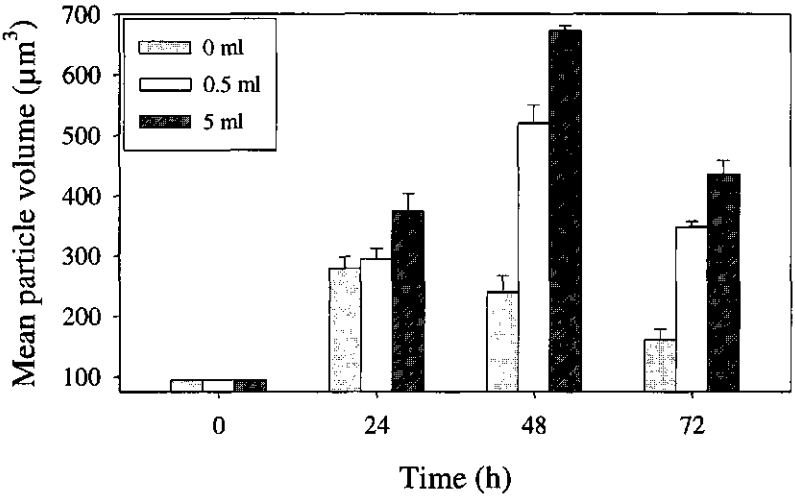


Figure 3.10: Effect of different amounts of filtered water from a *Daphnia* culture (0, 0.5 and 5ml) on the mean particle volume (µm³) of *Scenedesmus acutus* as a function of time (h). Error bars indicate 1 SD (n = 3).

The course of the mean particle volumes of incubations without *Daphnia* water, and of incubations that had received either 0.5 ml or 5.0 ml water from a high density *D. magna* culture was examined during 72 h. Mean particle volumes were maximal after 48 h incubation (Fig. 3.10). The decrease in MPV after 48 h could be a result of the increased algal biomass that affected growth and cell volume (§*3.2.2*) and reduced the activity of the *Daphnia* water

(§3.2.4). The decrease in the *Daphnia* water treatments was relatively stronger than in the controls. Moreover, at identical amounts of *Daphnia* water added (5 ml) an enormous difference in MPV between the two experiments occurred. In the latter experiment, the MPV was twice as high as in the former experiment. This could be due to differences in the algal physiology, despite the fact they were harvested from the chemostat, as the MPV in controls showed already a 21% difference. Initial densities were different with $2.5 \cdot 10^4$ particles·ml^{-1} and $4.4 \cdot 10^4$ particles·ml^{-1} in the former and latter experiment, respectively. However, these densities do not seem to be the explanation, because then one would expect the opposite (§3.2.4). Other factors involved may be the *Daphnia* density, size and activity.

3.2.6 *Daphnia density and body-size*

When incubation water from 200 *Daphnia*·l^{-1} was serially diluted, the induction of colonies decreased linearly and disappeared at *ca.* 50 *Daphnia*·l^{-1} (Lampert *et al.*, 1994). Moreover, starved *Daphnia* and *Daphnia* homogenate induced no colonies in *Scenedesmus*. Hence, the induction of colonies depends on the density of actively feeding *Daphnia*.

Several studies have reported reductions in the feeding when animals were exposed to chemicals released from conspecifics (Helgen, 1987), congeners (Matveev, 1993) or competitors and predators (Folt & Goldman, 1981). *Daphnia pulex* showed a remarkable decrease in clearance rate with increasing population density (unpublished data). This negative interference (Goser & Ratte, 1994), being the result of both chemicals and touch, could influence the production of colony inducing chemicals.

Test-waters from a *Daphnia magna* and a *Daphnia pulex* culture was used to examine the effect of incubation density on production of infochemicals. The *D. magna* (animals with a mean body length (± 1 SD) of 1.26 ± 0.22 mm) densities used were 0, 20, 50, 100, 200, and 400 ind.·l^{-1}. After 24 h at 20°C in the dark, water from these incubations was centrifuged during 5 min. at 4000 rpm and added to the test alga *S. acutus*. Colony formation expressed as mean particle volume increased significantly ($F_{5,18} = 43.8$; $P < 0.001$) with higher *Daphnia* densities (Fig. 3.11). Tukey's post-hoc comparison revealed three homogeneous groups: 1) '0, 20, 50'; 2) '20, 50, 100' and 3) '200, 400' *D. magna*·l^{-1}.

The other animal, *D. pulex* (animals with a mean body length (± 1 SD) of 1.23 ± 0.16 mm), was incubated at 0, 20, 40, 80, 120, 160, 200, 400 and 600 ind.·l^{-1}. After 21 h at 20°C in the dark, water from these incubations was filtered through a glass-fiber filter (GF52) and 5 ml filtrate was added to 45 ml fresh WC-medium inoculated with the test alga *S. acutus*. Again a significant increase ($F_{8,18} = 16.5$; $P < 0.001$) of mean particle volume with *Daphnia* density was observed. Tukey's test revealed three homogeneous groups: 1) '0, 20, 40, 80, 120, 160, 200'; 2) '200, 400' and 3) '400, 600' *D. pulex*·l^{-1}.

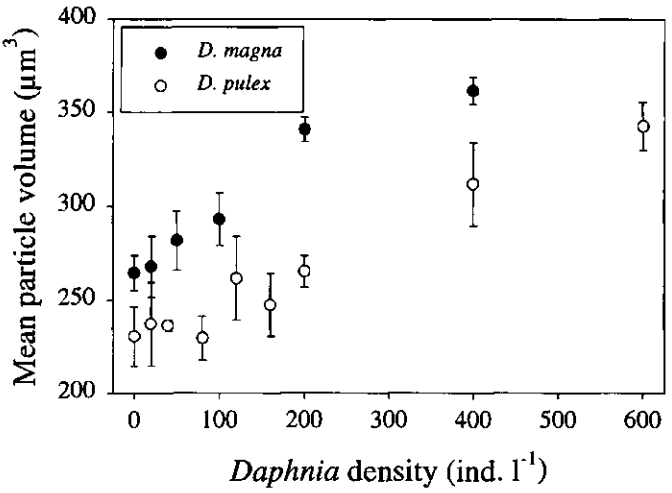


Figure 3.11: Effect of 5 ml water from cultures with different densities of *Daphnia magna* (filled symbols) and *Daphnia pulex* (open symbols) on mean particle volume (μm^3) in *Scenedesmus acutus*. Error bars indicate 1 SD (n = 3).

Starved animals have been shown to induce less colonies than well-fed ones (Lampert *et al.*, 1994). This suggests that the release of colony inducing compounds may be related to the activity of the grazer or more specifically to the amount of algae grazed upon. In a second experiment, the effect of medium from *Daphnia magna* cultures varying in body size and density on the colony formation in *S. acutus* was examined and related to the amount of algae harvested by the animals. Animals belonging to the same cohort of new-born *D. magna* were transferred into separate 100 ml tubes with *S. acutus* suspensions (~7.5 $mgC \cdot l^{-1}$) in WC-medium. After 24 h, the daphnids were removed from the test tubes, measured and placed in new tubes containing fresh food suspensions. The densities used were 0, 20, 50, 100, 200 and 400 daphnids per liter. Initially and after 24h the algal concentrations in the test tubes were measured in the range 3.0 - 20.0 ESD using the Coulter Multisizer II. Algal losses (AL) were calculated for each vessel according:

$$AL = (\text{biovolume}_{\text{control}} - \text{biovolume}_{\text{treatment}}) \times 3.9 \cdot 10^{-7} \text{ (in mg C)}.$$

The medium was then filtered through a glass-fiber filter (GF52) and 5 ml was used as test-water in a biotest with *S. acutus* as test-alga (run in triplicate). After 48 h of incubation, the mean particle volume, as a measure for colony size, was determined.

Colony formation in *S. acutus* significantly increased with *Daphnia* density and with body size (Fig. 3.12). The dose-response of colony formation seemed to reach a plateau at *ca.* 200 *Daphnia*$\cdot l^{-1}$. However, body-size seems more linearly related to colony formation (Figs. 3.12 & 3.14).

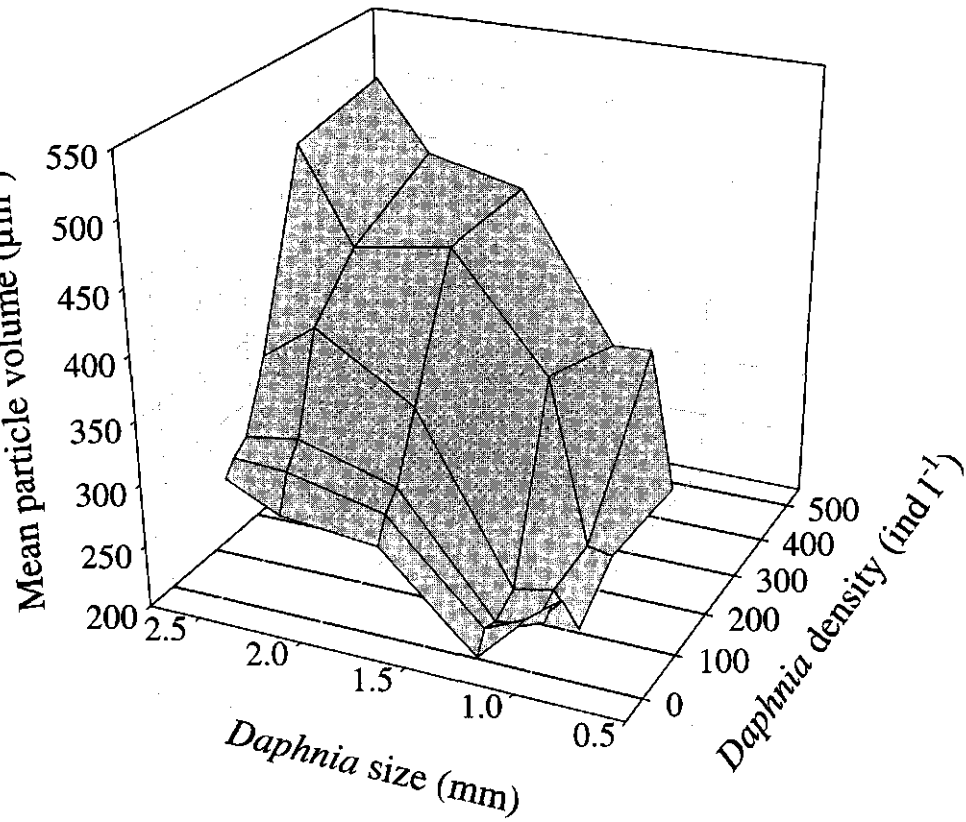


Figure 3.12: Effect of 5 ml water from cultures with different densities and size classes of *Daphnia magna* on mean particle volume (μm^3) in *Scenedesmus acutus*.

In both cases, increase in size or in density results in an increase in animal biomass. Hence, similar effects on colony formation were expected. However, colony formation seems not related to biomass, but to the feeding activity of the animals, as starved animals induced no colonies (Lampert *et al.*, 1994). The amount of algae grazed by the daphnids per vessel (AL, in $mgC \cdot l^{-1}$) was calculated and plotted against the mean particle volumes obtained in the biotests. The colony formation appears significantly correlated with the algal loss (AL) in a positive manner meaning that higher algal grazing loss resulted in more colonies (Fig. 3.13).

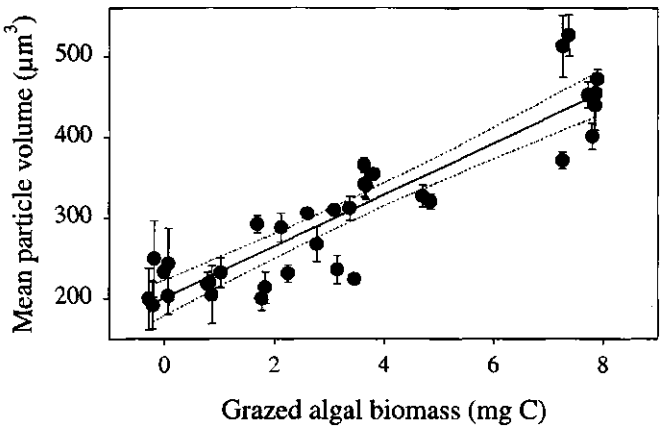


Figure 3.13: Relationship between *Scenedesmus* losses due to grazing and the colony formation in the test-alga *Scenedesmus acutus* induced by chemicals associated with grazing. Solid line represents linear regression (MPV = 200.85 + 31.948 × GAB; $r^2 = 0.826$).

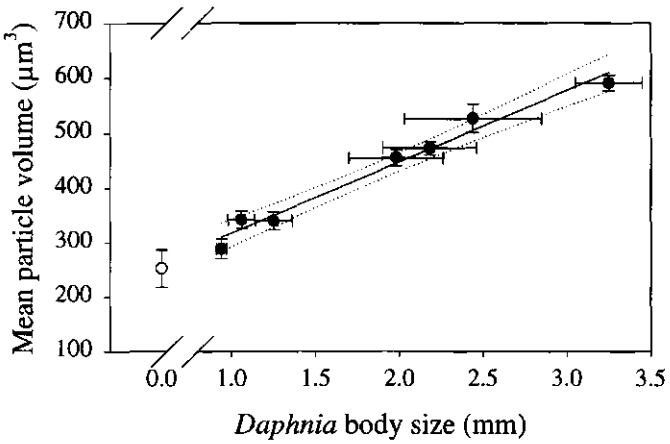


Figure 3.14: Effect of 5 ml water from cultures of *Daphnia magna* with different size classes on mean particle volume (μm^3) in *Scenedesmus acutus*. Error bars indicate 1 SD (n = 3). Solid line represents linear regression MPV = 187.3 +129.9 × BL ($r^2 = 0.976$). Open symbol (o) represents the control that was left out of the regression analysis.

The highly significant linear correlation between the mean particle volume and the body-size of *Daphnia* at a fixed density of 400 animals per liter (Fig. 3.14) suggests that the algae were proportionally grazed to the body-size of the animals. However, the feeding rate (FR) of *Daphnia* can be described by a power function of the body-size (BL) according to the equation (Lampert, 1987): $FR = a \times BL^b$

The exponent of the power function *b* usually lies between 2 and 3, but varies with environmental conditions with food as an important factor (Lampert, 1987). The values of a and

b in the power function were estimated in an experiment with *D. magna* (200 animals per liter) fed with the green alga *S. acutus* and revealed a the power equation $CR = 0.109 \times BL^{2.829}$ (r^2 = 0.992) (own unpublished data).

Reaching a plateau at *ca.* 100-200 *Daphnia*·l^{-1} (Fig. 3.12) may be explained by a production of the inducing chemicals probably not proportional to the *Daphnia* density, instead of reaching the saturation point of the algal physiological response rate. Since the latter would approximate a mean number of cells per colony of 8 rather than the observed 5. The production of colony-inducing chemicals could be limited as a result of depletion of available food at the higher *Daphnia* densities. The amount of food grazed by the differently sized *Daphnia* was plotted against the densities used (Fig. 3.15). This revealed that at 100 *Daphnia* per liter and above an animal size of ~ 1.7 mm all the available food had been grazed upon (food conditions were $1 \cdot 10^7$ µm^3·ml^{-1} for 0.94 to 1.71 mm and $2 \cdot 10^7$µm^3·ml^{-1} for 1.98 to 2.44 mm animals).

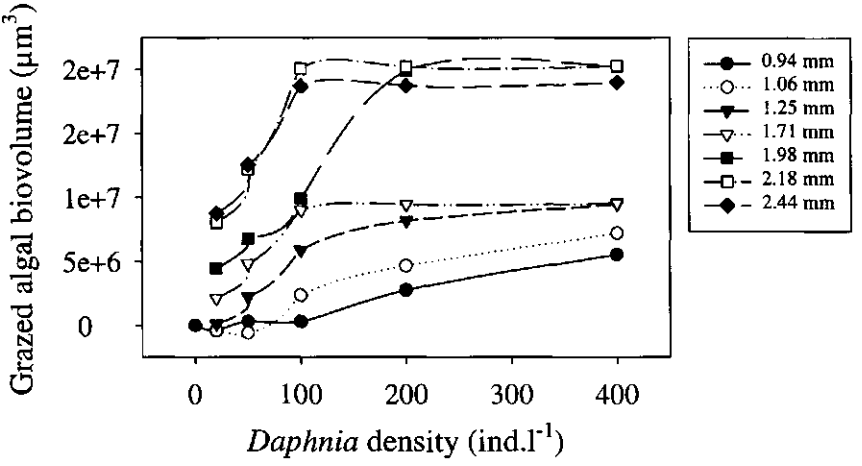


Figure 3.15: Amount of algae grazed by differently sized *Daphnia* at various densities.

3.2.7 Food concentration

Water from incubations with high algal grazing losses induced more colonies in *Scenedesmus* than water with low grazing rates and hence contained more inducing chemicals (see § *3.2.6*). The amount of available food to *Daphnia* may be an important factor affecting the production of colony inducing chemicals. To examine the effect of food concentration on the production of colony inducing chemicals, *D. pulex* (2.28 ± 0.28 mm) was incubated for 24 h at a fixed density of 300 animals·l^{-1} on different amounts of *Scenedesmus* in RT-medium. Food suspensions ranged from 0 to 48.8 mg C· l^{-1} *S. acutus*. After 24 h incubation at 20°C in the dark, water from these cultures was filtered through glass-fiber filters (GF52) and 5 ml filtrate was added to 100 ml Erlenmeyer flasks containing 45 ml *S. acutus* in WC-medium. The batches

were incubated for 48 h on a rotating shaking device (80 rpm.) at 20°C in continuous light of 125 $\mu mol \cdot m^{-2} \cdot s^{-1}$. Colony formation was determined by Coulter analysis and expressed as mean particle volumes (μm^3) (Fig. 3.16). One-way ANOVA of mean particle volumes after 48 h incubation indicated significant differences between treatments ($F_{10,22} = 15.3$; $P < 0.001$). Tukey's test revealed that no colony formation was induced below an initial food concentration of 3 mg $C \cdot l^{-1}$.

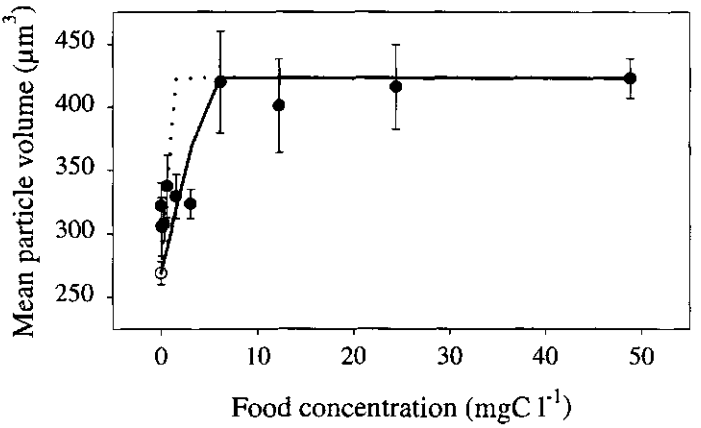


Figure 3.16: Effect of food concentration for 300 *Daphnia pulex* l^{-1} on colony formation in *Scenedesmus acutus*. Error bars indicate 1 SD (n = 3). The solid line represents calculated MPV based on the amount of food grazed, the dotted line when food is inexhaustible (below 7.9 mg C l^{-1}).

The clearance rate (CR) of 300 *D. pulex* per liter around the incipient limiting level (ILL ~0.2 mg $C \cdot l^{-1}$) was determined at 0.67 $ml \cdot ind.^{-1} \cdot h^{-1}$. Below the ILL the CR was considered independent of the food concentration [food], above the ILL the CR decreases according to $1/[food]$. The amount of food processed is considered equal to the amount ingested, the total algal loss (AL) was calculated from AL = CR × [food] × 300 × 24 (in µg C). Using the relation between MPV and algal biomass grazed (Fig. 3.13), but with the control particle volume as y_0 (MPV = 268.8 + 31.948 × AL), the calculated colony formation was in close match with the measured values (Fig. 3.16 *solid line*). The relatively high "threshold-level" was caused by complete removal of all food at low concentrations. Assuming an inexhaustible food pool at these low food concentrations the threshold-level for colony formation drops close to the ILL for this animal (Fig. 3.16 *dotted line*).

A simple model, based on the relationships between body-size and grazing, density and grazing, and between the grazed amount of algae and colony formation (Fig 3.13), was used to calculate mean particle volumes (MPV). The food concentration (7.9 mg $C \cdot l^{-1}$) and animal densities and sizes were similar as in the experiment of *§3.2.6* (Fig. 3.12). Calculated MPV

reached a plateau as a result of complete food depletion (Fig. 3.17). Comparison of calculated and measured MPV at similar densities and animal body sizes revealed that the model explained 75% of the variance (Fig. 3.18).

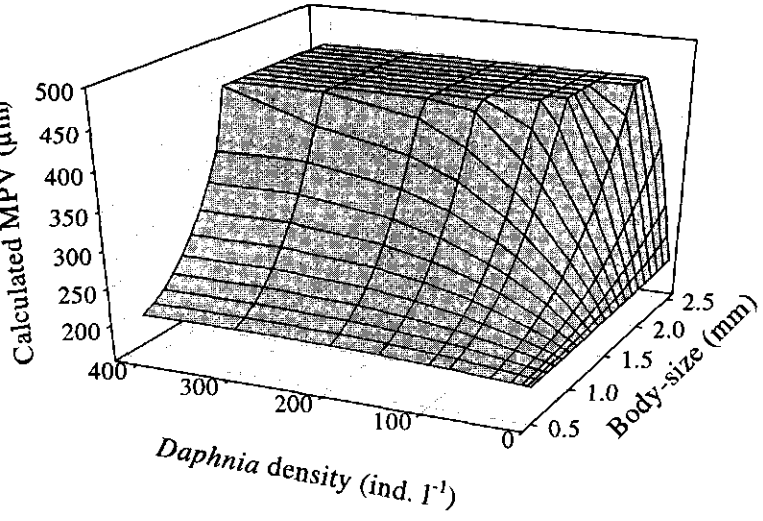


Figure 3.17: Calculated mean particle volumes (MPV, in µm³) in *Scenedesmus* as a function of *Daphnia* body-size (mm) and density (ind.·l⁻¹).

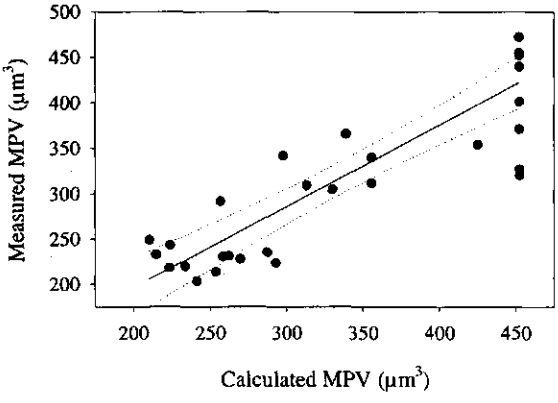


Figure 3.18: Calculated vs. measured mean particle volumes (µm³). Solid line represents linear regression ($r^2 = 0.751$), dotted lines the 95% confidence intervals.

3.2.8 *The biotest*

Experiments with the test-alga *Scenedesmus acutus* to evaluate the effect of various test-waters on growth and morphology will be performed according to the following protocol for a biotest.

THE BIOTEST:

Inocula of the test-alga *Scenedesmus acutus* Meyen will be obtained from a chemostat that is run on slightly modified WC-medium in continuous light of at least 100 $\mu mol \cdot m^{-2} \cdot s^{-1}$ at a constant temperature of 20°C and with a dilution rate of 1.2 day^{-1}. The test-algae will be incubated for 48 hours in 100 ml cellulose-plug stoppered Erlenmeyer flasks containing 50 ml medium with 10% v/v test-water at 20°C on a shaking device (80 rpm) in continuous light (125 $\mu mol \cdot m^{-2} \cdot s^{-1}$). Incubations will be run at least in triplicate with an initial density below $5 \cdot 10^4$ $particles \cdot ml^{-1}$. Test-water should be filtered through glass-fiber filters or through thoroughly rinsed cellulose-nitrate filters. Initially and after 48 hours the algal size distributions and densities will be determined using an electronic particle counter (if necessary the number of cells per colony will be determined using a microscope).

3.3 BIOTESTS

The grazing-associated colony inducing infochemicals may originate from the algae, from the grazers or from both. Infochemicals should be reliable and predictable, and inform the algae about the presence of active grazers (Vet & Dicke, 1992). One possible origin of infochemicals is the animal's digestive system as starved animals produce less inducing chemicals than well-fed *Daphnia* (Lampert *et al.*, 1994). On the other hand, the infochemicals could originate from the algae. During the "sloppy" grazing process, algal cell contents could be released into the environment, although algal homogenates have been reported to be ineffective as colony-inducing agent for *S. acutus* (Lampert *et al.*, 1994).

The observations of possible enhanced growth (*see* Table 2.1) and altered morphology may suggest a role of growth substances, such as auxins (Bradley, 1991; Evans & Trewavas, 1991). In fact, auxins have been found to affect *Scenedesmus* morphology (Nagy-Tóth, 1964; *in* Nagy-Tóth *et al.*, 1992).

3.3.1 *Effect of starvation and food type*

In the first biotest series, the effect of actively feeding and starved *Daphnia* was examined. Twenty well-fed *D. pulex* (G-clone, obtained from the culture collection at the Max-

Planck Institute for Limnology, Plön, Germany) were transferred in 100 ml WC-medium without food. After 24 h the animals were transferred into 100 ml fresh medium, but again without food. This procedure was repeated after 48 and 72 hours. After 72 hours, 10 animals were placed in 50 ml *S. acutus* suspension (10^7 $\mu m^3 \cdot ml^{-1}$; ~4 mg $C \cdot l^{-1}$), while the other 10 animals were placed in a suspension with spherical polystyrene beads (∅ = 15 µm, 10^7 $\mu m^3 \cdot ml^{-1}$). After every 24 hours, medium was filtered and used as test-water in the biotests. As positive control served incubations that had received 5 ml filtered water from a well-fed *D. pulex* culture with a density of 200 animals$\cdot l^{-1}$. Controls were algal suspension without water from a *Daphnia* culture. Controls and treatments were run in quadruplicate.

Medium from *Daphnia* incubations without food did not induce the formation of *Scenedesmus* colonies, neither did medium from a *Daphnia* culture which had been fed polystyrene beads (Table 3.4). Growth rates based on algal biovolumes of *Scenedesmus* populations in standard WC-medium or in medium with water from either starved or well-fed *Daphnia* were similar ($F_{2,33} = 0.19$; $P = 0.827$) and on average 1.70 ± 0.10 day^{-1}.

The mean particle volumes of *Scenedesmus* in treatments with water from *Daphnia* fed polystyrene beads were significantly larger than in the controls without *Daphnia* water. However, no difference with treatments that had received water from an incubation of beads without *Daphnia* was observed. Moreover, the mean particle volume was significantly lower than those of treatments with water from well-fed *Daphnia* cultures. Thus *Daphnia* need to feed actively on digestible food to produce the colony-inducing chemicals.

Table 3.4: Effect of medium from *Daphnia* fed with *Scenedesmus* (Positive control), from starved *Daphnia* (No food) and from animals that have been fed with either algae *(S. acutus)* or polystyrene particles (Beads) after a 72 h starvation period on colony formation in *Scenedesmus acutus* expressed as mean particle volume (μm^3) ± 1 SD (n=4). 'Control' represents algal incubations without the addition of test water.

	24 h	48 h	72 h	96 h
Control	293.6 (17.2)^A	228.7 (32.5)^A	247.3 (28.7)^A	221.0 (5.5)^A
No food	362.6 (13.4)^B	260.0 (24.1)^A	243.8 (14.7)^A	---
Pos. Control	489.0 (19.7)^C	504.8 (43.4)^B	500.5 (18.5)^B	432.7 (30.3)^B
S. acutus	---	---	---	419.0 (40.8)^B
Beads	---	---	---	307.2 (4.5)^C
Beads control	---	---	---	309.4 (21.1)^C
one-way ANOVA	$F_{2,9} = 136.4$ $P < 0.001$	$F_{2,9} = 78.0$ $P < 0.001$	$F_{2,9} = 188.3$ $P < 0.001$	$F_{4,15} = 50.3$ $P < 0.001$

To investigate the effect of algal food-type on production of colony-inducing chemicals, a biotest was performed with medium from *Daphnia* cultures that had been fed with different algae. Equal amounts (i.e. 10^7 $\mu m^3 \cdot ml^{-1}$) of the following algal strains were used as food for *Daphnia*:

- *Scenedesmus acutus* Meyen MPI,
- *Chlamydomonas reinhardtii* Dangeard NIVA-CHL 13,
- *Cyclotella* sp. Kützing NIVA-BAC 8,
- *Rhodomonas lacustris* Javornicky NIVA-8/82 and
- *Microcystis aeruginosa* Kützing strains NIVA-CYA 43, CYA 140 and CYA 228/1.

All algae except *S. acutus* from the Max-Planck Institute for Limnology (Plön, Germany) were obtained from the Norwegian Institute for Water Research (NIVA, Norway). Twenty *D. magna* of the same cohort were incubated without any food for 48 h to empty their guts. Then, they were transferred into 100 ml suspensions of each food type and incubated for 24 h in the dark at 20°C. Medium from each incubation was filtered through a glass-fiber filter (GF52) and used as separate treatments in the biotest, which was run in quadruplicate. The results are presented in figure 3.19.

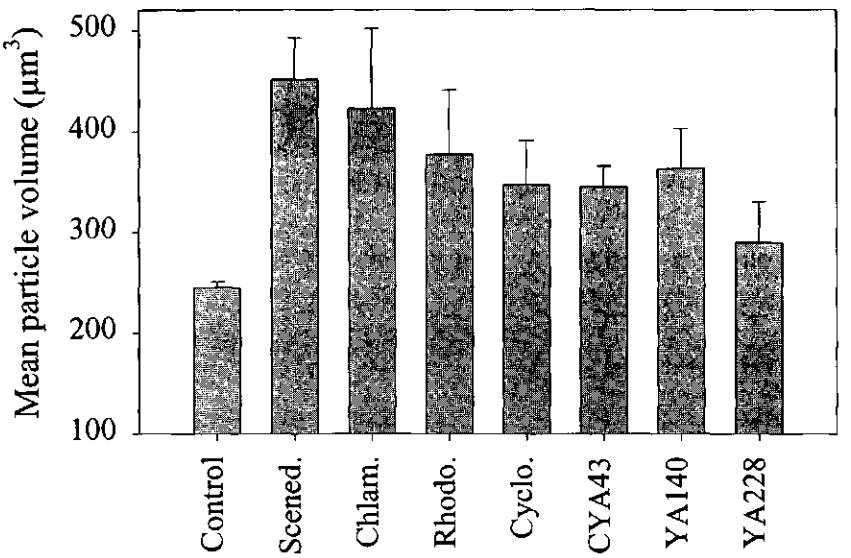


Figure 3.19: Effect of water from *Daphnia* cultures fed different algal food on colony formation in *Scenedesmus acutus* expressed as mean particle volume (μm^3). Error bars indicate 1 SD (n = 4).

Because of non-homogeneous treatment variances, no ANOVA was performed. Nevertheless, figure 3.19 clearly shows that the mean particle volume of all treatments was

enhanced compared to the control. However, when strain CYA 228/1 had been the food, the effect seemed less pronounced. Most likely this was due to the lower feeding activity on this strain (Van der Grinten *et al., in press*). These experiments suggest that *Daphnia* need to feed on digestible food, not strictly on *S. acutus* to induce colonies, whereas the quality of the grazable food algae seems of minor importance (Fig. 3.19).

3.3.2 Algal constituents

The previous experiments indicate that possibly algal constituents may be involved as colony-inducing agents. Therefore, the effect of algal constituents on colony formation in *S. acutus* was examined after exposure to 5 ml test-water from *S. acutus* homogenate (4 mg $C \cdot l^{-1}$) resuspended in WC-medium. In an additional treatment test-algae were exposed to 5 ml from a heat-killed *S. acutus* suspension (4 mg $C \cdot l^{-1}$). Other treatments were run with 5 ml test-water from homogenated *Cryptomonas pyrenoidifera* Geitler NIVA 2/81 (4 mg $C \cdot l^{-1}$) resuspended in WC-medium or with 5 ml from a heat-killed *C. pyrenoidifera* suspension (4 mg $C \cdot l^{-1}$). As a positive control (i.e. a control in which colony formation should occur) served algal cultures that were exposed to 5 ml from a *Daphnia* culture (200 animals per liter) after 24 h of feeding on *S. acutus* (4 mg $C \cdot l^{-1}$). Controls without test-water and all treatments with test-water were incubated in quadruplicates.

In a second biotest the effect of auxins on *S. acutus* was examined. Indole-3-acetic acid (IAA), phenylacetic acid (PAA) and p-chlorophenoxyacetic acid (CPA, all chemicals from Sigma) were dissolved separately in ethanol (analytical grade) to a concentration of 10 $g \cdot l^{-1}$. Of each solution 50 µl was added in triplicate to different 50 ml *S. acutus* suspensions (final concentration of 10 $\mu g \cdot ml^{-1}$). As a positive control 5 ml from a 200 animals per liter *Daphnia* culture with 50 µl ethanol was used.

Filtered medium from heat-killed and homogenated *S. acutus* and *C. pyrenoidifera* suspensions did not induce colony formation in *S. acutus* (Table 3.5). Auxins had little effect on colony formation too. Moreover, growth rates were not affected by the addition of auxins (Table 3.5). Therefore, the colony-inducing chemicals are probably not constituents of the algae themselves. Moreover, *Daphnia* needed to feed to induce colonies in *Scenedesmus*; starved animals induced no colonies, which is in agreement with observations made by Lampert *et al.* (1994) and Van Donk *et al.* (1999). *Daphnia* confronted with less edible algae such as the filamentous cyanobacterium *Oscillatoria*, induced no colonies in *Scenedesmus*, but feeding on edible algae such as *Scenedesmus* or *Microcystis* did induce colonies (Van Donk *et al.*, 1999).

Table 3.5: Effect of medium from heat-killed (Boiled.) and homogenated algae (n = 4) and medium with auxins (n = 3) on colony formation, expressed as mean particle volume (MPV, in μm^3 ± 1 SD), in *S. acutus*, including growth rates (μ, day^{-1} ± 1 SD) of auxin-treatments and *F*- and *P*-values of the one-way ANOVAs. Similar symbols ^{A,B} within a column indicate homogeneous groups that are not significantly different at the 95% level (Tukey's test).

Treatment	MPV (μm^3)	Treatment	MPV (μm^3)	Growth rate (d^{-1})
Control	283.6 (22.0)^{A}	Control	240.8 (14.7)^{A}	1.40 (0.05)^{A}
Pos. control	575.1 (49.3)^{B}	Pos. control	549.2 (55.1)^{B}	1.41 (0.06)^{A}
Sa-homogenate	242.8 (20.1)^{A}	Ethanol	275.0 (7.5)^{A}	1.45 (0.08)^{A}
Sa-boiled	257.5 (23.4)^{A}	IAA	247.4 (7.4)^{A}	1.41 (0.08)^{A}
Cr-homogenate	248.1 (12.9)^{A}	PAA	208.8 (13.0)^{A}	1.45 (0.05)^{A}
Cr-boiled	228.0 (6.7)^{A}	CPA	240.1 (6.9)^{A}	1.41 (0.04)^{A}
one-way ANOVA	$F_{5,18} = 109.3$, $P < 0.001$		$F_{5,12} = 71.1$, $P < 0.001$	$F_{5,12} = 0.43$, $P = 0.820$

These results indicate that the colony inducing substances originate from the food-grazer interaction, i.e. from active grazers feeding on digestible food. The origin of these infochemicals remains, however, unsolved. Bacteria associated with both grazers and algae may play an important part in modifying or production of the infochemicals or precursors of it.

3.3.3 Organic Carbon

The organic carbon concentrations in *Daphnia* water are higher than in standard WC-medium (CHAPTER 2). Organic carbon sources could increase cell dimensions of *S. acutus* (Nagy-Tóth *et al.*, 1992) and hence influence the mean particle volumes. Nagy-Tóth *et al.* (1992) examined the effect of several carbon sources on the morphology of *S. acutus*, but did not report any formation of eight-celled coenobia. They discussed whether the dominance of unicells in the presence of glucose was a peculiarity of their strain or just the result of bubbling. Interestingly, glucose has been reported to induce coenobia in the non-spiny *Scenedesmus obliquus* UTEX 393 (Trainor, 1964). Glucose being the major building block of cellulose could be released from the algal predator *Daphnia,* which is a sloppy feeder that may release 10-17% of the ingested carbon (Lampert, 1978). The colony-inducing chemical is most likely an organic molecule too (Lampert *et al.*, 1994). Therefore the effect of several organic carbon sources on growth and morphology of *S. acutus* was examined. The compounds tested were glucose, fructose, urea, ascorbic acid and citric acid (all at a final concentration of 100 $\mu g \cdot l^{-1}$), while as additional controls two series with either 0.5 or 5 ml

from a *Daphnia* culture were run. The initial cell density was 2.8 (± 0.4)·10^4 particles·ml^{-1} (i.e 4.0 (± 0.6)·10^6 $\mu m^3 \cdot ml^{-1}$). The test was run for 48 h in quadruplicate.

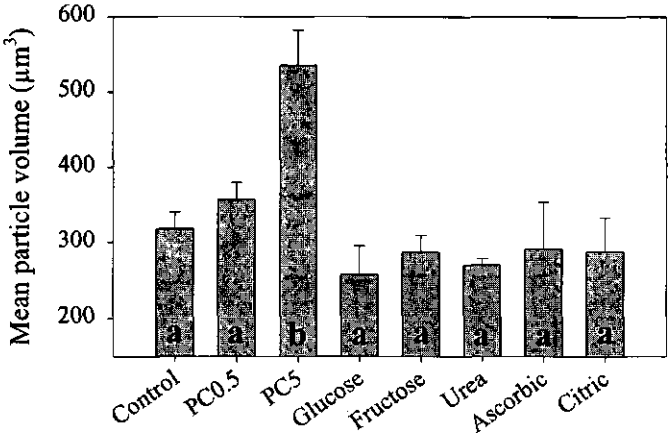


Figure 3.20: Effect of several organic carbon sources added to *Scenedesmus acutus* suspensions in a final concentration of 100 $\mu g \cdot l^{-1}$. PC (=Positive Controls) represent incubations that received either 0.5 or 5 ml from a *Daphnia* culture. Error bars represent 1 SD (n = 4).

The addition of different organic compounds did not result in any formation of eight-celled coenobia (Fig. 3.20), but the addition of medium from a *Daphnia* culture did ($F_{7,24}$ = 23.4; $P < 0.001$).

One major difference between '*Daphnia* water' and the separate organic compounds is that the former contains a mixture of numerous compounds. The negative result with glucose could be explained from the absence of another compound necessary to induce coenobia. It remains, however, fairly impossible to determine what compound or compounds are additionally necessary, but perhaps something energetic (Mur, pers. comment). "*It could also be possible for the Daphnia factor to be a combination of nutrients, both the organic compound which has received attention and inorganic materials which would also be released. It would seem that the supplies of nitrogen and phosphorus are sufficient with or without Daphnia, but the latter could be providing a specific nutrient in a different form, e.g. ammonium rather than nitrate*" (Trainor, 1998). Gons (1977) demonstrated the importance of the redox-state of the N-source, due to the high N-content of *Scenedesmus* cells up to almost 11% of the dry-weight. Moreover, the efficiency for conversion of absorbed energy into biomass is influenced by the nature of the N-source, and is higher with urea and ammonium (~0.27) than with nitrate (~0.19-0.23) (Gons & Mur, 1975; Gons, 1977).

Therefore, a biotest was performed to analyse more detailed the possible role of ammonium, urea and glucose on growth and morphology in *S. acutus*. The biotest was run in quadruplicate with an initial density of 2·10^4 particles·ml^{-1} according the scheme:

Control: standard WC medium (control)

I. standard WC medium + 0.5 mg·l^{-1} NH_4Cl + 0.5 mg·l^{-1} Urea

II. standard WC medium + 0.5 mg·l^{-1} NH_4Cl + 0.5 mg·l^{-1} Glucose

III. standard WC medium + 0.5 mg·l^{-1} Urea + 0.5 mg·l^{-1} Glucose

IV. WC with NH_4Cl as N-source (14.0 mg N·l^{-1})

V. WC with Urea as N-source (14.0 mg N·l^{-1})

VI. WC with 1:1 Urea and NH_4Cl (14.0 mg N·l^{-1})

VII. WC with NH_4Cl as N-source + 0.5 mg·l^{-1} Glucose

Again no effect of glucose or of different N-sources on growth and morphology of *S. acutus* was detected (Fig. 3.21).

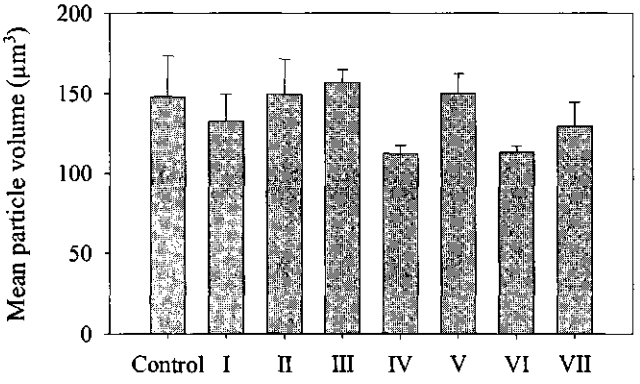


Figure 3.21: Effect of different N-sources and glucose on the mean particle volume (µm^3) in *Scenedesmus acutus*. Error bars represent 1 SD (n = 4). For explanation of I-VII see text.

S. acutus exhibited excellent growth and growth rates were identical regardless the addition of N, the redox-state of N or the presence of glucose ($F_{7,24}$ = 1.25; P = 0.315). Although one-way ANOVA on the mean particle volumes indicated significant differences ($F_{7,24}$ = 4.98; P = 0.001), Tukey's test revealed that this was caused by lower MPV in two treatments. In no treatments the MPV was significantly larger than in control populations and qualitative microscopic analysis revealed unicell dominance in all cultures. Hence, neither glucose nor ammonia and urea seem involved in the formation of colonies in *S. acutus*.

3.3.3 Surface active compounds: a key to identification of the colony inducing chemicals?

The interesting phenomenon of colony formation in cultures with extractables from cellulose-nitrate filters was further examined. Glucose, being the major building block of cellulose, appeared ineffective as a colony-inducing chemical (see § *3.2.4* and *3.3.3*). The effect of cellulose (Sigma, S-3504) and pectin (Sigma, P-8471) was investigated in a biotest in

which the chemicals were added separately in concentrations of 1 $mg \cdot l^{-1}$ to *S. acutus* suspensions. As positive control served incubations with filtered water from a well-fed *Daphnia* culture. However, these compounds could not induce colony formation in *S. acutus* (Table 3.6).

Table 3.6: Effect of cellulose and pectin on mean particle volume (in μm^3 ± 1 SD; n = 4) of *Scenedesmus acutus*. One-way ANOVA: $F_{3,12} = 70.4$; $P < 0.001$.

Treatment	Mean particle volume
Control	318.9 (36.4) A
Daphnia water	561.9 (13.2) B
Cellulose	276.6 (21.6) A
Pectin	277.0 (48.0) A

Apparently, other chemicals are released from the filters. More detailed information about the chemical composition of the used membrane filters was supplied by employees of the producing company (Schleicher & Schuell). Extractable compounds may reduce the filter dry-weight by 1.5% (pers. comm. Ir. S.P. Verboon, Schleicher & Schuell Nederland BV). Besides cellulose, also ethylacetate, methylketones, and detergents are present in the filters. More detailed information about the latter group was not delivered because they determine the pore-width of the filters. Nevertheless, based on this information a biotest could be performed in which 11 substances were tested of which substances V to XI represent commercially available detergents. Of each chemical, listed below, 10 µl was added to 50 ml *S. acutus* suspensions. As controls served incubations that received no test water, whereas positive controls had received 5 ml filtered medium (GF52) from a *Daphnia* culture. The biotest was run in triplicate.

The chemicals tested are:

I. Ethylacetate
II. 2-butanon
III. Di-isopropylketone
IV. Iso-butylmethylketone
V. FFD6
VI. Epos
VII. MSD
VIII. Brei 35
IX. Laurylsulphate
X. Na-dodecyl-sulphate
XI. Etran

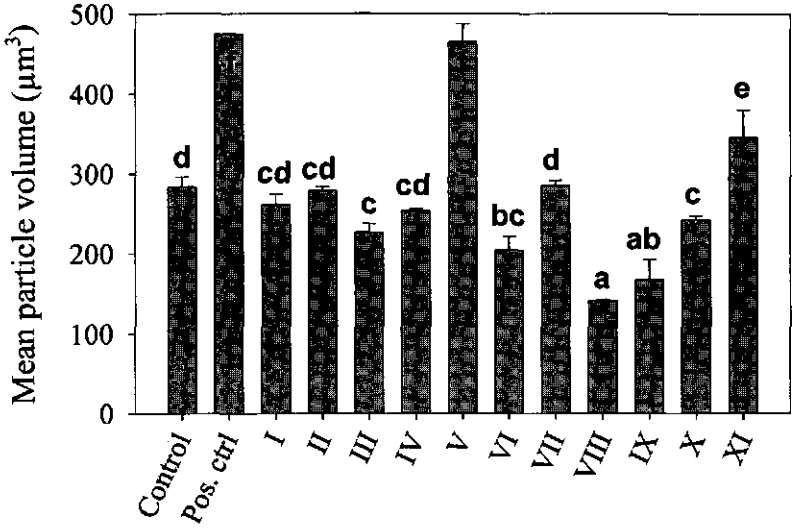


Figure 3.22: Effect of potential membrane-filter extractables (I-XI; for explanation see text) on mean particle volume (μm^3) of *Scenedesmus acutus*. 'Pos. ctrl. ' represents a treatment with water (10% v/v) from a *Daphnia* culture. Error bars indicate 1 SD (n = 3).

One-way ANOVA indicated significant differences ($F_{12,26} = 126.2$; $P < 0.001$) among the mean particle volumes of *S. acutus* populations after exposure to several potential membrane-filter extractables (Fig. 3.22). Tukey's test revealed that substances V was not different from the positive control (i.e. incubations with water (10%v/v) from a *Daphnia* culture in which colony formation was expected). Microscopic analysis showed colony formation in both the positive control and chemical 'V', with mean number of cells per colony (± 1 SD, n = 3) of 3.56 (0.12) and 4.08 (0.17), respectively. No colony formation was observed in the controls with 1.31 (0.19) cells per aggregate. The chemical 'V' was FFD6, which is a detergent supplied by SKALAR that consists of water and of 45-47% of Sodiumdodecyl difenyl-oxide disulfonates. The latter substance is highly artificial and will certainly not be exuded by *Daphnia*. Moreover, this detergent will probably contain several by-products (Dr. T. Van Beek, Dept. Organic Chemistry, Univ. Wageningen, pers. comm.).

Also chemical 'XI', sodium-dodecyl-sulphate, differed significantly from control populations. Both chemicals were examined further in an additional experiment, whereby both chemicals were added at different concentrations:

FFD6 in 0, 0.02, 0.1, 0.2 and 1.0 $\mu l \cdot ml^{-1}$

Na-dodecyl-sulphate in 0, 5, 10, 50 and 100 $\mu g \cdot ml^{-1}$

After 48 h, *Scenedesmus* populations exposed to the highest concentration Na-dodecyl-sulphate were aggregated in enormous aggregates consisting of hundreds of cells. These particles were by far too large to be measured with an electronic particle counter. Hence, the

mean particle volume determined with the Coulter Multisizer II appeared not significantly different from the controls (Fig. 3.23; Panel A).

The number of cells per colony was significantly enhanced, but at the highest concentrations counts were impossible (Fig. 3.23; Panel B). The large multicelled aggregates resulted in underestimation of the algal biovolume and thus in lower volume based growth rates. Growth rates (± 1SD) at the highest concentration were 1.15 (0.03) d^{-1}, whereas the mean growth rates at the other concentrations was 1.75 (0.05) d^{-1}. Volume based growth rates of *S. acutus* exposed to different concentrations of FFD6 were similar with a mean (± 1SD) of 1.71 (0.06) d^{-1}. Both the mean particle volume (Fig. 3.23; Panel C) and the number of cells per colony (Fig. 3.23; Panel D) were significantly larger when *S. acutus* was exposed to FFD6. Both chemicals significantly promoted colony formation in *S. acutus* MPI. One similarity is that both substances contain similar groups, i.e a Na-dodecyl-group.

The previous experiment revealed that concentrations of 0.2 $\mu l \cdot ml^{-1}$ and 10 $\mu g \cdot ml^{-1}$ for FFD-6 and Na-dodecyl-sulphate, respectively, were sufficient to induce colonies in *S. acutus* MPI. Therefore, these concentrations were chosen to examine the effect on three strains of the spined *S. subspicatus*.

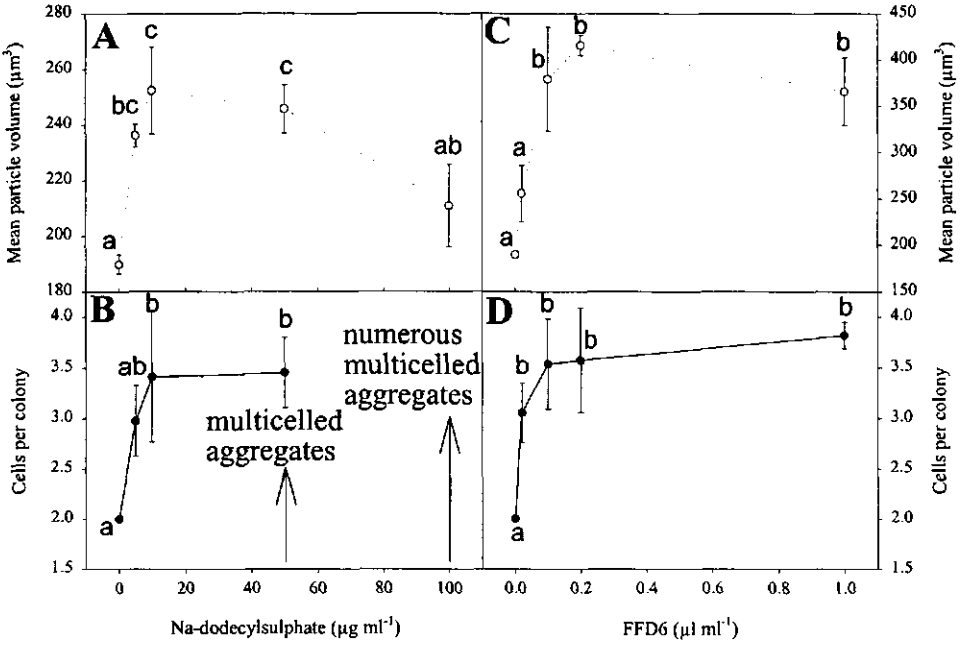


Figure 3.23: Mean particle volume (μm^3) and mean number of cells per colony of *Scenedesmus acutus* exposed for 48 h to different concentrations of detergents (Na-dodecyl-sulphate, Panels A&B; FFD6, Panels C&D). Error bars represent 1 SD (n = 3). Similar symbols a...c indicate homogenous groups that are not significantly different (Tukey's test; $P = 0.05$).

The strain *S. subspicatus* NIVA-CHL 55 was obtained from the Norwegian Institute for Water Research (NIVA), strain RWTH was provided by Dr. Brigitte Goser at the University of Aachen (Germany), whereas strain UTEX 2594 was obtained from the University of Texas Culture Collection. The different strains were cultured for 48 h in triplicate in standard WC medium (controls), in medium with 5 ml from a *Daphnia pulex* culture and in medium with either FFD-6 or Na-dodecyl-sulphate (treatments). The initial algal density was $2.5 \cdot 10^4$ particles·ml^{-1}. As algal control served incubations with *S. acutus* MPI.

In control series with *S. acutus* MPI, colony formation was induced by all three treatments, but not in the controls in standard WC medium (Fig. 3.24). By contrast, in the spined *S. subspicatus* strains, colony formation was only induced by Na-dodecyl-sulphate (Fig. 3.24).

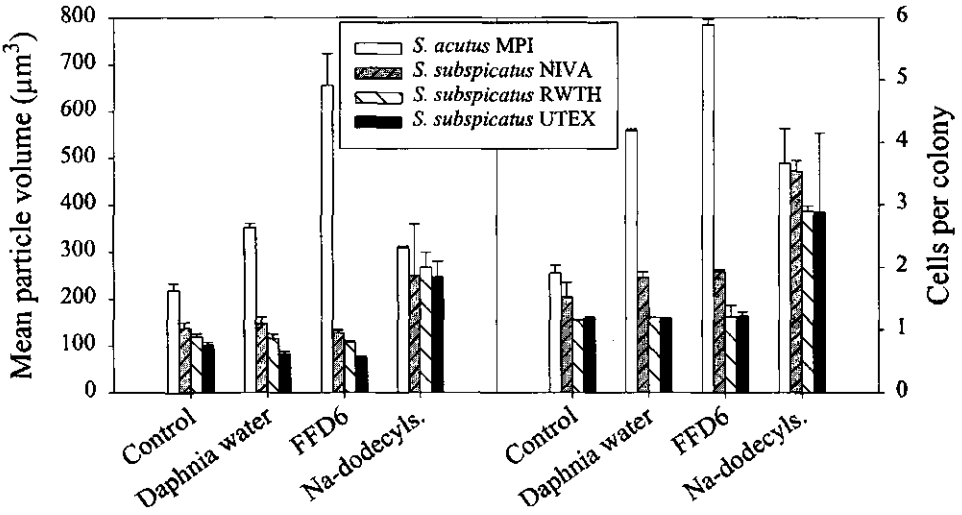


Figure 3.24: Colony formation, expressed as mean particle volumes (left panel) and mean number of cells per colony (right panel) in the non-spiny *Scenedesmus acutus* and three strains of the spined *Scenedesmus subspicatus* after 48 h incubation with either filtered medium from a *Daphnia* culture or in the presence of detergents FFD6 (0.2 µl·ml^{-1}) or Na-dodecylsulphate (10 µg·ml^{-1}). Error bars indicate 1 SD (n = 3).

Another interesting surface active compound broadly used to keep *Daphnia* out of the surface film is 1-hexadecanol ($CH_3(CH_2)_{14}CH_2OH$) or cetyl alcohol. In a recent paper, high levels of cetyl alcohol decreased survival and reproduction in *Daphnia*, but the mechanism remained unclear (Desmarais, 1997). As at high concentration cetyl alcohol will not only appear at the surface, its strong surfactancy may affect the available algal food by clogging it into aggregates. The effect of cetyl alcohol on *Scenedesmus* was examined at a low dose of

100 µg per 50 ml algal suspension and at a high dose of 1 mg per 50 ml. However, the latter concentration contained too much cetyl alcohol particles for electronic particle counting.

After 48 h of incubation, *Scenedesmus* had formed colonies in incubations that had received water from a *Daphnia* culture, and in both the low and high dose cetyl alcohol treatments (Table 3.7). In the presence of 1 mg cetyl alcohol, numerous large aggregates were observed. About 10% of the populations consisted of aggregates with more than 8 cells, compared to only 2% in the *Daphnia* water treatments.

Table 3.7: Effect of cetyl alcohol (100 µg and 1 mg added to 50 ml) on the mean number of cells per aggregate (± 1 SD; n = 4) and mean particle volumes (μm^3; ± 1 SD; n = 4).

Sample	Cells per colony	Mean particle volume
Control	1.38 (0.17) ^{A}	168.5 (18.1) ^{A}
Daphnia water	2.82 (0.21) ^{B}	301.8 (15.4) ^{B}
100 µg cetyl alcohol	2.22 (0.60) ^{AB}	223.8 (25.5) ^{C}
1 mg cetyl alcohol	3.03 (0.54) ^{B}	ND
F- and *P*-values	$F_{3,12} = 12.1$; $P = 0.001$	$F_{2,9} = 44.3$; $P < 0.001$

Although cetyl alcohol may clog algae together and thereby affect the food availability to *Daphnia*, this appears to occur only at relatively high doses. Filtering the medium from a *Daphnia* culture with cetyl alcohol prior to experimentation will remove most if not all cetyl alcohol. Moreover, no cetyl alcohol has been added to *Daphnia* cultures used for the studies presented in this thesis. Therefore, *Daphnia*-induced colony formation is not the result of cetyl alcohol.

Resuming, the discovery of artificial colony-inducing compounds may provide organic chemists with a useful clue to search more orientated for active groups and to solve piece by piece the *Daphnia*-infochemical puzzle.

3.4 ZOOPLANKTON AND COLONY FORMATION IN *S. ACUTUS*

In the previous sections the effect of medium from *Daphnia* cultures on colony formation in *Scenedesmus acutus* was examined. In this section medium from different herbivorous zooplankton such as *Ceriodaphnia* (§3.4.1), *Simocephalus* (§3.4.2), *Spirostomum* (§3.4.3), and carnivorous zooplankton such as *Bythotrephes* and *Leptodora* (§3.4.4) was added to the cultures of *Scenedesmus*. The main objective was to examine the effect on *Scenedesmus* morphology in order to reveal whether the response is restricted to *Daphnia*,

whether it is a general herbivore effect or a zooplankton effect including carnivorous species. Also included in this section is a biotest with medium from a fish culture (§3.4.5).

3.4.1 Ceriodaphnia reticulata

The relatively small cladoceran *Ceriodaphnia* (± 0.7 mm) was incubated for 24 h in RT-medium with equal amounts of algae (*S. acutus* 10^7 $\mu m^3 \cdot ml^{-1}$) at densities of 0, 40, 100, 200, 400, 800 and 1600 animals per liter. After 24 h the algal disappearance rates were determined from decrease in algal volumes, followed by filtration of the medium that was used as test water in a biotest. Colony formation after 48 h incubation, expressed as mean particle volumes (in μm^3), was significantly promoted in incubations that had received medium from *Ceriodaphnia* incubations ($F_{7,16} = 58.6$; $P < 0.001$). Tukey's test revealed three homogeneous groups: 'Control, 0, 40, 100'; '200, 400' and '800, 1600' animals per liter. The mean particle volume reached a plateau of ~500 μm^3 at incubation densities above 800 *Ceriodaphnia* per liter (Fig. 3.25). This could imply that the physiological response maximum of the algae was reached. The algae at the highest *Ceriodaphnia* densities were reduced to 38% of the initial concentration hence overexploitation of the algal resource seemed not the explanation.

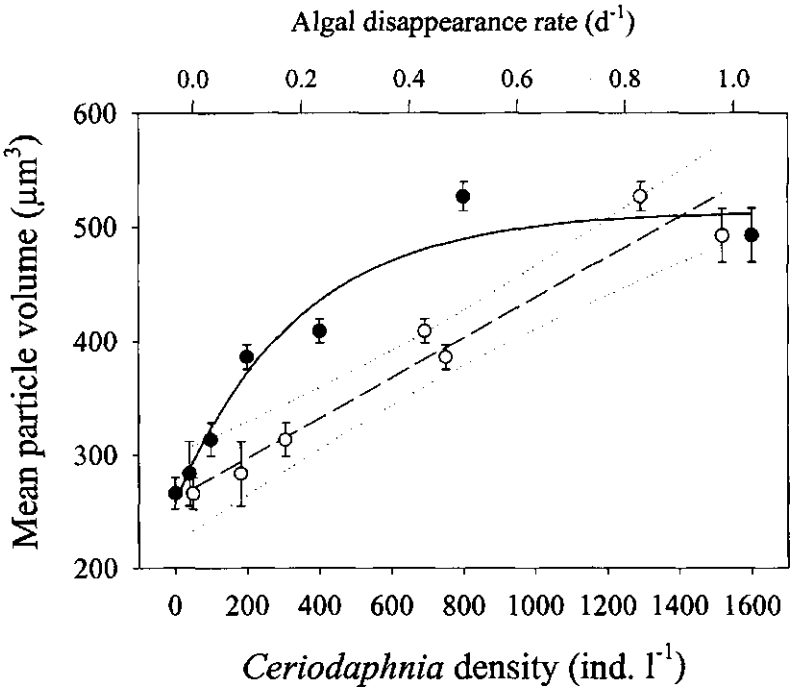


Figure 3.25: Colony formation in *Scenedesmus acutus*, expressed as mean particle volumes (μm^3), as a function of *Ceriodaphnia reticulata* incubation density (●) and algal loss due to grazing (O). The dashed line represents linear regression, the dotted lines the 95% confidence intervals.

Crowding effects in the incubations could also account (partly) for the flattening as touch and interference at high densities may affect the grazing activity. As a result of crowding, the clearance rates were depressed at high animal densities. Clearance rates were 92 $\mu l \cdot animal^{-1} \cdot h^{-1}$ at 40 *Ceriodaphnia* per liter but had dropped to 25 $\mu l \cdot animal^{-1} \cdot h^{-1}$ at 1600 *Ceriodaphnia* per liter. The grazing loss of the algae (DR) was significantly correlated with the observed colony formation (MPV) according the linear relationship: MPV = 270.2 + 265.0 * DR ($r^2 = 0.942$) (Fig. 3.25).

3.4.2 Simocephalus vetulus

The cladoceran *Simocephalus* (± 2.1 mm) was incubated for 24 h in RT-medium with equal amounts of algae (*S. acutus* 10^7 $\mu m^3 \cdot ml^{-1}$) at densities of 0, 20, 50, 100, 200, 400, and 800 animals per litre. One-way ANOVA on both the number of cells per colony ($F_{8,27} = 48.7$; $P < 0.001$) and the mean particle volumes ($F_{8,27} = 29.7$; $P < 0.001$) indicated significant differences. Tukey's test revealed that medium from the *Simocephalus* incubations triggered colony formation in *Scenedesmus* (Table 3.8).

Table 3.8: Effect of medium from *Simocephalus vetulus* cultures varying in density on colony formation in *Scenedesmus acutus*, expressed as mean particle volumes (MPV) and number of cells per colony (means ± 1 SD; n = 4). Similar symbols $^{A...E}$ within a column indicate homogeneous groups (Tukey's test; $P = 0.05$).

Sample	MPV (μm^3 ± 1 SD)	Cells per colony (± 1 SD)
Control	196.1 (19.3) ^{A}	1.40 (0.09) ^{A}
Daphnia water	333.2 (18.1) ^{D}	3.32 (0.25) ^{E}
0 *S. vetulus* l^{-1}	219.1 (26.8) $^{A\ B}$	1.70 (0.21) ^{B}
20 *S. vetulus* l^{-1}	254.8 (13.2) ^{BC}	2.18 (0.16) ^{C}
50 *S. vetulus* l^{-1}	265.4 (4.0) ^{C}	2.52 (0.12) ^{D}
100 *S. vetulus* l^{-1}	268.7 (12.1) ^{C}	2.12 (0.15) ^{C}
200 *S. vetulus* l^{-1}	241.9 (18.5) ^{B}	2.03 (0.21) ^{C}
400 *S. vetulus* l^{-1}	276.6 (16.8) ^{C}	2.62 (0.28) ^{D}
800 *S. vetulus* l^{-1}	347.5 (22.4) ^{D}	3.52 (0.25) ^{E}

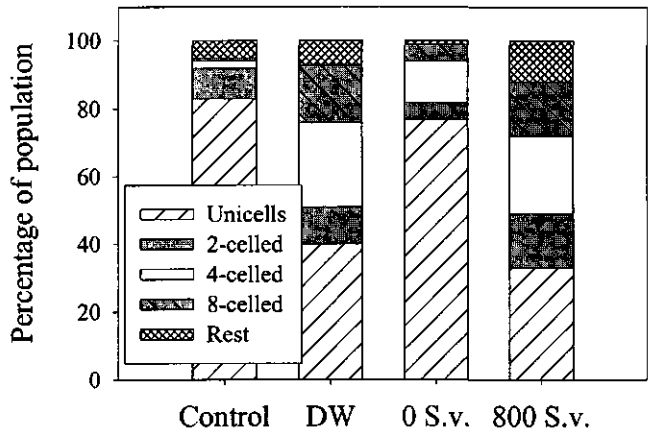


Figure 3.26: Percentage of unicells, 2-, 4-, and 8-celled coenobia in *Scenedesmus acutus* populations in the absence (Control & 0 S.v.) and presence of medium from *Daphnia* (DW) and *Simocephalus* (S.v.) cultures.

3.4.3 Spirostomum ambiguum

The freshwater ciliate *Spirostomum ambiguum* was transferred from a concentrated culture into WC-medium with *S. acutus* as food (10^7 $\mu m^3 \cdot ml^{-1}$) at densities of 0, 250, 1000, 2000 and 5000 per litre. After an incubation period of 4 days at 20°C in the very low light, the medium was filtered and used as test water in a biotest. As positive control served medium from an incubation of 100 *D. magna* per liter (~1.7 mm).

One-way ANOVA indicated significant differences ($F_{6,14} = 92.9$; $P < 0.001$), however, Tukey test revealed that this was due to the positive control with *Daphnia* water (Table 3.9). Medium from the *Spirostomum* incubations had no effect on the mean particle volumes of *Scenedesmus* cultures.

Inspection of *Spirostomum* revealed the appearance of green particles inside the animals indicating the ciliates were feeding on the algae.

3.4.4 Bythotrephes and *Leptodora*

Both cladocerans are carnivorous species, primarily feeding on small zooplankton. The animals were collected from Petrusplaat water-reservoir (The Netherlands) and transferred into RT-medium with *Ceriodaphnia* as food. Based on the experiment with *Ceriodaphnia* (§ 3.4.1) no *Scenedesmus* was added to prevent an effect of this species. Moreover, to all incubations, including the one without carnivorous zooplankter, 50 *Ceriodaphnia* were added. *Bythotrephes longimanus* was incubated at densities of 0, 50, 100, 200 and 500 per liter, *Leptodora kindtii* at a density of 100 animals per liter. After 24 h incubation in the dark at 15°C the medium was filtered and used as test water in a biotest. An additional treatment was

run with filtered water from the Petrusplaat since the reservoir contained also *Daphnia*. As positive control served incubations with medium from a laboratory *Daphnia* culture, as negative control for the latter incubations with medium from incubations of algae without *Daphnia*.

Table 3.9: Effect of medium from cultures of the freshwater ciliate *Spirostomum ambiguum* (*Sp.*), the carnivorous cladocerans *Bythotrephes longimanus* (*By.*) and *Leptodora kindtii* (*Le.*), and the fish *Leuciscus idus* (Fish) on the mean particle volumes (MPV ± 1SD, n= 3, in μm^3) of *Scenedesmus acutus*. GF represents additional control with filtered medium, Dno-*S.a.* = control with medium from algal culture without zooplankton, *Daphnia* water = positive control with medium from a *Daphnia* culture, Petrusplaat = treatment with water from the Petrusplaat. Similar symbols ($^{A...E}$) indicate homogeneous groups (Tukey's test).

Spirostomum ambiguum		*Bythotrephes & Leptodora*	
Sample	MPV	Sample	MPV
Control	203.9 (3.2) ^{A}	Control	283.7 (9.7) $^{A\,D}$
Daphnia water	278.7 (6.9) ^{B}	Control-GF	261.7 (29.3) $^{A\,C}$
0 *Sp.* l^{-1}	200.1 (2.4) ^{A}	Dno-*S.a.*	244.4 (12.7) $^{A\,C}$
250 *Sp.* l^{-1}	192.0 (11.2) ^{A}	0 *By.* l^{-1}	265.9 (37.8) $^{A\,C\,D}$
1000 *Sp.* l^{-1}	195.4 (4.3) ^{A}	50 *By.* l^{-1}	208.8 (6.2) ^{B}
2000 *Sp.* l^{-1}	189.1 (5.2) ^{A}	100 *By.* l^{-1}	229.7 (14.9) $^{B\,C}$
5000 *Sp.* l^{-1}	187.0 (1.9) ^{A}	200 *By.* l^{-1}	198.3 (14.7) ^{B}
Leuciscus idus		500 *By.* l^{-1}	191.2 (4.2) ^{B}
Control	221.1 (23.4) ^{A}	100 *Le.* l^{-1}	242.2 (6.2) $^{A\,C}$
Fish	186.3 (16.9) ^{A}	Petrusplaat	316.3 (11.3) ^{D}
Daphnia water	429.6 (67.6) ^{B}	*Daphnia* water	403.6 (12.0) ^{E}

The one-way ANOVA indicate significant differences in mean particle volumes among treatments ($F_{10,22} = 36.5$; $P < 0.001$). Colony formation was significantly promoted in the *Daphnia* water treatment (positive control), but not in the other treatments. Mean particle volumes of *Scenedesmus* exposed to medium from *Bythotrephes* incubations appeared significantly smaller than in the controls (Table 3.9).

3.4.5 *Leuciscus idus*

A freshwater fish (*Leuciscus idus*) was well fed with *Daphnia* prior to transfer into 10 liter WC-medium. After 24 h incubation without food the medium was filtered and used as test water in a biotest. Treatments with medium from a culture of 200 *Daphnia magna* per litre (3.0 ± 0.2 mm) served as positive control. Medium from the fish incubation induced no colonies in *Scenedesmus* (Table 3.9), but the positive control did ($F_{2,9} = 38.4$; $P < 0.001$).

3.5 DISCUSSION

The morphological response of *Scenedesmus acutus* to zooplankton mediated chemicals is related to the amount of algae grazed upon. The type of food seems unimportant as long as it is digestible. Animals fed with ingestible, but indigestible particles, produced no colony-inducing chemicals, neither did starved animals. *Scenedesmus* did not respond to algal homogenates, thus the infochemical originates in the grazer, but most probably as a residual of the digestive process. Moreover, water that had contained carnivorous zooplankton evoked no colony formation. The differences seem directly related to the grazers diet, which, in fact, is not uncommon in the aquatic world. Crucian carp increased its body depth in response to predators (Pike, Perch) with a piscivorous diet. By contrast, perch fed chironomids had no effect (Brönmark & Petterson, 1994). Fathead minnows showed only a fright reaction to pike that had been fed with minnows, but not to pike fed with swordtails (Mathis & Smith, 1993). Also snails (Crowl & Covich, 1990) and sea anemones (Howe & Harris, 1978) showed different responses related to the predators diet. Identification of predators per se could be more advantageous than having to identify all predator species (Brönmark & Petterson, 1994). Thus a response to a general herbivore cue should be adaptive in habitats with variable grazing pressure from a zooplankton assemblage with various herbivores.

CHAPTER 4

Nutrient-status and colony formation in *Scenedesmus acutus*

Parts of this chapter are based on:
Lürling, M. & Van Donk, E. *submitted to Freshwater Biology*

"The integrative nature of the cellular physiology of phytoplankton is a clear indication that the organisms have the ability to track the changing external environment and to make adaptive changes which enhance their chances of survival"

- G.P. Harris 1986

4.1 INTRODUCTION

Several factors may influence the ecomorph expression in *Scenedesmus* species. *Scenedesmus* strains are known to produce unicells and coenobia (e.g. Chodat, 1926; Uherkovich, 1966; Trainor, 1998). In nature, the non-spiny *Scenedesmus acutus* mostly exists as coenobia and eight-celled colonies may be common (Krienitz, 1987). However, in most laboratory cultures, *S. acutus* exists as unicells, although cultures dominated by four-celled coenobia may also occur. Formation of either unicells or coenobia may depend on factors such as length of photoperiod (Steenbergen, 1975), pH (Trainor & Roskosky, 1967), temperature (Trainor, 1992a,b; 1993), nutrients (Ramos-Cárdenas & de Lara-Isassi, 1985; Holtmann & Hegewald, 1986), age of the culture, initial cell density and the presence of a grazer may be involved (Egan & Trainor, 1989; Hessen & Van Donk, 1993; Lampert *et al.*, 1994).

The number of cells per colony in *Scenedesmus* is also related to the amount of energy stored in the parent cell (Šetlík *et al.*, 1972) and may be directly proportional to growth rates (Siver & Freeda, 1982). Gavis *et al.* (1979) observed high proportions of eight-celled *S. quadricauda* at growth rates of 0.9 – 1.0 day^{-1}, but not below 0.6 day^{-1}. They used nitrate-limited cultures, in which at strong N-limitation cultures were dominated by four-celled coenobia. Eight-celled coenobia appeared at moderate limitations (Gavis *et al.*, 1979). Siver & Trainor (1981) showed that control of the unicellular stage in *Scenedesmus* was complex, but proposed that nitrogen, as ammonium, was the main factor involved. They did not report any growth rates, but observed high proportions of unicells at high light intensities and temperatures and thus presumably at higher growth rates. In the non-spiny *S. acutus*, at high growth rates the cultures were either dominated by unicells in the absence or by four- and eight-celled coenobia in the presence of water from a *Daphnia* culture (Lampert *et al.*, 1994; Lürling, 1998). The mean number of cells per colony (± 1 SD) in *S. acutus* cultured under various nutrient conditions appeared 1.43 ± 0.22 (Sterner & Smith, 1993; Lampert *et al.*, 1994; Lürling & Van Donk, 1996; 1997a;b).

Most culture media contain relatively high amounts of nitrogen and phosphorus compared to natural waters. In most natural waters the type of nutrient limitation likely to occur is N- or P-limitation, that may exhibit a high spatial and temporal variability (Butler *et al.*, 1989). Nutrient limitation is not a rare phenomenon restricted to oligotrophic waterbodies, but may even occur around algal blooms in eutrophic lakes. For example, both P-limitation and N-limitation have been observed in hypertrophic lakes (Sommer, 1989; Van Donk *et al.*, 1993). Thus, in freshwater pelagic systems algae may not only experience variations in abundance of grazers, but also nutrient changes (Sommer *et al.*, 1986). High grazing on small edible algae could favor larger algae that have a size-refuge from grazing (Sommer *et al.*, 1986) by the regeneration of nutrients (Lehman, 1980; Sterner, 1989) thereby influencing competition among algae (Elser *et al.*, 1988). Also nutrient conditions (Sommer, 1988) and

fluctuations (Harrison & Turpin, 1982) may affect phytoplankton succession. These fluctuations may not only influence the community structure of the phytoplankton, but also the chemical composition and morphology of the algae present, which may influence the ingestibility and digestibility of algal cells and, hence, may affect zooplankton growth (Van Donk *et al.*, 1997).

Holtmann & Hegewald (1986) did not observe formation of eight-celled coenobia in *S. pectinatus* in the laboratory in five different media, but found the same alga in the field occurring with over 50% in this typical eight-celled morph. Moreover, cell dimensions were considerably larger in *S. pectinatus* from the field. The mean cell length and width in the laboratory were 17.7 and 3.7 µm, but in the field they were 30.3 and 4.6 µm, respectively (Holtmann & Hegewald, 1986). Since bulk elements, such as carbon, vary little with growth conditions (Goldman & McCarthy, 1979) and may make up about 54% in *Scenedesmus* (Sterner, 1993), cells of *S. pectinatus* in the field could have contained around 30% more carbon than in the laboratory.

When growing in a water-body or laboratory cultures, *Scenedesmus* cells will compete for several resources, including carbon, nitrogen, phosphorus and light. Most laboratory media, however, are low in carbon content, such as Z8 (Skulberg & Skulberg, 1990; Hessen & Van Donk, 1993), Chu (Lampert *et al.*, 1994) and WC (Lürling, 1998), or even lack an (in)organic carbon source, such as Bristol's and medium 7 (Egan & Trainor, 1989a,b,c; Ramos-Cárdenas & de Lara-Isassi, 1985) (*see* Table 4.1).

Together with often relatively high algal densities this may result in carbon limited culture conditions, which could affect growth and morphology. In contrast, in most natural waters inorganic carbon rarely appears to be a limiting nutrient (e.g. Schindler, 1971; Schindler *et al.*, 1972; Goldman *et al.*, 1972), with average concentrations above 20 mg inorganic-C per liter (Goldman *et al.*, 1974).

Grazing is one of the most important loss-processes among algae (Reynolds *et al.*, 1982; Sterner, 1989). Therefore, one might expect that a strong selection pressure exist on the development of traits that reduce mortality through grazing. The most obvious way to withstand grazing pressure is through morphological changes such as size or cell wall shape (Lehman, 1988; Van Donk *et al.*, 1997). In the presence of *Daphnia,* unicellular *Scenedesmus* are triggered into colonies presumably in order to reduce their vulnerability against grazing (Hessen & Van Donk, 1993; Lürling & Van Donk, 1996; CHAPTER 7). However, so far this response has only been demonstrated under nutrient-replete conditions. But is this response still possible under nutrient limitation because of reduced or arrested cell divisions due to a lack of building blocks? Would nutrient-limited *Scenedesmus* form colonies despite a reduced growth rate, or would there be a switch to another 'strategy'? One alternative 'strategy' may be the digestion-resistance hypothesis proposed by Van Donk and Hessen (1993). They provided evidence that P-starved *Selenastrum* and *Scenedesmus* pass largely intact through the

Different P-levels affected growth and morphology in *S. acutus* (Fig. 4.9).

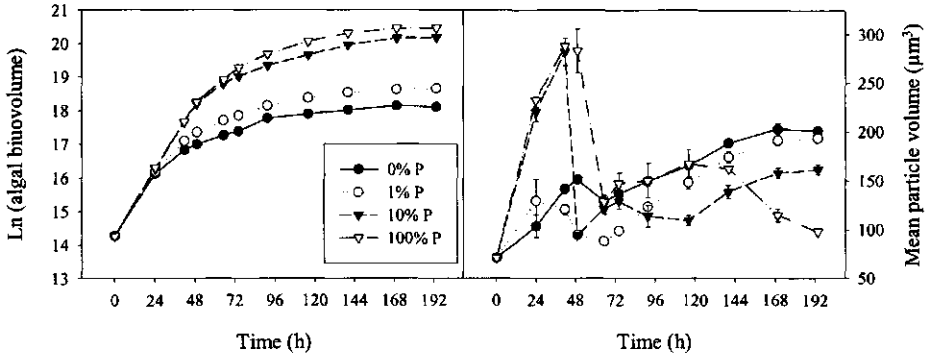


Figure 4.9: Growth (Panel A) and mean particle volumes (µm³; Panel B) of *S. acutus* in medium varying in P-content (0-100% P). Error bars indicate 1 SD (n = 3).

Initially particle volumes increased at both 100% and 10% P and about 20% two- and 10% four-celled coenobia were observed. After two days, the majority of the populations cultured at 10, 1 and 0% P consisted of unicells with means (± 1SD) of 79% (10), 85% (4) and 86% (6), respectively. In populations cultured in standard medium (100% P), the proportion of unicells was 74% (12). Moreover, the composition of these 100% P populations showed more fluctuation during the course of the experiment (Fig. 4.10). After 5 days, unicells had dropped to 50% and eight-celled coenobia (10%) were observed (Fig. 4.10), but the latter disappeared in the consecutive days while concomitantly the proportion of unicells increased.

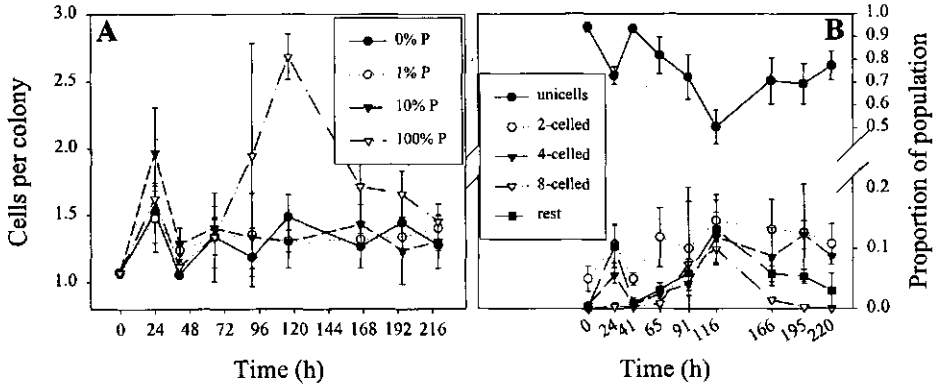


Figure 4.10: Number of cells per colony in *S. acutus* populations cultured at different P-levels (Panel A) and the proportion of unicells, two-, four- and eight-celled coenobia in 100% P (Panel B). The rest group represents three-, five-, six-, seven-, and multicelled (>8) coenobia. Error bars indicate 1 SD(n = 3).

4.4.3 *Effect of* Daphnia *infochemicals on P-limited* Scenedesmus

After one week, algae were taken from the various incubations grown at different P-levels (§*4.4.2*) and transferred into six flasks with corresponding medium, of which three contained also water from a *Daphnia* culture (10% v/v). Since the *Daphnia* water contained phosphorus, P-levels were higher than 0%, 1% and 10% P and approximately 10, 11 and 20% of the standard amount. The initial algal density was $3 \cdot 10^4$ particles·ml^{-1}. For a period of four days samples were taken daily from each flask and analyzed for algal densities and particle volumes in the range 3.0 –20.0 µm equivalent spherical diameter using a Coulter Multisizer II (100 µm capillary). An increase in the mean particle volumes was interpreted as colony formation. For statistical comparison mean particle volumes may be used since the mean particle volumes are highly correlated with the mean number of cells per colony (Lampert *et al.*, 1994; CHAPTER 3). However, since increased cell size seems to be a general phenomenon of chlorophytes under P-limitation, subsamples were taken after 48 and 72 h, fixed with 10% Lugol's solution and the number of cells per colony determined by microscope. The effect of *Daphnia* water at different P-levels on the mean particle volume was analyzed applying repeated measurements ANOVA. The mean number of cells per colony was compared using two-way ANOVA per date.

Growth of *Scenedesmus* differed among populations cultured at different P-levels in the absence (controls) or presence of medium from a *Daphnia* culture (Fig. 4.11).

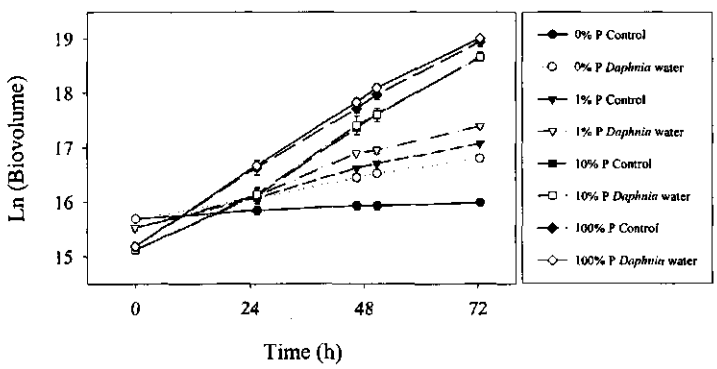


Figure 4.11: Growth of *Scenedesmus acutus* at different P-concentration varying from 0% to the normal amount in WC medium (100%) in the absence (filled symbols) and presence of medium from a *Daphnia* culture (open symbols).

The two-way ANOVA on volume based growth rates determined over the first three days, indicated significant *Daphnia* water effect ($F = 214.9$; $P < 0.001$), P-concentration effect ($F = 5074$; $P < 0.001$) and interaction ($F = 76.6$; $P < 0.001$). The individual factors were tested against the interaction and revealed no *Daphnia* water effect ($F = 2.80 < F_{crit} = 10.1$),

but a dominant P factor ($F = 66.2 > F_{crit} = 9.28$). Thus, growth rates were influenced most by the amount of P in the medium. However, since *Daphnia* water was a significant factor too, separate *t*-tests (two-tailed) were performed. At 0% and 1% P the addition of *Daphnia* water had significantly enhanced the growth rate (Table 4.6), as a result of higher P-levels in the *Daphnia* water treatments.

Table 4.6: Growth rates (μ, ± 1 SD) for *S. acutus* at different P-levels in the absence (control) or presence of medium from a *Daphnia* culture, including *t*- and *P*-values (n = 3).

P-levels	Growth control	Growth *Daphnia* water	*t*-value	*P*-value
0%	0.099 (0.016)	0.370 (0.010)	25.1	**< 0.001**
1%	0.516 (0.003)	0.623 (0.014)	13.3	**< 0.001**
10%	1.175 (0.006)	1.182 (0.028	0.41	0.699
100%	1.253 (0.030)	1.275 (0.005)	1.23	0.285

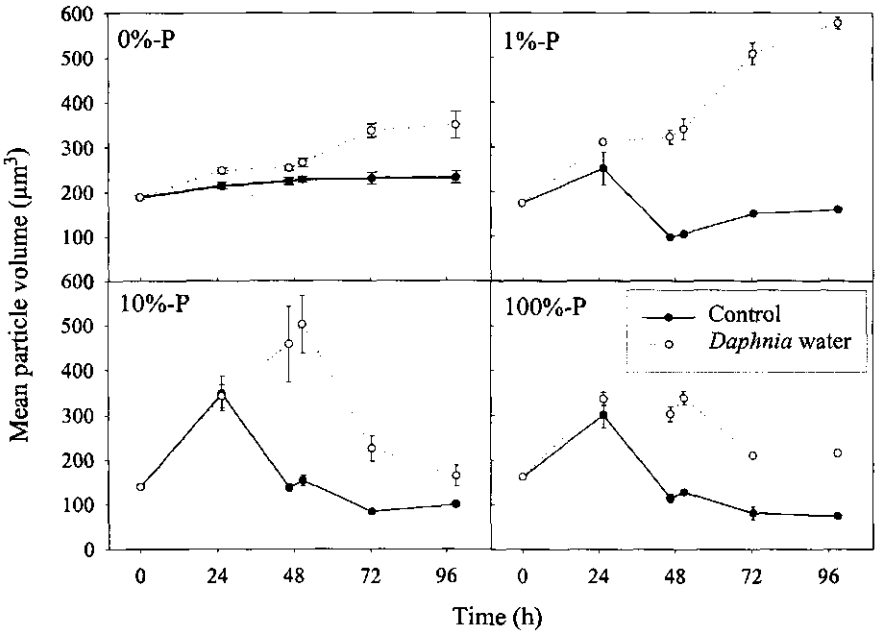


Figure 4.12: The mean particle volume (μm^3) of *Scenedesmus acutus* cultured in media varying in P-content (0 to 100% of normal WC medium) in the absence (filled symbols) and in the presence of medium from a *Daphnia* culture (open symbols). Error bars indicate 1 SD (n = 3).

The addition of *Daphnia* water not only affected growth at low P-levels, but also influenced the morphology of the cells. At all four P-levels a significant increase in the mean

particle volume as a result of the addition of *Daphnia* water was observed (Fig. 4.12). The ANOVA with repeated measurements (time) indicated a significant *Daphnia* water effect (F = 578.4; P = 0.002) and a significant P effect (F = 81.1; $P < 0.001$), but also a significant interaction ($F_{12,24}$ = 38.7; $P < 0.001$). Therefore, the individual factors were compared with the interaction which revealed a significant difference between the interaction and *Daphnia* water ($F = 14.9 > 4.4$), but not for P-level ($F = 2.1 < 3.1$) and time ($F = 2.2 < 2.9$). Thus, the *Daphnia* water factor was most dominant.

The *Daphnia* water significantly enlarged the mean number of cells per colony (Table 4.7). Two-way ANOVA indicated a significant *Daphnia* water effect on the number of cells per colony after 48 h (F = 86.9; $P < 0.001$) and 72 h (F = 50.1; $P < 0.001$).

Table 4.7: Mean number of cells per colony (± 1 SD; n = 3) of *S. acutus* after 48 h and 72 h incubation at four different P-levels (100% = standard amount of P).

48h	Control	*Daphnia* water	72h	Control	*Daphnia* water
100%	1.50 (0.18)	2.87 (0.76)	100%	1.60 (0.32)	3.16 (0.21)
10%	1.96 (0.22)	3.73 (0.70)	10%	1.95 (0.55)	1.72 (0.38)
1%	1.59 (0.18)	4.02 (0.70)	1%	1.71 (0.27)	3.57 (0.51)
0%	1.12 (0.08)	3.17 (0.51)	0%	1.35 (0.11)	2.66 (0.52)

4.4.4 Concluding

Under P-limitation, in the absence of medium from a *Daphnia* culture, S. *acutus* remained unicellular. However, even under considerable P-limitation, the formation of eight-celled coenobia may be induced by infochemicals present in the filtered medium from a *Daphnia* culture (*see* 0% and 1%-P in Table 4.7 and Fig. 4.12). Because the formation of colonies is not a process of simple aggregation of free-living cells, but a result of vegetative reproduction, *Daphnia* induced colony formation may be acting as long as cell division in *Scenedesmus* is not hampered.

4.5 EFFECT OF MEDIUM STRENGTH ON INDUCED COLONY FORMATION

In this experiment the test alga *S. acutus* was cultured in WC-medium varying in strength from 1% of normal composition to 500% according the scheme: 1, 10, 25, 100, 250 and 500%. Three replicate cellulose-plug stoppered 100 ml Erlenmeyer flasks contained 50 ml of algal suspension in the different media. An additional series of replicate flasks per medium type contained test-algae in WC-medium with 10% (v/v) test water from a *D. pulex* culture (300 animals per liter). WC-media with test water deviated from control flasks due to the addition of test water and had strengths compared to normal of 10.9, 19, 32.5, 100, 235 and 460%. The conductivity of the different media varied between 6 and 1190 $\mu S \cdot cm^{-1}$. Initial

algal density was 12000 particles·ml^{-1} (~13000 cells·ml^{-1}). Initially, and after 17, 24, 43, 49, 65 and 72 h, cell densities and size distributions were determined using a Coulter Multisizer II (100 µm capillary). The number of cells per colony was determined microscopically initially as well as after 24, 48, and 72 h by counting at least 100 particles (unicells and colonies) in subsamples preserved in Lugol's fixative.

Different strength of the WC medium resulted in significantly different volume based growth rates among treatments. Repeated measurement ANOVA indicated significant differences among controls and treatments with *Daphnia* water ($F_{1,2}$ = 22.8; P = 0.041) and among different media ($F_{5,10}$ = 197.4; P < 0.001). The interaction (*Daphnia* factor × medium type) was also significant ($F_{5,10}$ = 6.6; P = 0.006), but testing the individual factors against the interaction revealed that medium type was the most dominant factor (F = 29.9 > F_{crit} = 3.7) influencing growth rate. Between 24 and 48 hours significant colony formation could be observed (Fig. 4.13 and 4.14). The repeated measure ANOVA on the mean particle volumes (Fig. 4.13) revealed a significant *Daphnia* factor ($F_{1,2}$ = 690.7; P = 0.001) and medium type effect ($F_{5,10}$ = 21.5; P < 0.001), but no interaction effect ($F_{5,10}$ = 2.5; P = 0.104).

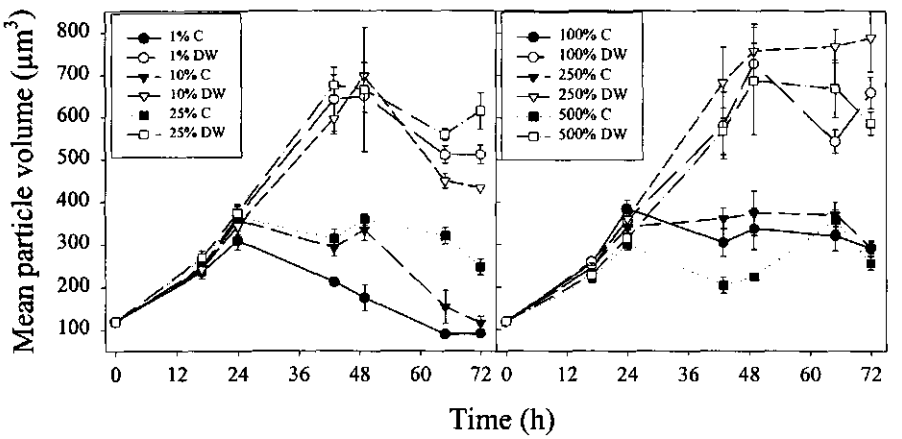


Figure 4.13: Course of the mean particle volume (µm^3) of *Scenedesmus acutus* cultured in the absence (C) or presence of medium from a *Daphnia* culture (DW) in WC medium with different salt levels varying from 0.01 times (1%) to 5 times (500%) the normal amount (100%). Error bars represent 1 SD (n = 3).

However, repeated measure ANOVA on the mean number of cells per colony (Fig. 4.14) revealed only a significant *Daphnia* factor effect ($F_{1,2}$ = 395.8 ; P = 0.003), but no medium type effect ($F_{5,10}$ = 2.4; P = 0.109) and no interaction ($F_{5,10}$ = 0.9; P = 0.502). This means that regardless the strength of the medium colony formation is induced, but that the size of the colonies, expressed as mean particle volumes, strongly depends on the amount of available resources. This becomes clear when the mean particle volumes and the mean

number of cells per particle are plotted against each other (Fig. 4.15). The slope of the overall regression: log (mean particle volume) = 2.299 + 0.755 × log (cells colony^{-1}) is < 1, which indicates lower cell volume in coenobial cells. Similar observations were made by Lampert *et al.* (1994) and in this thesis (CHAPTER 3). Linear regressions per medium strength were all significant (Table 4.8).

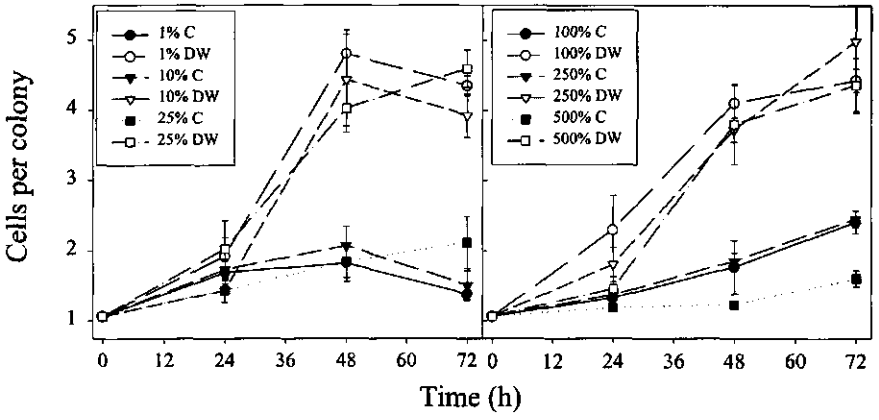


Figure 4.14: Course of the number of cells per colony in *Scenedesmus acutus* cultured in the absence (C) or presence of medium from a *Daphnia* culture (DW) in WC medium with different salt levels varying from 0.01 times (1%) to 5 times (500%) the normal amount (100%). Error bars represent 1 SD ($n = 3$).

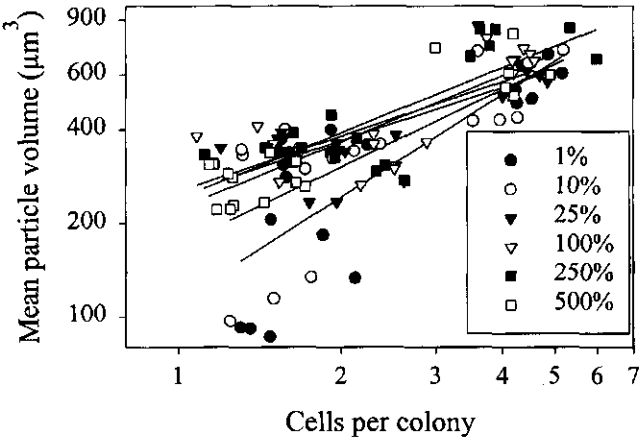


Figure 4.15: Relation between the mean particle volume and the number of cells per colony in *Scenedesmus acutus* cultured in WC medium varying in composition from 0.01 times (1%) the normal amount of salts to 5 times (500%). Solid lines represent linear regressions.

Testing the regressions on significant differences among medium strength revealed that but only the differences in regression between the 1% and 25% and between 1% and 100% medium were significant. Thus, the size of *S. acutus* cells (in volume-units) cultured in 1% medium was significantly lower than the size of cells in the 25% and 100% medium.

Table 4.8: Regressions of mean particle volume (MPV) on mean number of cells per colony (C/C) of *Scenedesmus acutus* cultured in medium varying in strength from 1% to 500% (i.e.0.01 to 5 times the standard amount of nutrients added), including r^2 of the regressions and *P*-values of *t*-tests for distinguishing significant differences between regression lines.

Medium	Regressions:		*P*-values					
Strength	log(MPV) = ... × log(C/C)	r^2	1%	10%	25%	100%	250%	500%
1%	...= 2.055 + 1.096	0.646	XXX	0.363	**0.038**	**0.041**	0.110	0.090
10%	...= 2.234 + 0.827	0.494		XXX	0.308	0.332	0.604	0.607
25%	...= 2.395 + 0.596	0.527			XXX	0.932	0.507	0.391
100%	...= 2.404 + 0.585	0.563				XXX	0.550	0.432
250%	...= 2.382 + 0.697	0.630					XXX	0.941
500%	...= 2.349 + 0.709	0.803						XXX

In the absence of *Daphnia* water, both the mean particle volume ($r = 0.64$; n = 54; $P < 0.001$) and the individual cell volume ($r = 0.47$; n = 54; $P < 0.001$) were significantly correlated with the growth rates. In the presence of *Daphnia* water, the correlation was less strong between the mean particle volume *vs.* growth rate ($r = 0.30$; n = 54; $P = 0.014$) and cell volume *vs.* growth rate ($r = 0.32$; n = 54; $P = 0.009$). No correlation between growth rate and the number of cells per colony was detected ($r = 0.05$; n = 108; $P = 0.304$). Also when only data for 48 and 72 h were used and the set was separated in controls and *Daphnia* water treatments, no correlation was detected ($r = 0.15$; $P = 0.191$ and $r = 0.21$; $P = 0.109$, respectively, n = 36).

Summarizing, different medium strength affected growth and cell size in *S. acutus*, but had no effect on *Daphnia*-induced colony formation. Coenobia were induced at all strengths in the presence of *Daphnia* water, whereas the controls at all strengths remained unicellular.

4.6 MEDIA & UREA

Scenedesmus is mostly found in eutrophic waters where it appears to thrive (Trainor, 1998). Using a high salt medium (Bristol's) Siver & Trainor (1981; 1983) demonstrated that unicells were formed in UTEX 2533 in response to ammonium and the internal carbohydrate content. In standard Bristol's they reported only 7% unicells and thus 93% coenobia, whereas the addition of ammonium (7.8 $mg \cdot l^{-1}$) and glycolic acid (39 $mg \cdot l^{-1}$) resulted in 7% coenobia (Siver & Trainor, 1983). Based on these data one could expect *S. acutus* MPI to be colonial in

Bristol's. Later the Trainor laboratory developed a low salt medium (Medium 7) with nutrient levels similar to those encountered in nature to stabilize colony formation in the laboratory (Trainor, 1998). In the previous section, the modified WC (*see* Table 3.1) was used at different strengths. Here, a comparison will be made with two other media, one a high nutrient medium (Bristol's) and the other a low nutrient medium (Medium 7).

Moreover, Wiltshire & Lampert (*in prep.*) proposed that urea would be the colony inducing substance, but that the C:N ratio of the alga is important too. Urea has been shown NOT to induce coenobia in Chu 12 (Lampert *et al.*, 1994; own unpublished data) and WC medium (CHAPTER 3; *see* Figs. 3.20 and 3.21), but the urea concentration used may have been too low. Therefore, an experiment was performed with *S. acutus* cultured in WC-medium, Bristol's medium and Medium 7 (Controls), in the three media with medium from a *Daphnia* culture added (*Daphnia* water) and in additional series with urea (14 mg $N \cdot l^{-1}$) added. The initial densities were 14500 particles$\cdot ml^{-1}$ ($1.65 \cdot 10^6$ $\mu m^3 \cdot ml^{-1}$) in Bristol's and 21200 particles$\cdot ml^{-1}$ ($1.69 \cdot 10^6$ $\mu m^3 \cdot ml^{-1}$) in WC and medium 7.

Colony formation, as indicated by the mean particle volume, had occurred in the presence of *Daphnia* water in all three media (Fig. 4.16). Two-way ANOVA indicated a significant treatment effect ($F = 371.0$; $P < 0.001$), a significant medium effect ($F = 9.70$; $P = 0.001$) and a significant interaction ($F = 17.1$; $P < 0.001$). However, testing the individual factors against the interaction revealed a significant treatment effect, but no medium effect.

Although the number of cells per colony was higher after two days in all media and treatments than at the start of the experiment, colony formation was significantly promoted in the *Daphnia* water treatments (Fig. 4.17). Numerous eight-celled coenobia were detected in the *Daphnia* water treatments, but not in the controls and urea treatments.

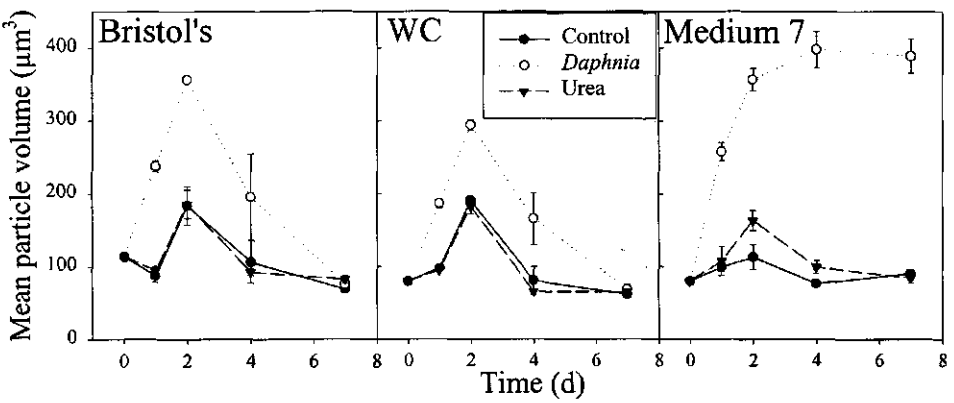


Figure 4.16: Effect of *Daphnia* water and urea on the mean particle volume in *Scenedesmus acutus* cultured in three different media. Error bars represent 1 SD (n = 3).

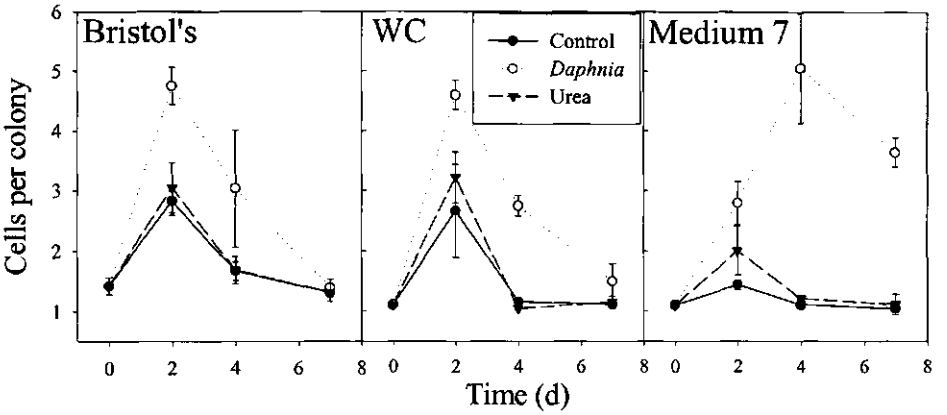


Figure 4.17: Effect of *Daphnia* water (*Daphnia*) and urea on the mean number of cells per colony in *Scenedesmus acutus* cultured in three different media. Error bars represent 1 SD (n = 3).

Analogous to §4.5 no colony formation was observed in control population of *S. acutus* cultured in media varying in strength. However, *Daphnia*-induced colony formation was observed in all media, regardless their strength. By contrast, no colony formation was induced by urea, which has also been observed in CHAPTER 3 (§3.3) and reported in literature (Lampert *et al.*, 1994).

4.7 DISCUSSION

Many *Scenedesmus* strains may grow as coenobia or unicells under various conditions (f.e. Trainor, 1964; Trainor & Shubert, 1974; Holtmann & Hegewald, 1986). It was proposed that nitrogen, as ammonium, could be the main factor in controlling unicell formation (Siver & Trainor, 1981). However, *S. acutus* is also primarily unicellular in medium without ammonium as N-source as shown by literature data. The mean number of cells per colony (± 1 SD) in *S. acutus* cultured under various nutrient conditions appeared 1.43 ± 0.22 (Sterner & Smith, 1993; Lampert *et al.*, 1994; Lürling & Van Donk 1996; 1997a;b). In contrast, eight-celled coenobia may be produced rapidly when this strain of *S. acutus* is cultured in medium with water from a *Daphnia* culture (Lampert *et al.*, 1994; Lürling, 1998). The algal predators *Daphnia* are sloppy feeders. They ingest more food than they assimilate and as a result many products are released from *Daphnia* (Peters, 1987), including organic carbon (Lampert, 1978), cyclic AMP (Francko & Wetzel, 1982), phosphorus (Peters & Rigler, 1973) and ammonium and amino acids (Gardner & Miller, 1981). Several of these excretion products have been tested, but all appeared negative in inducing colonies (Lampert *et al.*, 1994; Lürling, 1998; CHAPTER 3).

Although in one specific treatment with reduced nitrogen up to 16% eight-celled coenobia were formed in *S. acutus,* nitrogen does not seem to be the main factor controlling morphology. The addition of water from a *Daphnia* culture resulted in populations dominated by the eight-celled morph, but both ammonium and urea appeared ineffective as colony inducing agent (Lampert *et al.*, 1994; §3.3 and §4.6). Although recently proposed (Wiltshire & Lampert, *in prep.*), all experimental resuls were in direct conflict with the urea hypothesis. Ecologically a response to urea is unlikely as a defense, simply because other organisms, such as fish, excrete urea as well. One would expect *Scenedesmus* to respond to a general algal grazer chemical, rather than to a general animal substance. Since *S. acutus* did not respond to fish or to urea under several conditions, but did to *Daphnia* water (§3.3, §3.4 and §4.7), urea is definitely not the factor. Perhaps that urea under specific conditions may act as a morphogenetic factor, similar to glucose, that has been reported to induce coenobia in a non-spiny *Scenedesmus* too (Trainor, 1964), but could also not be confirmed by others (Nagy-Tóth *et al.*, 1992; CHAPTER 3). Also the latest chemical analyses do not support the urea-hypothesis. According to the chemical analysis the *Daphnia* factor is an olefinic low-molecular-weight carboxylic acid (Von Elert, *in press*; Von Elert & Franck, *in press*).

The effect of different nutrient conditions on the morphological development of *S. acutus* seems to differ from that of several spined *Scenedesmus*. Whereas coenobial cell number in spined *Scenedesmus*, like *S. quadricauda*, may be affected by nutrients, *S. acutus* seems insensitive and to remain mainly unicellular. However, within-species variation may be considerable. Statistical analysis of calculated colony sizes of five non-spiny strains of *Scenedesmus* cultured in five different media (Holtmann & Hegewald, 1986; presented in their Table 3) revealed no medium effect but a significant strain effect. Moreover, *S. pectinatus* strains that occurred in the field as *ca.* 50% eight-celled coenobia, failed to produce this morph in the laboratory under various nutrient conditions (Holtmann & Hegewald, 1986). Unfortunately, Holtmann & Hegewald do not discuss this phenomenon, neither the difference in individual cell size.

In a 35 days investigation of the growth and morphology in non-treated *S. acutus* populations, eight-celled coenobia were found only in very low numbers, but four-celled coenobia appeared when cultures reached the stationary phase (*see* CHAPTER 2). During the course of that experiment non-limiting conditions probably have been changed into nutrient-(N) and light-limiting conditions. Above an intracellular C:N ratio of about 12, *S. acutus* occurred mainly as unicells and colony formation seemed rare. The non-linear relationship between atomic C:N ratio and growth rate is in agreement with data for the same strain obtained by Sterner (1993). The number of cells per colony in *Scenedesmus* seems closely related to the amount of energy stored or protoplasm produced in the parent cell (Šetlík *et al.*, 1972) and may be directly proportional to growth rates (Siver & Freeda, 1982). At high growth rates the formation of eight-celled coenobia has been reported to occur (Šetlík *et al.*,

1972; Gavis *et al.*, 1979). The latter observed high proportions of eight-celled *S. quadricauda* at growth rates of 0.9 – 1.0 day^{-1}, but not below 0.6 day^{-1} (Gavis *et al.*, 1979). Combination of the results presented in the figures 4.6 and 4.7 suggests that induced colony formationmay occur at growth rates of approximately 0.1 d^{-1} and more (*see* Fig. 4.7). In the presence of medium from a *Daphnia* culture, cultures were dominated by eight-celled coenobia. The intracellular C:N ratios were, however, similar as were growth rates. Siver & Trainor (1983) demonstrated unicell/colony formation to be independent of growth rates by altering the medium composition. Also for the *S. acutus* strain used in this study formation of eight-celled coenobia was independent of growth rate, and occurred in the presence of *Daphnia*-water. In the absence the cultures were dominated by unicells. No trade-off between colony formation and growth rate was detected, which has also been observed by Hessen & Van Donk (1993) and Lampert *et al.* (1994). One could, however, expect no colony formation to occur when cell division is arrested since in *Scenedesmus* a parent cell divides inside the parental cell wall into several daughter cells that may be released as coenobium or single cells (Van den Hoek *et al.*, 1995). This could be the case in P-starved *S. acutus* where a smaller increase in the mean particle volume was detected after the addition of *Daphnia* water than at higher P-levels (*see* §4.4). However, growth rates were non zero and microscopy revealed that even in strongly P-limited cells grazer-induced coenobia formation had occurred. The significantly enhanced growth rates at low P–levels after the addition of *Daphnia* water was caused by the addition of P with this water.

With *S. armatus* the addition of extra P to a dilute medium stimulated the production of unicells within three days, although growth rates were not affected (Shubert & Trainor, 1974). It was hypothesized that at low P-levels (i.e 1% and 0%) after a few days coenobia would be formed, however, populations remained unicellular with a tendency of increased proportion unicells with declining P-levels.

Since the addition of a buffer resulted in similar pH among treatments, colony formation did seem independent of pH. Also the strength of the medium seems unimportant (§4.5 and §4.6). The availability of dissolved inorganic carbon may become limiting to *Scenedesmus* cultures when the carbon dioxide flux between the air outside and inside the experimental vessel is hampered (*see §3.2.2 & 3.2.3*). Under carbon limitation *S. acutus* cells were significantly smaller. However, cellulose plugging ensured sufficient diffusion that replenishment of carbon dioxide could occur during a period of 48 h, which is sufficient to examine *Daphnia*-induced colony formation in non-spiny *Scenedesmus*. Literature data on the cell volume of the same strain of *S. acutus* cultured in chemostats revealed a mean cell volume (± 1 SD) of 67 (15) μm^3 (Lampert *et al.*, 1994; Lürling & Van Donk, 1996; 1997a,b; De Lange & Van Donk, 1997). Most probably all these cultures were carbon/light limited due to high algal biomass. The cell volume of around 60 μm^3 in flasks with parafilm or silicon

rubber sealing (*§3.2.2*) is close to the value obtained from literature. That high algal densities may cause smaller cells is also observed when biotests performed by Lampert *et al.* (1994) and Lürling (1998) are compared. The former used heavy inocula of $1.25 \cdot 10^5$ cells·ml^{-1}, while the latter used inocula below $5 \cdot 10^4$ cells·ml^{-1}. The mean particle volumes in both studies differ significantly (*t*-test: $t = 7.9$; df = 9; $P < 0.001$) with mean values (± 1 SD) of 142 (11) and 253 (30) µm^3, respectively. The effect of carbon availability on cell size will undoubtedly affect the colony size, as cells are bigger. A highly significant correlation exists in *S. acutus* between the mean particle volume and the mean number of cells per colony (Lampert *et al.*, 1994; CHAPTER 3). With an individual cell volume of 60 µm^3 this yields an eight-celled coenobium of 285 µm^3. However, when unicells have a cell volume of 200 µm^3 the eight-celled coenobia will be 962 µm^3. The difference will of course also be reflected in cell dimensions that could affect their susceptibility to grazers. Lampert *et al.* (1994) found no differences in the uptake of unicells and colonies by *Daphnia*. Under their culturing conditions eight-celled coenobia will have hardly exceeded dimensions of 30 × 20 µm and would still have been ingestible by small 1 mm *Daphnia*. Relatively small eight-celled coenobia in *S. acutus* with mean dimensions of 24 × 19 µm were also found by Lürling & Van Donk (1997c) when they used heavy inocula of $1.4 \cdot 10^5$ cells·ml^{-1}. By contrast, when low inocula were used *S. acutus* eight-celled coenobia with dimensions of 57 × 30 µm and up to 65 × 40 µm were observed (Lürling, 1998; CHAPTER 2). These large coenobia could confront even the largest grazers with ingestibility problems (cf. Burns, 1968).

Increased carbon levels had no effect on colony formation both in the absence and presence of medium from a *Daphnia* culture neither had the strength of the used medium. Cell volume was increased at higher carbon levels, but reduced at lower medium strengths. Thus, the availability of carbon seems important in determining the cell size, not in determining the amount of cells per colony.

Although only short-term experiments were employed, no effects of carbon, nitrogen, phosphorus and medium strength on colony formation in *S. acutus* were detected. The cultures remained unicellular even at cell densities far above *ca.* 1000 cells ml^{-1}. Hence, low cell density as unifying principle for unicell development in the genus *Scenedesmus* (Egan & Trainor, 1989b) does not seem so necessary in several *Scenedesmus* strains. The non-spiny *S. obliquus* (Lürling, 1999) and several other *Scenedesmus* spp. occur as unicells in the laboratory (CHAPTER 8) even at high densities and for long-term periods. *S. abundans* from the field formed unicells in the laboratory (Fott, 1968) and also *S. armatus* seemed to occur mainly as unicells (Tukaj *et al.*, 1996). Despite that unicells may be common in *Scenedesmus* (e.g. Swale, 1967; Fott, 1968; Trainor, 1979; Holtmann & Hegewald, 1986; Krienitz, 1987; Egan & Trainor, 1989a,b; Trainor & Egan, 1990; Hessen & Van Donk, 1993) they are but only occasionally reported from nature. This led to a hypothetical seasonal life history of

Scenedemus with unicells occurring in early spring (Egan & Trainor, 1989a). But why would unicells occur only in spring especially since sufficient literature data exist on unicellular *Scenedesmus* under a wide range of nutrients and cell densities? Trainor (1979) observed that unicells disappeared when incubated in dialysis sacks in the field or when cultured in pond water in the laboratory. Interestingly, in another study ten years later the same strain produced unicells in the same pond water (Egan & Trainor, 1989a). We now have sufficient evidence that grazers are involved in *Scenedemus* plasticity, by both selective grazing on small, unprotected morphs and chemical induction of large protected morphs. Perhaps, grazers in the pond may account for the different observations by Trainor (1979) and Egan & Trainor (1989a). *Scenedesmus* unicells have been reported in a sewage oxidation pond (Mattoni *et al.*, 1965), where the number of large grazers may be low, as well as in river water when effluent from a sewage plant was added (Shubert & Trainor, 1974).

CHAPTER 5

Daphnia-induced colony formation in *Scenedesmus acutus*: Ecomorph expression at different temperatures

Parts of this chapter are based on:
Lürling, M. & Van Donk, E. *submitted to Journal of Phycology*

"Wij noemen planten, die wij niet eten of noodig hebben onkruid; streken waar wij niet leven kunnen wildernis; wij zoeken aan alle dingen die zijde op, die eene betrekking heeft op ons en vergeten daarbij, dat het niet de waarheid der natuur is, maar onze eigenliefde, die ons zulk een verkeerd oordeel over de wereld buiten ons ingeeft."

- G.H. Rissik 1860

5.1 INTRODUCTION

In aquatic systems algae experience rapid nutrient and light changes, temperature fluctuations and variations in abundance of grazers (Sommer *et al.*, 1986). The environmental variables change at different temporal and spatial scales and algae may respond in different ways (Harris, 1986). Many algae are notoriously plastic in morphology, growth and biochemical composition. Changing environmental conditions may favor different clones of the same species that may lead to the replacement of one clone by the other (Wood & Leatham, 1992), or changed environmental conditions may alter a specific trait within one clone, which is defined as phenotypic plasticity (Schlichting, 1989; West-Eberhard, 1989).

One of the commonest freshwater green algae, *Scenedesmus*, has been shown extremely phenotypically plastic i.e. all species exhibit an extensive morphological variability (Trainor, 1991; 1998). An ordered sequence of ecomorph development has been reported in *Scenedesmus armatus* Chodat (Trainor, 1992a), in *S. communis* Hegewald (Trainor, 1992b) and in *S. subspicatus* Chodat (Trainor, 1993). These morphological changes are considered a cyclomorphosis (*sensu* Black & Slobodkin, 1987) driven by nutrients, temperature or a chemical cue from a grazer. In the presence of the grazer *Daphnia* (Crustaceae) unicellular *Scenedesmus* are triggered into colonies to reduce their vulnerability against grazing (Hessen & Van Donk, 1993; Lürling & Van Donk, 1996). The phenomenon of grazer-induced colony formation has been demonstrated in both spined *S. subspicatus* (Hessen & Van Donk, 1993) and non-spiny *S. acutus* Meyen (Lampert *et al.*, 1994). Various nutrient conditions, in the absence of *Daphnia*, did not affect colony size in *S. acutus* and cultures remained dominated by unicells (*see* CHAPTER 4). However, one important environmental factor that was considerably constant in these studies was the temperature of 20 – 22°C.

Temperature may have a pronounced effect on *Scenedesmus* growth and morphology (Trainor, 1998). Metabolic processes related to photosynthesis and biosynthesis are profoundly affected by temperature (Rhee & Gotham, 1981; Davidson, 1991). At low temperatures the rate of carbon fixation and cell division may be reduced (Morgan & Kalff, 1979; Davidson, 1991), and excess of photosynthates may be accumulated as starch (Coesel & Wardenaar, 1990), thereby increasing the amount of carbohydrates per cell, whereas protein and lipids may decrease (Aaronson, 1973).

Temperature may not only have a clear effect on algal growth rate (Goldman & Carpenter, 1974; Harris, 1986), but it may also influence drastically the morphological appearance of cells. In general, cell size seems to increase with lower temperatures (e.g. Morgan & Kalff, 1975; 1979; Rhee & Gotham, 1981; Trainor, 1992b; 1995; 1998). Moreover, *S. subspicatus* appeared coenobial at 10°C, while unicells were dominant at 22°C (Trainor, 1993). In *S. communis* populations, at 22°C the proportion of eight-celled coenobia was less than 5% (Egan & Trainor 1989), but at 10°C over 80% eight-celled coenobia occurred (Trainor, 1992b). By contrast, at 10°C *S. kisii* Hortobagyi remained completely unicellular. In

this species both coenobia and spine formation were suppressed at low temperature (Trainor, 1995). In *S. quadricauda* (Turpin) Brébisson cultures were dominated by unicells at 20°C, but by four-celled coenobia at 30°C (Steenbergen, 1978).

Additionally, temperature may affect the response of *Scenedesmus* to infochemicals released from grazers as physiological processes of the algae may be altered. Temperature has been reported to result in pronounced effects on defensive morphology in *Daphnia* (Hanazato, 1991), growth of *Daphnia* exhibiting a predator-avoidance strategy (Sakwińska, 1998) and their vulnerability to predation (Dodson & Wagner, 1996). Moreover, temperature may affect degradation and production of colony-inducing chemicals. The production of colony inducing chemicals seems directly related to the amount of food processed by grazers such as *Daphnia*. Since the temperature effect on food ingestion by *Daphnia* results in an optimum (Lampert, 1987), and has a positive effect on excretion (Peters & Rigler, 1973), the production of infochemicals is most likely influenced by temperature as well.

5.2 ECOMORPH EXPRESSION AT DIFFERENT TEMPERATURES

5.2.1 Methods

An inoculum of exponentially growing unicellular *S. acutus* was derived from the chemostat and was transferred into 300 ml cellulose-plug stoppered Erlenmeyer flasks containing 150 ml of medium. Each batch culture contained 134 ml autoclaved WC medium, 1 ml algal inoculum and either 15 ml additional WC medium filtered through a glass fiber filter (controls) or 15 ml filtered test water (treatments). The test water was produced prior to the experiment by allowing 200 adult *Daphnia magna* Straus to feed for 24 h on a 1.0 l suspension of *S. acutus* (*ca.* 10^5 particles·ml^{-1}; i.e. *ca.* 3.5 mgC·l^{-1}) in WC medium. The batch cultures were incubated at four different temperatures 9.5°; 16.5°; 24° and 29°C. The cultures were shaken manually once a day and continuously illuminated from above by fluorescent cool-white tubes (Osram L 36W/21-840) at 125 µmol·m^{-2}·s^{-1}. Controls and treatments were run in quadruplicates for 28 days. The initial algal density was 12000 particles·ml^{-1} (*ca.* 14000 cells·ml^{-1}). Algal densities and particle size distributions were determined routinely in the size range 3.0 - 20.0 µm ESD (equivalent spherical diameter) using a Coulter Multisizer II (100 µm capillary). For each replicate the number of cells per colony was determined microscopically by counting at least 100 aggregates (i.e. unicells as well as coenobia) in a subsample preserved in Lugol's fixative. Growth curves were fitted by non-linear regression using the regression wizard in SigmaPlot 4.0 program according the model:

$$\text{Ln (biovolume)} = y_0 + a \times (1 - \exp(-r \times t))$$

where t = time (d) and r = intrinsic rate of population increase (d^{-1}). Maximal growth rates (μ_{max}, d^{-1}) were based on the increase in algal volume (V) over the first two days according the equation: $\mu_{max} = \ln(V_t/V_0) \times \Delta t^{-1}$. Estimated μ_{max} were compared using two-way ANOVA,

with temperature and absence/presence of *Daphnia* water as the two fixed effects. The population growth rates (*r*) were compared applying two-tailed *t*-tests.

5.2.1 *Results*

Growth of *S. acutus* was similar in populations cultured in standard medium (controls) or in medium with filtered water (10% v/v) from a *Daphnia* culture (treatments), but differed among populations cultured at different temperatures (Fig. 5.1; Table 5.1).

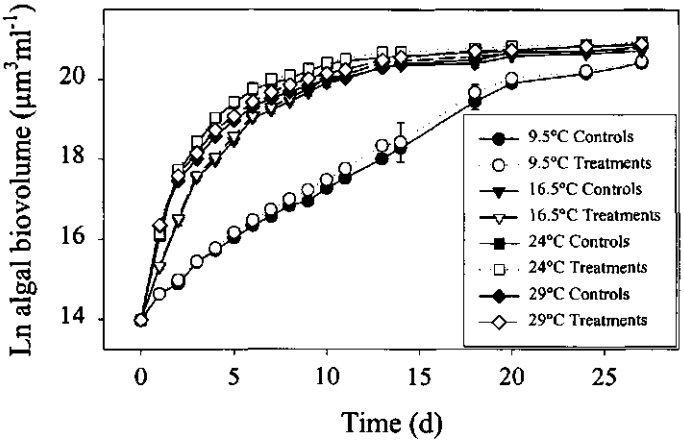


Figure 5.1: Growth of *Scenedesmus acutus*, expressed as ln of biovolume, cultured for four weeks at four temperatures in the absence (filled symbols, Controls) or presence of medium from a *Daphnia* culture (open symbols, Treatments). Error bars indicate 1 SD (n = 4).

Table 5.1: Intrinsic rates of population increase ($r \pm 1$ SE) obtained from non-linear regression using the model Ln (biovolume) = $Y_0 + a \times (1 - \exp(-r \times t))$, with r = intrinsic rate of population increase (d^{-1}), including r^2 of the fit.

Temp.	*Treatment*	r (d^{-1})	r^2
9.5°C	*Control*	0.0406 (0.0057)	0.993
9.5°C	*Daphnia* water	0.0510 (0.0053)	0.995
16.5°C	Control	0.2292 (0.0057)	0.999
16.5°C	*Daphnia* water	0.2263 (0.0062)	0.998
24°C	Control	0.3394 (0.0147)	0.994
24°C	*Daphnia* water	0.3406 (0.0144)	0.994
29°C	Control	0.2844 (0.0210)	0.984
29°C	*Daphnia* water	0.2934 (0.0203)	0.986

The population growth rates (r) of 0.05 d^{-1} at 9.5°C and 0.23 d^{-1} at 16.5°C were significantly different from each other and from growth at the higher temperatures. Growth at 24°C ($r \approx 0.34\ d^{-1}$) or 29°C ($r \approx 0.29\ d^{-1}$) was not significantly different (Table 5.2).

Table 5.2: *P*-values of two-tailed *t*-tests performed on the estimated intrinsic rates of population increase (r) among treatments (C = controls, *Dw* = *Daphnia* water).

Temp.	9.5-C	9.5-*Dw*	16.5-C	16.5-*Dw*	24-C	24-*Dw*	29-C	29-*Dw*
9.5-C	XXXX							
9.5-*Dw*	0.256	XXXX						
16.5-C	**<0.001**	**<0.001**	XXXX					
16.5-*Dw*	**<0.001**	**<0.001**	0.756	XXXX				
24-C	**<0.001**	**<0.001**	**0.002**	**0.002**	XXXX			
24-*Dw*	**<0.001**	**<0.001**	**0.002**	**0.002**	0.956	XXXX		
29-C	**<0.001**	**<0.001**	0.064	0.056	0.098	0.092	XXXX	
29-*Dw*	**<0.001**	**<0.001**	**0.038**	**0.034**	0.338	0.130	0.774	XXXX

The two-way ANOVA on exponential growth rates (μ_{max}) revealed a significant temperature effect ($F = 3553$; $P < 0.001$), a significant *Daphnia* water effect ($F = 17.4$; $P < 0.001$), but no interaction effect ($F = 0.58$; $P = 0.631$). Separate two-tailed *t*-tests for μ_{max} showed significantly different growth rates among control and treatment populations at three of four temperatures (Table 5.3). Not only maximal growth rate was affected during the first days, but also *Scenedesmus* morphology was changed drastically in the treatment populations at warmer temperatures (16.5°, 24° and 29°C). In the first two days colony formation was promoted by *Daphnia* water (Fig. 5.2). In the treatment populations a rapid formation of four-celled and eight-celled coenobia could be observed followed by a subsequent recovery of unicell abundance (Fig. 5.2). The control populations were dominated by unicells. However, when populations aged the dominance of unicells in the control populations gradually decreased to about 20-25%, while the proportion of four-celled coenobia concomitantly increased to about 50-60% (Fig. 5.2). After 14 days, population composition seemed to stabilize and was more or less comparable between control and treatment populations (Fig. 5.2). This process is clearly temperature dependent and at 9.5°C not only four-celled coenobia, but also eight-celled coenobia were formed that made-up 20% of control- and 35% of treated populations (on day 18). At the end of the experiment, populations were more or less comparable.

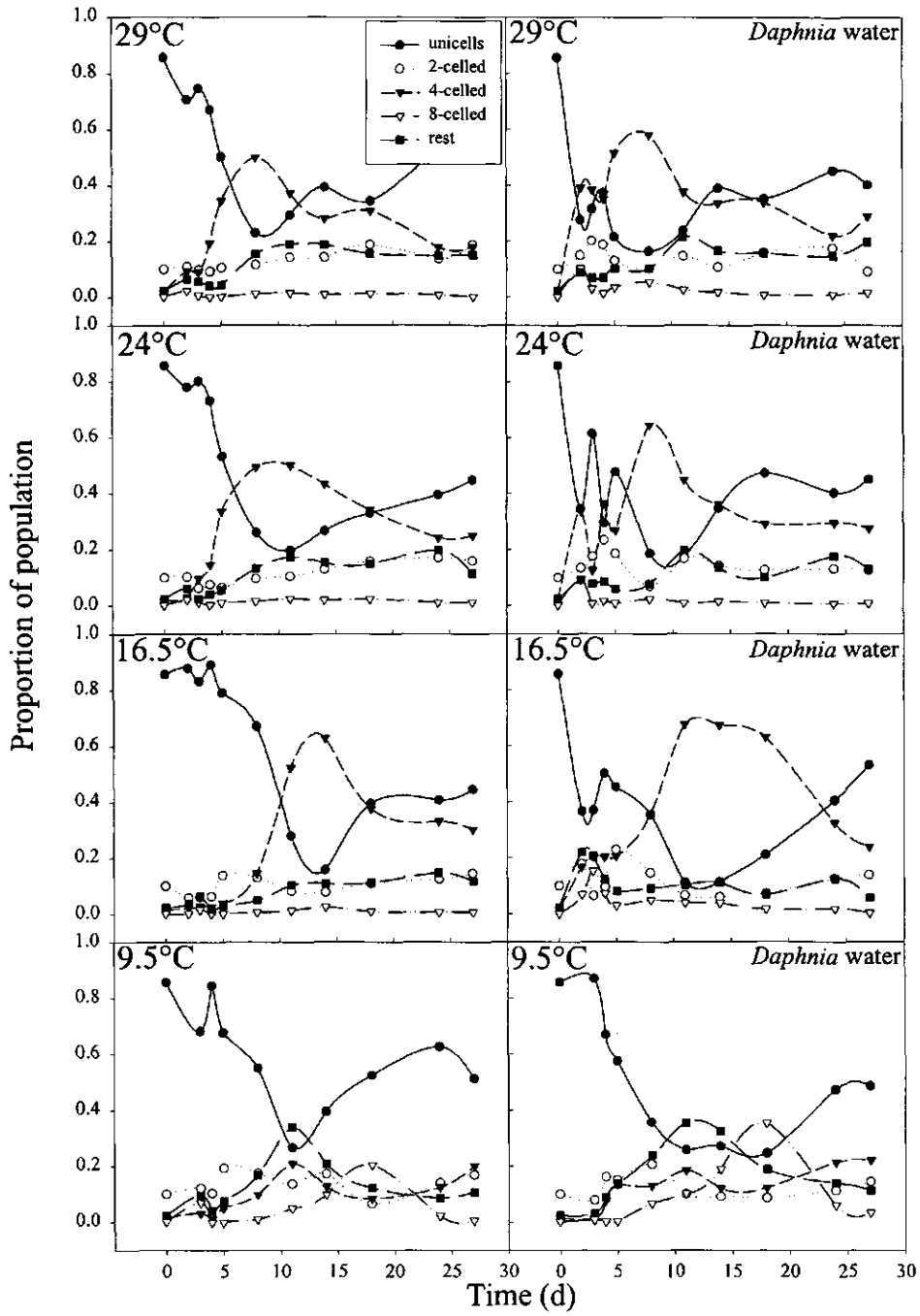


Figure 5.2: Proportion of unicells, two-, four-, and eight-celled coenobia in *Scenedesmus acutus* populations grown for four weeks at four temperatures in the absence (left panels) and presence of medium (10% v/v) from a *Daphnia* culture (right panels). The rest-group represents three-, five-, six-, seven- and multicelled aggregates.

Table 5.3: Maximal growth rates (μ_{max}, d^{-1}, ± 1 SD; n = 4) of *Scenedesmus acutus* cultured at four temperatures in the absence (Control) or presence of *Daphnia* water (*Daphnia* water), including *t*- and *P*-values of *t*-tests.

Temp.°C	Control	*Daphnia* water	*t*	*P*
9.5	0.453 (0.033)	0.496 (0.011)	2.48	**0.048**
16.5	1.232 (0.057)	1.263 (0.036)	0.93	0.387
24	1.839 (0.015)	1.874 (0.015)	3.27	**0.017**
29	1.729 (0.030)	1.797 (0.011)	4.26	**0.005**

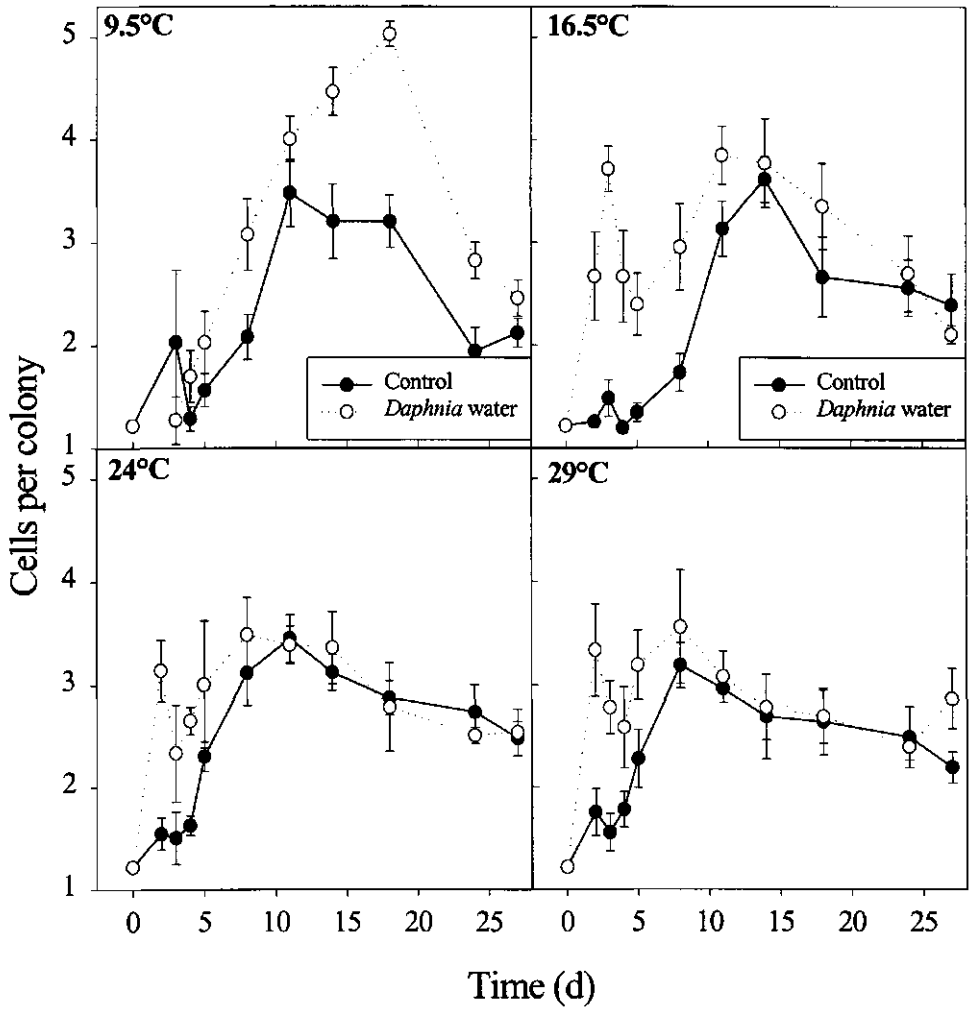


Figure 5.3: The mean number of cells per colony in *Scenedesmus acutus* cultured for four weeks at four temperatures in the absence (Control) or presence of medium from a *Daphnia* culture (*Daphnia* water). Error bars indicate 1 SD (n = 4).

The rapid morphological response of *Scenedesmus* in the treatments is also reflected in the mean number of cells per colony (Fig. 5.3) and in the mean particle volume (Fig. 5.4). The mean particle volume remained larger in treated populations compared to the controls throughout the entire experiment. The mean number of cells per colony in control and treated populations depended on the temperature. At the two warmest temperatures (24° and 29°C), only during the weeks 1-2 the treatments contained more cells per colony than the controls. At 16.5°, however, during 2-3 weeks colony size in the treatments was larger, whereas at 9.5°C this period lasted almost 4 weeks. Individual cell size, as reflected in cell volume, also appeared larger in treatments than in control populations although differences were small (Fig. 5.5). During 2 weeks the differences in cell volume among cells cultured at 9.5°C and at higher temperatures were considerable. However, after 18 days cell volume became comparable among populations.

Populations never consisted solely of unicells and two-, four- or eight-celled coenobia, but always contained a fraction of three-, five-, six-, seven-celled coenobia and even some aggregates with more than eight cells (all indicated as rest-group in Fig. 5.2). At 9.5°C numerous cells and coenobia had irregular shapes, while this was not observed at higher temperatures where the majority of coenobia were isofacial, alternating coenobia.

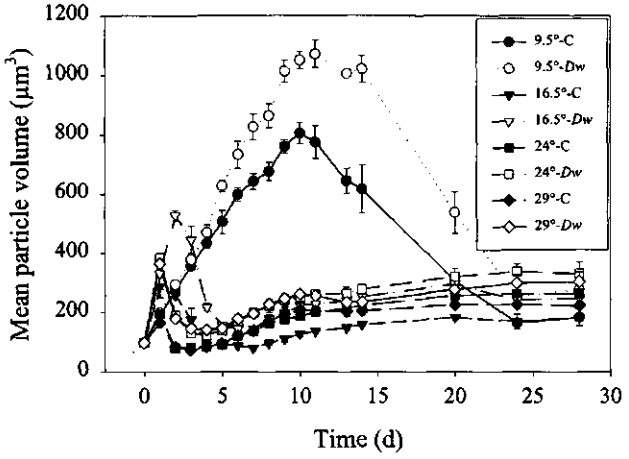


Figure 5.4: The mean particle volume (µm³) of *Scenedesmus acutus* populations cultured for four weeks at four temperatures in the absence (Control) or presence of medium from a *Daphnia* culture (*Daphnia* water). Error bars indicate 1 SD (n = 4).

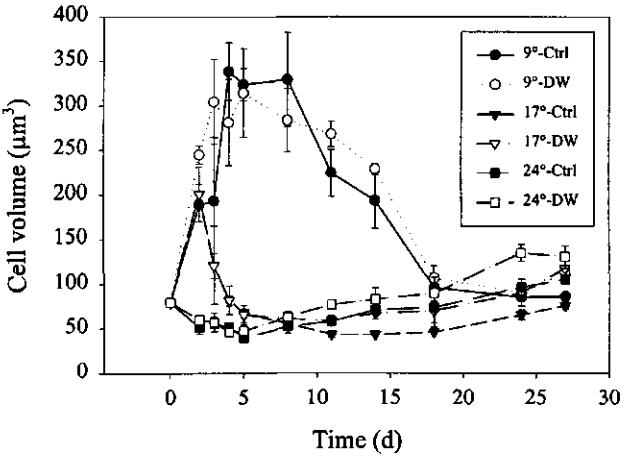


Figure 5.5: The mean cell volume (μm^3) of *Scenedesmus acutus* cultured for four weeks at different temperatures in the absence (Control) or presence of medium from a *Daphnia* culture (*Daphnia* water). Error bars indicate 1 SD (n = 4).

5.3 EFFECT OF TEMPERATURE ON GROWTH AND GRAZER-INDUCED COLONY FORMATION

Temperature affects the growth rate of the algal populations (e.g. Cloern, 1977; Harris, 1986; Ojala, 1993). Therefore, the mean number of cells per colony, the mean cell volume and the mean colony volume at the end of the exponential growth phase were used for comparison of the effects of temperature and infochemicals on morphology in *S. acutus*. Incubations run at 9.5°, 16.5°, 24° and 29°C were analyzed to the full extent in the previous section (§5.2). An additional series of experiments was run in quadruplicates at 11.2°, 13.5°, 14.7° and 17.1°C respectively. The experiments were run analogous to the experiments described in §5.2.

Growth rates (μ, day^{-1}) were determined during the exponential growth phase and were based on the number of cells (N) and on the algal volume (V) according the equation: $\mu = \ln (N_t/N_0) \times \Delta t^{-1}$. The number of doublings per day was calculated using growth rates based on cell numbers: $D_d = \mu_{cells}/\ln 2$.

Estimated growth rates and morphological characteristics were compared using two-way ANOVA, with temperature and absence/presence of *Daphnia* water as the two fixed effects.

Growth rates on the basis of cell numbers (Fig. 5.6) were significantly affected by temperature ($F = 299.0$; $P < 0.001$), but not by *Daphnia* infochemicals ($F = 1.61$; $P = 0.211$). No interaction effect was detected ($F = 0.98$; $P = 0.457$). On the basis of volume, however, besides a temperature effect ($F = 1880$; $P < 0.001$), growth rates were also influenced by *Daphnia* chemicals ($F = 6.94$; $P = 0.011$). Again no significant interaction effect was observed ($F = 1.12$; $P = 0.369$). Comparison of growth rates based on cell numbers with rates

based on volume revealed significant differences for controls ($F = 51.9$; $P < 0.001$) and for treatments ($F = 170.1$; $P < 0.001$). Only at the two highest temperatures volume based growth rates were not higher than cell number based growth rates. The number of doublings per day varied between 0.41 at 9.5°C and 2.3 at 24°C.

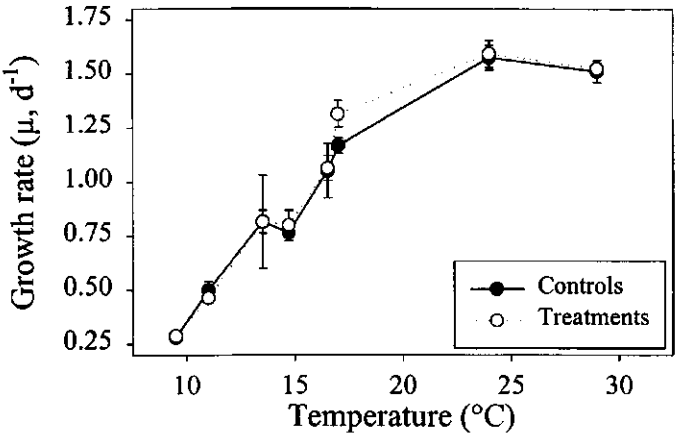


Figure 5.6: Effect of temperature on growth rates (μ, d^{-1}) of *S. acutus* in standard medium (Controls) and in medium with 10% (v/v) water from a *Daphnia* culture (Treatments). Error bars indicate 1 SD (n = 4).

The expression of morphotypes varied within a population in time, between populations in the absence and presence of *Daphnia* infochemicals, and at different temperatures (*see* Fig. 5.2). The number of cells per colony and the mean particle volume at the end of the exponential growth phase were used for comparison of the effects of temperature and infochemicals on colony formation in *S. acutus* (Fig. 5.7). Two-way ANOVA on the mean number of cells per colony at the end of the exponential growth phase indicated a significant temperature effect ($F = 36.7$; $P < 0.001$), a significant *Daphnia* water effect ($F = 445.5$; $P < 0.001$) and a significant interaction effect ($F = 3.84$; $P = 0.002$). The individual factors 'temperature' and '*Daphnia* water' were compared with the MS for the interaction. Both of the individual factors were significant compared with the interaction, therefore the individual factors dominate and the interaction may be ignored (Burke, 1998). Tukey's test revealed that the numbers of cells per colony in control populations at 9°C were similar to those in *Daphnia* water treated populations at warmer temperatures (Fig. 5.7).

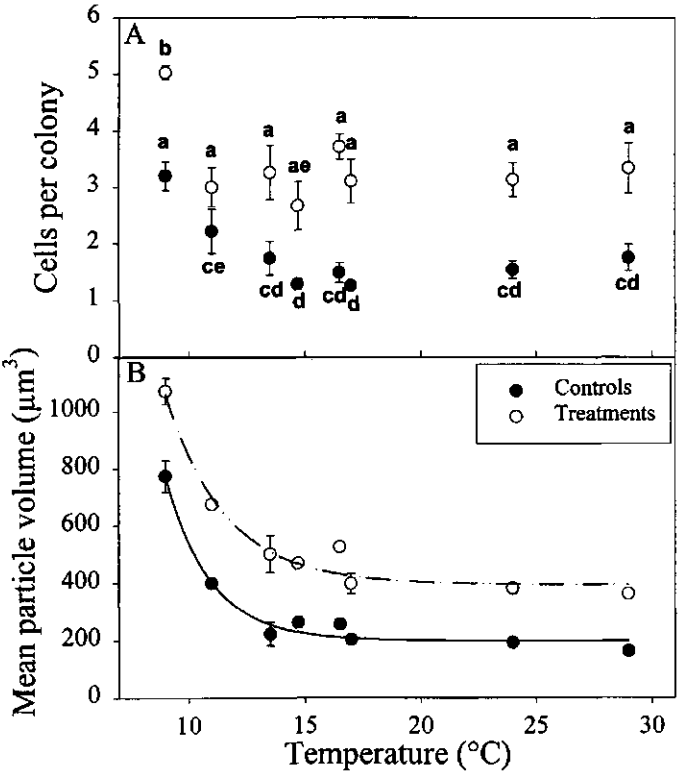


Figure 5.7: Effect of temperature and *Daphnia* infochemicals on colony size in *S. acutus* expressed as the number of cells per colony (upper panel) and the mean particle volume (μm^3, lower panel). Error bars indicate 1 SD (n = 4). Similar symbols in upper panel '**a...e**' indicate homogeneous groups that are not statistically different at the 95%-level (Tukey test).

The different incubation temperatures not only affected growth and colony development in *S. acutus*, but also had an effect on the individual cell size, expressed as mean cell volumes, at the end of the exponential growth phase (Fig. 5.8). Two-way ANOVA revealed a significant temperature effect ($F = 38.6$; $P < 0.001$), no effect of *Daphnia* water ($F = 0.74$; $P = 0.394$) and a significant interaction effect ($F = 4.93$; $P < 0.001$) on the mean cell size of *S. acutus*. The individual factors were tested against the interaction and the temperature appeared significantly different ($F = 7.83 > F_{crit} = 3.79$). Hence, temperature was the dominant factor. Since no statistically significant difference between cell volume of control and treatment populations was found, linear regression analysis was performed on all data. The cell volume was significantly reduced at higher temperatures (Fig. 5.8).

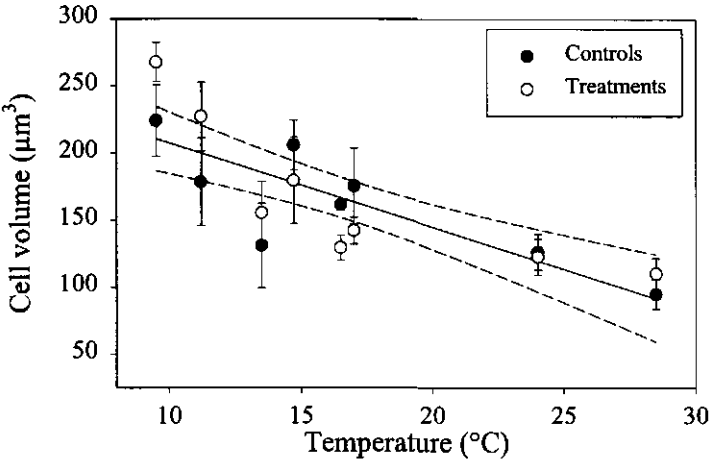


Figure 5.8: Effect of temperature on cell size in *S. acutus*, expressed as mean cell volume (μm^3), grown in standard medium (Controls) and in medium with 10% (v/v) water from a *Daphnia* culture (Treatments). Error bars indicate 1 SD (n = 4). Solid line represent linear regression: Cell volume = 270.14 - 6.266 × Temperature, $r = 0.812$; n = 16; $P < 0.001$. Dashed lines represent 95% confidence intervals.

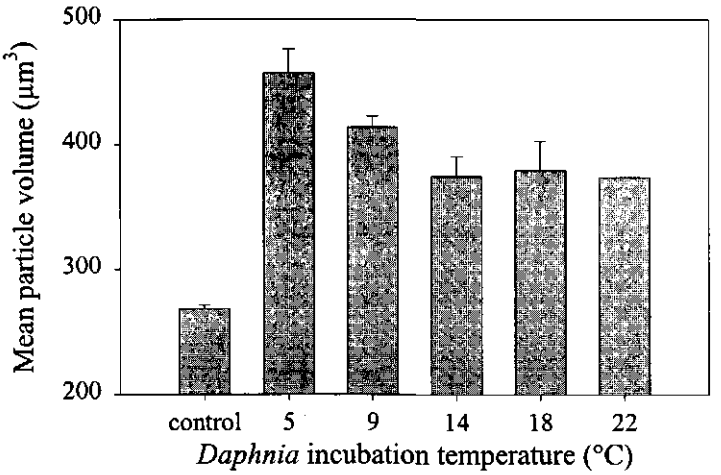


Figure 5.9: Colony formation in *S. acutus* expressed as the mean particle volume (μm^3, ± 1SD, n = 3) of populations exposed to filtered water (10%v/v) from *Daphnia* cultured at different incubation temperatures.

5.4 INFOCHEMICAL PRODUCTION AT DIFFERENT TEMPERATURES

Daphnia magna was exposed to 5 different temperatures of 5°, 9°, 14°, 18° and 22°C to examine the effect of temperature on the production of infochemicals. Twenty non-egg bearing animals (mean body length ± 1 SD: 1.68 ± 0.07 mm) were transferred into 100 ml

RT-medium with *S. acutus* as food (4.5 mg $C \cdot l^{-1}$) and stored at different temperatures in the dark. After 24 h the animals were transferred into new vessels with 100 ml food suspensions under the same conditions. Again after 24 h the medium was filtered and used as test-water in a standard biotest that was run in triplicate.

Medium from *Daphnia* incubations at different temperatures all contained colony inducing chemicals as the mean particle volume in all treatments was clearly higher than in the controls without water from a *Daphnia* culture (Fig. 5.9). No ANOVA was performed because the F_{max} test revealed heterogeneous variances ($F_{max} >> F_{crit}$), which was caused by the very low within group variance of the 22°C treatments.

5.5 DISCUSSION

Temperature had a clear effect on growth and morphological development in the green alga *Scenedesmus acutus*. Growth rate, colony size as well as individual cell size were affected by temperature. Moreover, *Daphnia*-induced colony formation appeared operating over a broad range of temperatures.

The *Scenedesmus* cell volume increased significantly at lower temperatures. Larger cell size of unicells and cells in colonies is not restricted to non-spiny *Scenedesmus*, but has also been observed in the spined *S. armatus* (Trainor, 1992a) and *S. communis* (Trainor, 1992b). This phenomenon could be a result of reduced growth as, in general, cells appear smaller at the maximum growth rate (Harris, 1986) which is influenced by the temperature according the van het Hoff's law. Cell volumes were measured at the end of the exponential growth phase, nevertheless at lower temperatures the total algal biomass appeared lower. Inasmuch algal biomass affects the availability of nutrients, smaller cell size at higher temperatures could be a result of carbon limitation as both higher cell densities and higher temperatures reduce the available carbon. Unicells and smaller colonies at higher temperatures may be advantageous because of a lower viscosity of the water.

The lower carrying capacity in treatment populations found in the experiment presented in CHAPTER 2 (*see* Fig. 2.1; Table 2.1) was not observed in the experiments reported here. The major difference between the experiments was that in the latter also the test-water from a *Daphnia* culture consisted of WC medium instead of RT medium.

The morphological expression at temperatures above 11°C was similar to the morphological development in *S. acutus* reported in CHAPTER 2. The formation of eight-celled coenobia at low temperatures, in the absence of infochemicals, has also been reported for the spined *S. subspicatus* (Trainor, 1993). The growth rates are in agreement with the growth rate for *S. communis* at 10°C (0.06 d^{-1}) and 22°C (0.44 d^{-1}) (Trainor, 1992). Moreover, the intrinsic rates of population increase at 16.5°C (0.23 d^{-1}) and 24°C (0.34 d^{-1}) are in good agreement with the growth rate of control populations at 20°C (0.31 d^{-1}) (Lürling, 1998). The number of

cells per colony in *Scenedesmus* is related to the amount of energy stored (Šetlík *et al.*, 1972) and may be proportional to growth rates (Gavis *et al.*, 1979; Siver & Freeda, 1982). However, Siver & Trainor (1983) demonstrated that unicell production was not determined by growth rate, but rather by the chemical composition of the medium. Gavis *et al.* (1979) observed at high growth rates eight-celled *S. quadricauda* coenobia, but not at low growth rates. In contrast, here, eight-celled coenobia were observed at high temperatures and high growth rates only in the presence of *Daphnia* water, but at low temperatures (and growth rates) also in the absence of *Daphnia* water. Thus, in *S. acutus* growth rates seem not to be a major factor in determining the colony size.

In the presence of *Daphnia* water more eight-celled coenobia were observed, the proportion of coenobia was larger at all four temperatures, and the coenobia formation occurred faster than in control populations. In the absence of *Daphnia* water when cultures had reached steady-state four-celled coenobia were formed analogous to the spined species *S. subspicatus* (Trainor, 1993), *S. communis*, and *S. komarekii* (Egan & Trainor, 1990). The medium from the *Daphnia* culture contained more organic carbon and ammonium (Lürling, 1998). Nagy-Tóth *et al.* (1992) examined the effect of several carbon sources on the morphology of *S. acutus*, but did not report any formation of eight-celled coenobia. Also ammonium and urea were ineffective as colony inducing agents (CHAPTER 3 and 4; Lampert *et al.*, 1994). Moreover, the combination of ammonium and organic carbon has been shown to favor unicell formation (Siver & Trainor, 1983). Nevertheless, the colony-inducing compound is probably an organic molecule with a low molecular weight (Lampert *et al.*, 1994). The chemical structure has not been resolved yet, but considerable progress has been made (Von Elert, *pers. comment*). The *Daphnia* factor may be characterized as an olefinic carboxylic acid (Von Elert & Franck, *in press*).

Initially, the *Scenedesmus* cell volume increased significantly at lower temperatures. At low temperature larger cell size of unicells and coenobial cells is not restricted to *S. acutus*, but has also been observed in *Scenedesmus sp.* (Rhee & Gotham, 1981), in the spined *S. armatus* (Trainor, 1992a) and *S. communis* (Trainor 1992b). *S. communis* colonies appeared 38.5 µm wide at 10°C, but only 11 µm at 22°C (Trainor, 1992b). Also four-celled *S. quadricauda* coenobia were differently sized at low (11°C) and high (35°C) temperature, with colony widths of 41.3 and 13.6 µm, respectively (Komárek & Ružička, 1969). In both *S. subspicatus* and *S. abundans* unicells were larger at a low temperature of 10°C than at a warm temperature of 25°C (*see* Table 6 on page 240 in Trainor, 1998). This phenomenon could be a result of reduced growth, as in general cells appear smaller at the maximum growth rate, which is influenced by the temperature (Harris, 1986). However, in this study, at cold temperature cell volumes gradually declined and became similar to those of cells cultured at warmer temperatures. Also Trainor (1998) reported a decline in unicell dimensions after a prolonged time at low temperature. Initially, at cold temperatures the total algal biomass was

lower compared to cultures at warmer temperatures, because of reduced growth. Inasmuch algal biomass affects the availability of resources smaller cell size at higher temperatures could be a result of carbon/light limitation due to high algal densities. As population density increased at low temperature cell size concomitantly declined. In previous experiments (*see* CHAPTER 4) it appeared that the availability of inorganic carbon played a significant role in determining the cell size of unicells in *S. acutus*, but was not involved in the amount of cells per colony. Thus, the availability of carbon/light might explain the differences in cell size, but not the phenomenon of colony formation at low temperatures, which seems a common response in *Scenedesmus* (Trainor, 1993). The response at low temperatures in the absence of a chemical trigger from a predator seems puzzling, because why invest in a defense when no predator is present? After 18 days, the control populations consisted of 20% of eight-celled coenobia (*see* Fig. 5.2). Formation of *S. acutus* coenobia at low temperature may be tolerable because of the higher viscosity of the water that reduces the size effect on sedimentation, but sinking velocities of coenobia still exceeded those of unicells (Conway & Trainor, 1972; CHAPTER 6). The coenobia formation at low temperatures in the absence of a chemical trigger released from grazers may indicate a general over-wintering strategy governed by *Scenedesmus*. In the fall negatively buoyant colonies may develop which settle to the sediment and over-winter. Here, at cold temperature and in low light or darkness coenobia will disintegrate (Dehning & Tilzer, 1989). In the dark, also the cell volume will decrease because of carbohydrate respiration (Morgan & Kalff, 1975). In spring, when temperatures are favorable, along with sufficient nutrients and low grazer abundancy, unicells are released. These data seem to support the concept of a seasonal life history as presented by Egan & Trainor (1989).

Small sized organisms with a large surface-to-volume ratio may grow more rapidly than larger organisms (Turpin & Harrison, 1980; Smith & Kalff, 1982). However, volume based maximal growth rates in *Daphnia* water treatments appeared somewhat higher than in the controls. Perhaps this is caused by the production of additional wall material necessary to cement cells together in a coenobium (Trainor, 1998). In previous studies, deflections in the growth curve were examined just prior to populations changing from unicellular to four-celled dominance indicative of cell death (Egan & Trainor, 1989; Trainor, 1993; Lürling, 1998). Also during the grazing process massive algal cell death might occur and cell contents could be released into the environment. However, both algal homogenates and auxins appeared to be ineffective as colony-inducing agent or growth stimulator (CHAPTER 3; Lampert *et al.*, 1994; Lürling, 1998). Thus, the colony inducing chemicals are probably not constituents of the algal cells. The flexibility of ecomorph expression and somewhat higher growth rates when grazing-associated chemicals are present strongly suggest that costs may be involved, because otherwise the defensive colonial form would be the norm (Dodson, 1989). Costs of colony formation were not reflected in growth rates, which has also been observed by Hessen & Van

Donk (1993) and Lampert *et al.* (1994), but may be attributed to sinking out of the euphotic zone. Small coenobia and unicells posses a better buoyancy than large coenobia (Conway & Trainor, 1972; *see* CHAPTER 6) and could maintain a position in the water column where conditions are favorable to support excellent growth (Siver & Trainor, 1981).

It was hypothesized that low temperature populations would be dominated by coenobia and warm water populations by unicells, because the size effect on sinking could be tolerable at low but not at high temperatures due to the viscosity of the water (Lampert *et al.*, 1994). Although still grazer-induced coenobia formation occurred at low temperature, under nutrient-replete conditions, unicells and coenobia were larger at the lower temperatures than at the warmer temperatures. Moreover, more coenobia occurred at lower temperatures. These differences in cell- and colony size will undoubtedly have an effect on settling velocities of the different morphs. However, as follows from Stoke's law, the difference in densities between alga and water might be reduced and might compensate (partially) for the size effect on sinking. These observations seem to support the hypothesis of Lampert *et al.* (1994). If expressed as the mean number of cells per colony, grazer-induced colony formation was highest at the lowest temperature. By contrast, predator-induced morphological defenses in *Daphnia*, such as spination (Havel, 1985), helmet length (Hanazato, 1991) and crest size (Grant & Bayly, 1981) all were significantly higher and larger at warmer temperatures. The weaker response at higher incubation temperatures could be a result of significantly higher algal biomass and hence relatively fewer active compounds. These high algal biomass coincide with the biomass in a previous experiment at which induced coenobia gradually disappeared from the treated populations (Lürling, 1998).

In nature *Daphnia* abundance may vary considerably during a season and will be lowest during winter and summer (Sommer *et al.*, 1986). There will be definitely numerous grazers other than *Daphnia* present in a water body of which several may trigger the unicell-colony transformation (Van Donk *et al.*, 1999). But a process as grazing will probably be reduced at low temperature (Burns & Rigler, 1967), thereby lowering the excretion of colony inducing compounds. Thus, grazer-induced colony formation could be less important during those periods. In the summer period, *Scenedesmus* could flourish, whereas in autumn at low temperature the initially larger and heavier cells and coenobia may sink to deeper water layers and over-winter. In spring a new population could be established, perhaps simply by wind-induced resuspension of a few small unicells, but what temperature is favorable? Wasmund (1992) determined optimal temperatures for growth of *S. abundans* and *S. obliquus* around 24°C or higher. In contrast, he observed the highest biomass of *S. abundans* and *S. obliquus* in winter-spring at temperatures from 1 to 14°C (Wasmund, 1992). In his study, Wasmund (1992) concluded that grazing was the most important factor determining the development of a *Scenedesmus* population in nature. Indeed, grazers may affect *Scenedesmus* populations by both selective feeding on small morphotypes and chemical induction of eight-celled coenobia

and even large multicelled aggregates (Hessen & Van Donk, 1993). However, *Scenedesmus* growth and morphology is influenced by several other factors such as nutrients, light and temperature (Trainor, 1998) that all could interact with the *Scenedesmus* grazer interaction. Moreover, *Scenedesmus* may not only display variability in colony formation, but also in the formation of spines, bristles, mucilage, cell wall thickness and biochemical composition that all could hamper ingestion and digestion by grazers. Illustrative is the apparent conflicting reports on digestibility of *Scenedesmus* cells. *Scenedesmus* is considered excellent food for *Daphnia* (Lampert, 1977), whereas others have reported digestion resistance of *Scenedesmus* (e.g. Horn, 1981; Levitan, 1987; Kerfoot, 1987). A major difference is that the latter observed *Scenedesmus* from the field and isolated cells from *Daphnia* feces, while the former used laboratory cultures. Is there a connection between the presence of grazers and the thickness of the cell wall or was the lake *Scenedesmus* perhaps P-limited? Under phosphorus limitation the cell wall of *Scenedesmus* is thickened (Tilberg *et al.*, 1984), a phenomenon also observed in *Chlamydomonas* (Van Donk *et al.*, 1997) that could result in digestion resistance (Van Donk & Hessen, 1993; Van Donk *et al.*, 1997). Thus, it does seem that *Scenedesmus* may have evolved several defensive strategies to resist grazing, i.e. either avoid ingestion or digestion, however, interactions with other important factors such as temperature and nutrients have to be kept in mind.

CHAPTER 6

Grazer-induced colony formation in *Scenedesmus*: Costs of being colonial?

Parts of this chapter are based on:
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"Evaluating the role of costs as a constraint in the evolution of inducible defenses continues to be a complex issue. Costs studies need carefully specify different types of costs, and rarely can all possible costs be considered."

- R. Tollrian & C.D. Harvell 1999

6.1 INTRODUCTION

Pelagic photoautotrophic organisms are confronted with conflicting allometries of selection pressure (Lehman, 1988). The dependence on light puts a constraint on the size of algae, because the algal cells have to remain in suspension preferably in the euphotic zone. Moreover, dissolved nutrients must pass through the semi-permeable membrane into the cell. Thus, algal cells have to remain small with a favorable surface-to-volume ratio enabling fast growth and low sinking loss (Reynolds, 1984). However, algae are not only subjected to sedimentation, but also to other loss processes such as grazing, wash out, parasitism and death that all may result in the disappearance of viable cells from the euphotic zone (Reynolds & Wiseman, 1982). In general, grazing and sedimentation appear the major loss processes operating (Reynolds *et al.*, 1982). Algae face the risk of mortality from an entire assemblage of grazers and an effective way to withstand grazing pressure is through increase in size (Lehman, 1988). Thus, in the pelagic one could imagine an adaptive trade-off between defensive large sized algae and competitive advantageous small sized organisms (Boraas *et al.*, 1998).

The grazer-induced colony formation in *Scenedesmus* may be interpreted as an anti-grazer defense to reduce their vulnerability against grazing (Hessen & Van Donk, 1993; Lürling & Van Donk, 1996; Van Donk *et al.*, 1999; *see* CHAPTER 7). One might expect costs associated with grazer-induced colony formation, because otherwise the defensive form would be the norm (Dodson, 1989). Potential costs involved with colony formation are:

1). Reduced nutrient and light harvesting expressed in lower growth rates.

2). Enhanced sinking.

Altered surface to volume ratios may influence the nutrient uptake by colonial cells and colonies may absorb less light energy per surface area as a result of the so called "package-effect" (Kirk, 1994), which could result in lower growth rates. However, no clear effects on growth rates have been observed yet (Hessen & Van Donk, 1993; Lampert *et al.*, 1994; Lürling & Van Donk, 1997a; Lürling, 1998).

In *Scenedesmus* a parent cell divides up, inside the parent cell wall, into a number of daughter cells, which subsequently form a new colony or fail to form a colony and become unicells (Van den Hoek *et al.*, 1995). Especially in the non-spiny *Scenedesmus* strains the path from parent cell to release of unicells or colonies is similar, since a coenobium is formed inside the mother cell that either rapidly disintegrates or remains colonial (Nilshammar & Walles, 1974; Trainor, 1998). Hence, no large differences in growth rates are to be expected between parent cells producing colonies or unicells, but costs may be more apparent between colonies and unicells. As differences may be small a sensitive method to estimate costs of induced colonies is desired. The good correlation between the Photosystem II (PSII) electron flow and the rate of C-fixation in algae (Kolber & Falkowski, 1993; Geel, 1997) and algal growth rate (Hofstraat *et al.*, 1994; Geel, 1997) indicates that estimation of costs may be derived from chlorophyll fluorescence. Therefore, the

PSII-efficiency was estimated during the unicell-colony transformation and among populations of induced colonies and non-induced unicells at different light intensities.

Faced with the problem of sinking out of the euphotic zone, costs may also be attributed to enhanced sedimentation rates of colonies as according to Stoke's law large particles sink faster than small ones (Reynolds, 1984). Since *Scenedesmus* unicells and small coenobia possessed a better buoyancy than large coenobia (Conway & Trainor, 1972) they may be able to maintain a position in the upper water layers where conditions are favorable to support good growth (Siver & Trainor, 1981). However, the biochemical composition of grazer-induced colonies may be slightly changed with less protein and somewhat higher amounts of fatty acids per unit dry-weight reducing their density and thereby their sinking (Lürling *et al.*, 1997; *see* CHAPTER 7). Therefore, sinking rates of *Scenedesmus* populations cultured in the absence (mainly unicellular) and in the presence of grazer (*Daphnia*) infochemicals were determined.

6.2 LIGHT-HARVESTING AND PSII-EFFICIENCY

6.2.1 Package-effect

Absorbance of intact cells will differ noticeably from that of dispersed thylakoid fragments, which is referred to as the package-effect (Kirk, 1994). This package-effect may exert a considerable influence on the light-harvesting capability of algae varying in size; specific absorption coefficients decrease with increasing cell sizes (e.g. Haardt & Maske, 1987; Sathyendranath *et al.*, 1987; Kirk, 1994). To examine the influence of the package-effect on specific absorption by *Scenedesmus*, four strains of *S. obliquus* Turpin were cultured in the presence and absence of *Daphnia* water in a standard biotest, which was run in triplicate. The specific absorption coefficient ($m^{-2} \cdot mg \cdot chl\text{-}a^{-1}$) was derived from absorption spectra, normalized per unit (mg) chlorophyll-a concentration at its red peak around 675 nm. The chlorophyll-a and phaeopigment concentrations were determined according to the Dutch standard method (NEN-6520) using a Beckmann DU-64 spectrophotometer.

The specific absorption coefficient decreased with increasing colony size of *Scenedesmus* (Fig. 6.1). Not only size, but also changes in pigment composition could account for some variation in the absorption. However, two-way ANOVA revealed no significant differences in chlorophyll-*a* content between *Scenedesmus* exposed to *Daphnia* water compared to controls ($F = 0.12$; $P = 0.736$). Hence, effects on growth may be expected as colonies absorb less light per unit biomass than unicells do.

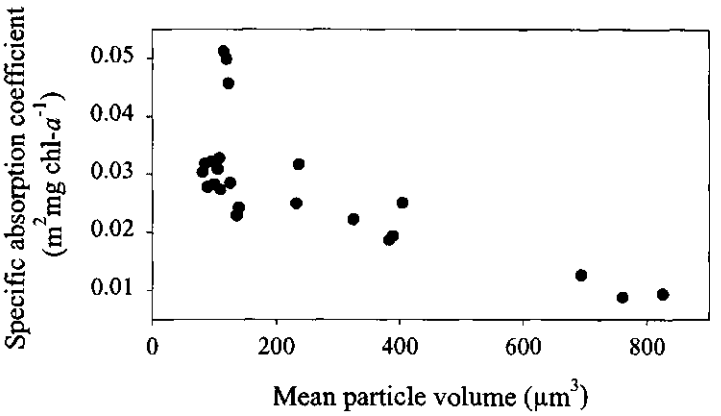


Figure 6.1: Relation between the specific absorption coefficient of *Scenedesmus* chlorophyll at the red maximum (670-680 nm) and colony size, expressed as mean particle volumes (μm^3).

6.2.2 PSII-efficiency

The green alga *Scenedesmus acutus* Meyen cultured in our laboratory in a 1.0 liter aerated chemostat on WC-medium (Guillard & Lorenzen, 1972). The chemostat was continuously illuminated at 100 $\mu mol \cdot m^{-2} \cdot s^{-1}$ provided by circular fluorescent tubes (Philips TLEM 40W/33RS) in a temperature controlled chamber at 20°C and with a dilution rate of 1.2 day^{-1}. Prior to the experiments, *S. acutus* from the chemostat were transferred into a 300 ml cellulose-plug stoppered Erlenmeyer flask containing fresh and sterile WC-medium. After 24 hours of adaptation to the desired light intensity of 120 $\mu mol \cdot m^{-2} \cdot s^{-1}$ algae from the Erlenmeyer flask were used as inocula in the experiments. In the experiments, *S. acutus* was transferred into 100 ml cellulose-plug stoppered Erlenmeyer flasks containing 50 ml of medium. Each batch culture contained *S. acutus* in autoclaved medium (controls) or in WC-medium with 5 ml membrane filtered water from a *Daphnia* culture (treatments). In a first experiment 4 replicates were used and 5 in a second experiment. The batch cultures were incubated for 3 days on a rotating shaking device (80 rpm.) at 20°C in continuous light of 120 $\mu mol \cdot m^{-2} \cdot s^{-1}$. The initial densities were $2.4 \cdot 10^4$ $cells \cdot ml^{-1}$ and $3.8 \cdot 10^4$ $cells \cdot ml^{-1}$ in the first and the second experiment, respectively. Algal densities and particle size distributions were determined in the size range 3.0-25.0 µm equivalent spherical diameter (ESD) using the Coulter Multisizer II (100 µm capillary). The number of cells per colony was determined microscopically by counting at least 120 aggregates (unicells as well as colonies) in a subsample preserved in Lugol's fixative. Cell dimensions (length and width, µm) were measured using a Leica Quantimet 500 MC image analyzer coupled with a light-microscope at 500 × magnification. Growth rates (μ, d^{-1}) were estimated from changes in algal biovolume (V) during the three day incubation period according to the equation: $\mu = \ln (V_t/V_0) \times \Delta t^{-1}$.

The efficiency of Photosystem II (PSII) electron transport (ϕPSII) was determined using a slightly modified version of the Xe-PAM fluorometer (Schreiber *et al.*, 1993; Geel *et al.*, 1997; Geel, 1997). The Xe-PAM is equipped with two halogen lamps for actinic and saturating light and a photodiode detector for detection. The white measuring light of the Xenon flash lamp for excitation is restricted to 400-560 nm by a 4 mm colour filter (Schott BG39). Emission is detected above 650 nm and the detector is protected from actinic and measuring light using two filters (Balzer R65 and Schott RG 645). The actinic light was adjusted with neutral density filters, the saturating light with an intensity of 5500 $\mu mol \cdot m^{-2} \cdot s^{-1}$ was filtered through a 650 nm short-pass filter (Balzer DT Cyan special). Irradiance was measured with a Skye photometer equipped with an integrating (PAR) quantum sensor (Geel *et al.*, 1997).

In the first experiment, the actinic light intensities used were 4.4; 8.5; 15.0; 33.5; 51.8; 97.7; 119.2; 171.5; 226 and 319 $\mu mol \cdot m^{-2} \cdot s^{-1}$. In the second experiment only actinic light of 120 $\mu mol \cdot m^{-2} \cdot s^{-1}$ was used. After dark adaptation for 30 minutes at 20°C, fluorescence of the algae (F0 and Fm) was measured in a temperature controlled DW2/2 oxygen electrode chamber (Hansatech, UK). Fluorescence nomenclature was according to Van Kooten & Snel (1990). At different actinic light intensities the actual (F) and maximal (F'm) fluorescence was measured and the mean of 5 measurements was used to calculate the efficiency of PSII e⁻-flow as (F'm - F)/F'm. Fluorescence measurements were performed with *Scenedesmus* at the end of the 3 day incubation period in experiment 1 and during the 3 day incubation period in experiment 2 at the beginning (t = 0), and consecutively after 24, 32, 48, 55 and 72 hours of incubation. Chlorophyll-*a* analysis was performed according to the Dutch standard method NEN-6520 using a Beckmann DU-64 spectrophotometer.

6.2.3 Results PSII-efficiency

- Colony formation:

In the first experiment, the exposure of *S. acutus* to water from a *Daphnia* culture significantly promoted the formation of colonies (Fig. 6.2). Both the mean particle volume ($t = 7.3$; $P < 0.001$) and the mean number of cells per particle ($t = 26.3$; $P < 0.001$) had significantly increased in treatments. The unicells were similarly sized in both controls and *Daphnia* water treatments with dimensions (mean length × width ± 1 SD) of 17.4 (2.2) × 5.1 (0.8) µm (n = 60) and 17.0 (1.9) × 5.7 (0.9) µm (n = 40), respectively. The dimensions of grazer-induced coenobia as observed in the treatments were on average 25.5 (3.4) × 22.1 (3.7) µm (n = 40) for four-celled and 37.2 (5.5) × 25.4 (3.2) µm (n = 40) for eight-celled coenobia. At the end of the experiment, no differences in the chlorophyll-*a* content among control and treated populations were observed ($t = 0.6$; $P = 0.546$) with a mean (± 1 SD) of 507 (73) $\mu g \cdot l^{-1}$.

In the second experiment, mean particle volumes of *S. acutus* were significantly larger in the presence of medium from a *Daphnia* culture (Fig. 6.2). The initial increase in volume in the controls reflects increased parental cell size. At the end of the experiment, the chlorophyll-*a* content

of control and treated populations were similar (t = 0.1; P = 0.956) with a mean (± 1 SD) of 588 (62) $\mu g \cdot l^{-1}$.

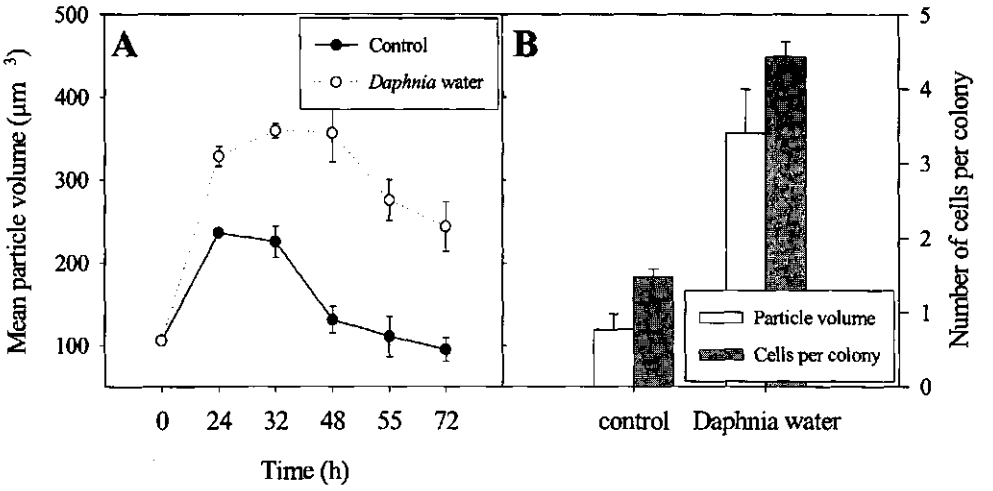


Figure 6.2: The course of the mean particle volume (μm^3) in cultures of *Scenedesmus acutus* grown in the absence (Control) and presence of filtered medium (10% v/v) from a *Daphnia* culture (*Daphnia* water) from the second experiment (left panel A) and both the mean particle volumes and mean number of cells per colony after 48 h in the first experiment (right panel B). Error bars represent 1 SD.

- **Growth rate**:

In both experiments examining the effect of morphology on PSII-efficiency, no significant differences in growth rates among populations cultured in the absence or presence of medium from a *Daphnia* culture were detected. In the first experiment, both control and treated populations expressed excellent growth with mean rates (± 1 SD; n = 4) of 1.39 (0.01) and 1.42 (0.03), respectively (P = 0.09). In the second experiment growth rates were 1.40 (0.03) and 1.43 (0.03) for control and treated populations, respectively (P = 0.06). The t-tests yielded low P-values due to small within group variation.

- **PSII-efficiency**:

No differences in ϕPSII of control and treated *S. acutus* were detected in both experiments (Figs. 6.3 & 6.4). The PSII-efficiency decreased with increasing light intensities as a result of increased non-photochemical quenching. In the second experiment, repeated measures ANOVA indicated no *Daphnia* water effect on ϕPSII (F = 0.80; P = 0.422), but a significant time effect (F = 143.5; P < 0.001). The ϕPSII had significantly increased during the course of the experiment, which could be the result of lower light intensities per alga due to higher algal biomass.

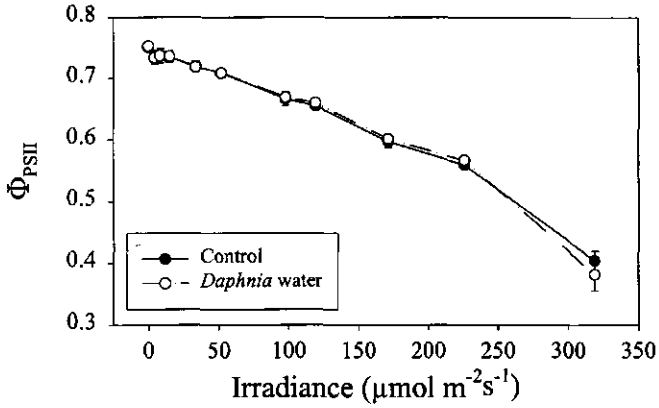


Figure 6.3: The quantum efficiency of PSII charge separation in the light (Φ_{PSII}) of unicellular (Control) and colonial (*Daphnia* water) *Scenedesmus acutus* at different light intensities. Error bars indicate 1 SD (n = 4).

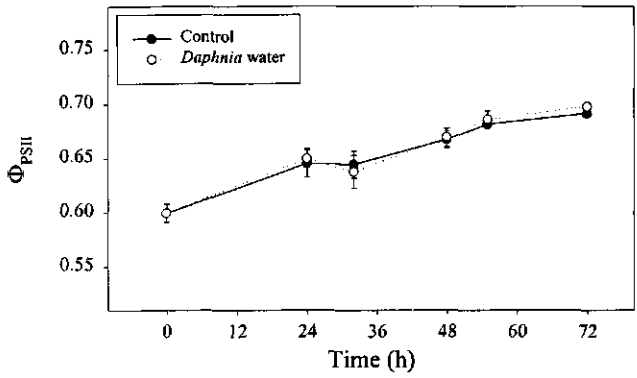


Figure 6.4: The quantum efficiency of PSII charge separation at 120 µmol·m^{-2}·s^{-1} (Φ_{PSII}) in time (h) of *Scenedesmus acutus* in the absence (Control) and presence (*Daphnia* water) of 10% (v/v) medium from a *Daphnia* culture. Error bars indicate 1 SD (n = 5).

6.3 METABOLIC COSTS REFLECTED IN GROWTH RATES OF INDUCED COENOBIA?

The gross growth rate (µ, day^{-1}) was estimated from changes in population density (N_0, N_t) and algal biovolume (V_0, V_t) between the start and the end of the biotests (the loss rate λ was assumed equal to zero) according to the equation: $\mu = \ln(N_t) - \ln(N_0) \times (\Delta t)^{-1}$.

Growth rates determined in the previous section were not significantly different among non-treated and treated cultures. For the analysis in this section, volume based growth rates of 14 biotest-experiments were joined. Statistical analysis revealed no differences between µ of control populations and µ of *S. acutus* cultured in the presence of water from a *Daphnia* culture (two-tailed t-test: $t = 1.66$; $p = 0.470$; df = 100). The within group variation was, however, considerable. The growth rates were on average (± 1 SD) 1.619 (0.200) d^{-1} for the controls and 1.648 (0.202) d^{-1} for

the treatments. Moreover, after examination of all biotests in this study one can only conclude that volume based growth rates in the presence of *Daphnia* water are not lower than in the absence.

6.4 SEDIMENTATION

6.4.1 Stoke's law

A constraint put on algal size is that the cells have to remain in suspension preferably in the euphotic zone. Below the compensation level, in the aphotic zone, respiration losses exceed gross photosynthesis. No growth occurs and eventually the cells may die because of a lack of the energy source light. Hence, algae should try to remain in suspension. Therefore, sedimentation may be a strong selective factor that favors the evolution of adaptations that reduce the sinking loss. According to the modified Stoke's equation:

$$v_{SED} = 2 \cdot g \cdot r^2 \cdot (\rho_a - \rho_m) \times (9 \cdot \eta \cdot \varphi_a)^{-1} \qquad (6.1)$$

in which v_{SED} = the sedimentation velocity ($m \cdot d^{-1}$), g = the earth's acceleration (9.8 $m \cdot s^{-2}$), r = radius of particle (m), ρ_a = density of algal particle ($kg \cdot m^{-3}$), ρ_m = density of medium ($kg \cdot m^{-3}$), η = dynamic viscosity ($kg \cdot m^{-1} \cdot s^{-1}$) and φ_a = form resistance of algal particle (-), algae can modify their size, density and/or form resistance to reduce sedimentation. The most important factor seems the size as the radius is squared in the equation. However, plotting literature data of the logarithm of average particle radius against the logarithm of mean sedimentation rates reveals a straight-line relationship, which is proportional to r rather than r^2 (Fig. 6.5). In fact, the exponent for r was 0.66 rather than 2, which is close to 0.7 as reported by Waite *et al.* (1992), suggesting that density and/or form resistance co-vary with size (Reynolds, 1984).

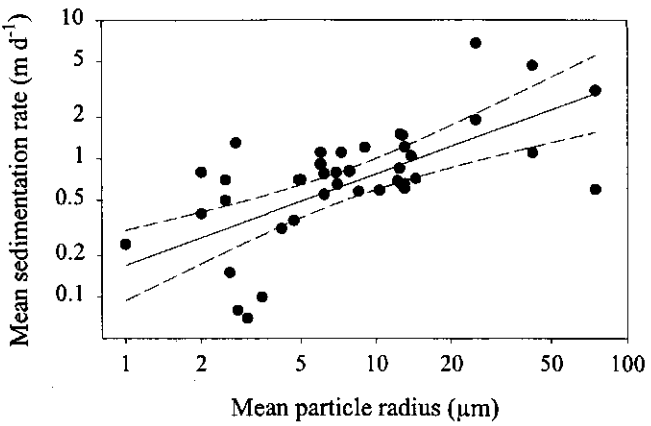


Figure 6.5: Log-log plot of v_{SED} ($m\ d^{-1}$) against corresponding r_a (µm) values. (Redrawn from data presented by Smayda, 1970; Titman & Kilham, 1976; Burns & Rosa, 1980; Reynolds, 1984; Waite *et al.*, 1992; Visser *et al.*, 1996). Straight line indicates linear regression log vSED = -0.772 + 0.661 × log r_a (r = 0.626; n = 42), dashed lines represent 95% confidence intervals.

6.4.2 *Sinking of Scenedesmus*

Sedimentation experiments were designed according the SETCOL procedure (cf. Bienfang, 1981). The sedimentation rate was calculated from the net change in vertical algal biomass distribution within a sedimentation column (Fig. 6.6) after a finite time:

The mean sinking velocity (v_{SED}, $m \cdot d^{-1}$) was calculated from the algal concentration at the settling region near the bottom (C_{sed}), the algal concentration remained in suspension (C_{sus}), the initial algal concentration (C_0), the height between the sampling ports (h) and the elapsed time (t) according to the equation:

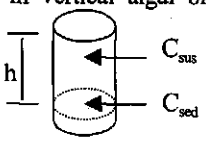


Figure 6.6: SETCOL

$$v_{SED} = (C_{sed} - C_{sus}) \times C_0^{-1} \times h \times t^{-1} \quad (6.2)$$

In a first experiment, sinking velocities were determined for *S. subspicatus* populations cultured in the absence and presence of one live *Daphnia*. A second experiment was conducted with unicellular *S. acutus* and mainly four-celled *S. communis*, while in a third and fourth experiment *S. acutus* populations cultured in the absence or presence of filtered medium form a *Daphnia* culture were used. The morphological characteristics of the strains used are presented in Table 6.1.

Table 6.1: The mean number of cells per colony (± 1 SD) and the mean particle volume (MPV in μm^3; ± 1 SD) for the *Scenedesmus* strains used in SETCOL-experiments.

Exp.	*Scenedesmus* strain	Cells per colony	MPV (μm^3)
I	*S. subspicatus* 'controls'	1.998 (0.221)	70.4 (3.3)
	S. subspicatus 'one live *Daphnia*'	5.142 (0.355)	217.2 (42.3)
II	*S. acutus*	1.124 (0.032)	58.6 (0.4)
	S. communis	3.450 (0.309)	844.5 (14.3)
III	*S. acutus* 'controls'	1.255 (0.032)	107.1 (4.0)
	S. acutus '*Daphnia* water'	3.172 (0.090)	476.8 (33.8)
IV	*S. acutus* 'controls'	2.455 (-)	193.3 (13.9)
	S. acutus '*Daphnia* water'	5.672 (-)	329.5 (21.4)

In all four experiments, the sinking velocities of the 'colonial' populations exceeded those of the 'unicellular' ones (Fig. 6.7). Significant differences per experiment were detected by *t*-tests. Separate *t*-tests revealed that in Exp. II, III and IV the sinking velocities of the colonial populations were significantly higher than in the unicellular populations (Table 6.2).

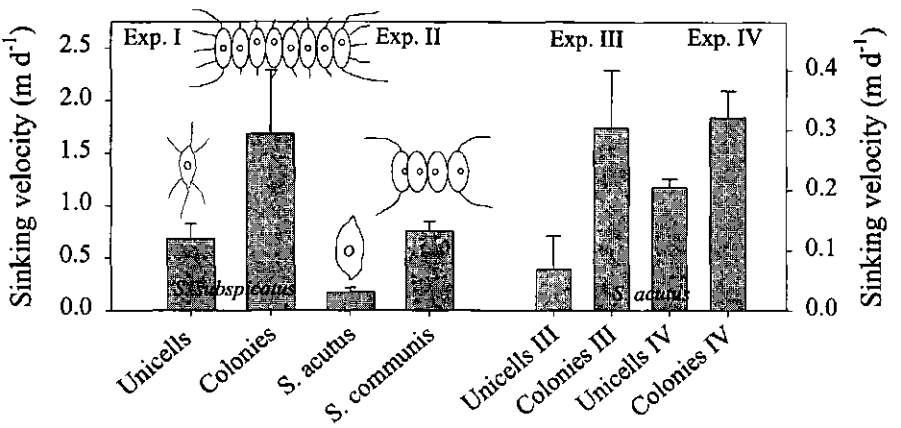


Figure 6.7: Sinking velocities ($m \cdot d^{-1}$) of unicellular and colonial *Scenedesmus* species in four SETCOL-experiments, including dominant morphotypes of strains used in Exp.I and II. Error bars indicate 1 SD.

In Exp. I, the high within group variance resulted in no statistically significant differences, but also in this experiment the colonial populations had higher sinking velocities. In *S. acutus*, the measured sinking velocities (v_{sed}) from Exp II, III and IV are correlated with both the mean numbers of cells per colony and the mean particle volumes (Table 6.2).

Table 6.2: *P*- and *t*-values of *t*-tests on the sinking velocities (v_{sed}) per experiment including results of regressions between v_{sed} of *Scenedesmus acutus* and the mean number of cells per colony (C/C) or the mean particle volume (MPV).

Exp.	*t*-value	*P*-value	Regression *Scenedesmus acutus*		Correlation (r) and *P*-value
I	2.77	0.109	v_{sed} - C/C:	$v_{sed} = 0.089 + 0.046 \times C/C$	$r = 0.832$; n = 5; $P = 0.040$
II	11.9	<0.001	v_{sed} –MPV:	$v_{sed} = 0.100 + 4.93 \cdot 10^{-4} \times MPV$	$r = 0.821$; n = 5; $P = 0.044$
III	3.73	0.014			
IV	4.98	0.003			

6.6 DISCUSSION

In larger cells or colonies the relatively smaller surface to volume ratio may reduce the rate of nutrient uptake (Reynolds, 1984), that could result in lower rates of growth (Banse, 1976). Reynolds (1984; Fig. 66) provides evidence that the maximal growth rates are significantly reduced in larger cells or colonies ($r = 0.531$; n = 16; $P = 0.017$), but below a particle volume of $\sim 10^4$ μm^3 this correlation is lost ($r \approx 0.25$; n = 13; $P \approx 0.2$). *Scenedesmus* generally has particle volumes below $\sim 10^4$ μm^3 and in this study no negative effect of colony size on growth was observed. As pointed out in § 6.1 this could be due to the fact that the path from parent cell to unicells or colonies in *S. acutus* is more or less similar and thus no large differences may be

expected. Moreover, between unicells and induced colonies no differences in the PSII-efficiency were observed. Especially at low light conditions differences were expected since theoretically costs would be measurable when no compensation is possible, for example when some resource, in this case light, is limiting. Although no differences in chlorophyll-*a* content between the two morphotypes was found, the pigment composition of induced colonies seems slightly different in a way that they are better adapted to low light which could explain that no effects on PSII-efficiency were found (Wiltshire, *unpublished data*).

The regression between the mean particle volume and the mean number of cells per colony has revealed a slope of less than 1 (*see* Fig. 3.1), which indicates that the individual cell size decreases with increasing colony size (Lampert *et al.*, 1994). However, this implies that when total algal volume is not reduced in *Daphnia* water treatments, the total cell number will be enhanced. By other means, cell multiplication will be enhanced, which is clearly demonstrated in the experiment with different *S. obliquus* strains where the number of doublings per day was significantly higher in three strains exposed to *Daphnia* water (§ *6.2.1*; *see* §*8.2.2.* and Fig. 8.4 therein). It could, therefore, be possible that the colony inducing substance is used as substrate for growth that has an effect on wall formation, because in a colony additional material is necessary in a cementing substance (Trainor, 1998). Siver & Trainor (1981; 1983) demonstrated that the unicell/colony transformation in *Scenedesmus* is independent of growth rate, but could be achieved by altering the chemical environment. In this study, chemicals excreted from the grazer *Daphnia* altered the environment. Apparently, no metabolic costs are associated with colony formation in the laboratory, which has also been reported by Hessen & Van Donk (1993) and Lampert *et al.* (1994) (Table 6.3). However, these reports seem in direct conflict with the unicell-superiority-hypothesis and the observation of competitive superior unicellular *Chlorella* (Boraas *et al.*, 1998).

Table 6.3: Growth rates (μ, d^{-1}; ± 1SD) of *Scenedesmus* cultured without (controls) or with filtered water from a *Daphnia* culture (treatments).

Scenedesmus	Temperature °C	Controls	Treatments
S. subspicatus[1]	20	0.35 (0.09)	0.39 (0.10)
S. subspicatus[1]	20	0.65 (0.06)	0.60 (0.11)
S. acutus[2]	22	1.32 (0.07)	1.35 (0.04)

[1]Hessen & Van Donk (1993)

[2]Lampert *et al.* (1994)

Faced with the problem of sinking out of the euphotic zone, costs of grazer-induced colony formation may also be attributed to enhanced sedimentation rates of colonies. Colonies had higher sinking rates than unicells, which has also been reported by Conway & Trainor (1972). The measured sinking rates (0.07 – 1.68 $m \cdot d^{-1}$) are in good agreement with literature data for *Scenedesmus* that vary from 0.07 to 1.1 $m \cdot d^{-1}$ (Titman & Kilham, 1976; Burns & Rosa, 1980;

Trainor & Egan, 1988; Visser *et al.*, 1996). The unicellular and the colonial *Scenedesmus* populations were not comprised of just unicells or eight-celled colonies, but consisted of a mixture of unicells and different coenobia (two- to eight-celled). Hence, sinking rates were an average of the population instead of one specific morph. Nevertheless, in *S. acutus* the sinking velocities increased with an increased colony size.

Although unicells possessed more buoyancy than colonies (Conway & Trainor, 1972) not only *Scenedesmus* unicells, but also forms with bristles, multispined coenobia and gametes are morphotypes with a greater resistance to sinking (Trainor, 1969; 1992; Lukavsky, 1991). Especially bristles reduce sinking in *Scenedesmus* (Conway & Trainor, 1972). These bristles may occur in spined and non-spiny *Scenedesmus* (Trainor & Burg, 1965; Burg & Trainor, 1967; Massalski *et al.*, 1974) and even in *S. acutus* (Marcenko, 1973), but no bristles were observed in the experiments. Thus probably size and density were the most important factors determining *S. acutus* sinking here.

Unicells may not only settle slower than colonies, but could also more easily be resuspended or moved to upper water layers. Sinking to deeper water layers could imply reduced growth as light and temperature may be lower. In a water body, light intensity (I) gradually decreases over depth (z) according the Lambert-Beer's law:

$$I_z = I_{in} e^{-kz} \tag{6.3}$$

where k = total vertical attenuation coefficient (m^{-1}), that is comprised of k_{water}, k_{humic}, k_{algae} and $k_{detritus}$ and z = depth (m). Since light provides the energy for growth, algal growth $\mu(I)$ is a function of the irradiance available for photosynthesis at a given depth:

$$\mu_I = \mu_{max} \frac{I}{K_I + I} \tag{6.4}$$

The K_I = the half-saturation light intensity for *Scenedesmus* (3.5 $W \cdot m^{-2} \approx 160$ $\mu mol \cdot m^{-2} \cdot s^{-1}$ according to Rhee & Gotham, 1981). The vertical position of an algal cell in time is determined by its size, shape and density according to: $\Delta z = v_{sed} \cdot \Delta t$, with $v_{SED} = 2 \cdot g \cdot r^2 \cdot (\rho_a - \rho_m) \times (9 \cdot \eta \cdot \varphi_a)^{-1}$. The effect of differently sized cells, with different settling rates, will result in different vertical positions in time and, hence, in differences in growth. Consider a hypothetical unicell and colony at the surface of water with a k of 2 m^{-1} and in 1000 $\mu mol \cdot m^{-2} \cdot s^{-1}$ light have settling rates of 0.05 and 1.0 $m \cdot d^{-1}$, respectively. After 12 h the theoretical growth rate of the colony will be reduced to 87% the rate of the unicell, because of sedimentation (Fig. 6.8).

Here growth was only affected by light with a constant incident irradiance and not limited by other resources, the vertical attenuation coefficient was assumed constant over depth, spectral shifts over depth did not occur and the v_{sed} was constant because the algae had a fixed morphology. Nevertheless, colonies will sink faster and even if they disintegrate after a certain period their vertical position may be different from unicells with lower sinking rates. Inasmuch colonies could experience lower growth rates than unicells, grazer-induced colony formation in *S. acutus* may have evolved because of the trade-off between sinking and reduced vulnerability to grazers.

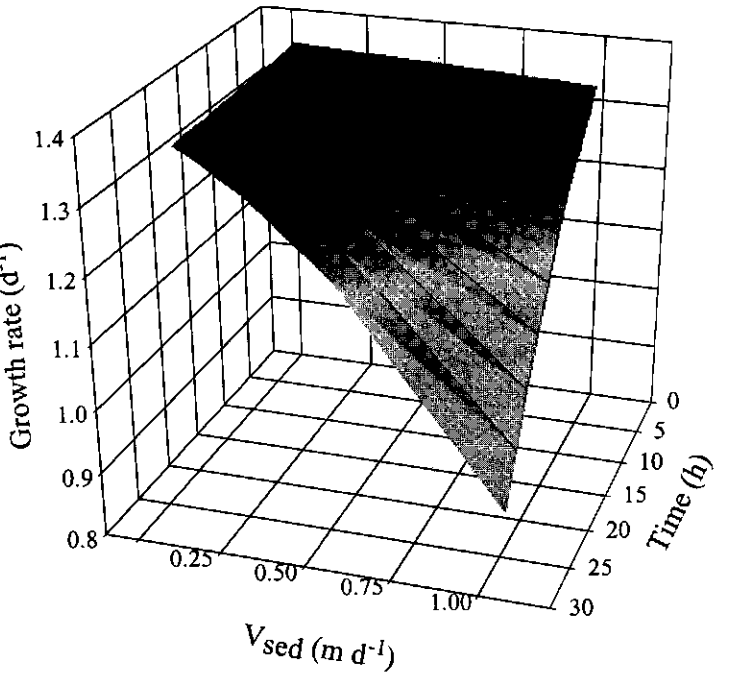


Figure 6.8: Computed growth rates (d^{-1}) in time (h) for cells with different settling velocities ($m \cdot d^{-1}$).

The trait colony formation is, however, only one of the potential anti-grazer defenses in *Scenedesmus* (Fig. 6.9). The genus *Scenedesmus* may be subdivided into two subgenera, *Scenedesmus* containing the non-spiny and *Desmodesmus* containing the spined species (Kessler *et al.*, 1997). Spines may be effective against small predators, whereas bristles of over 100 µm long may form a net that may discourage even larger grazers (Trainor & Egan, 1988). Thick cell walls and mucilage may give *Scenedesmus* resistance to digestion (Horn, 1981; Levitan, 1987; Van Donk & Hessen, 1993). Mucous could also be involved in the easy attachment of *Scenedesmus* to substrates thereby leaving their pelagic habitat (Otten & Willemse, 1988) analogous to flagellates that may adjust their recruitment to the water column in response to grazing pressure (Hansson, 1996). Some chemical compounds in *Scenedesmus* could be toxic to grazers and may even kill *Daphnia* (Boersma & Vijverberg, 1995).

A defense could also be constitutive under conditions when grazers are always present or when the environment is highly predictable (Dodson, 1989; Brönmark & Petterson, 1994).

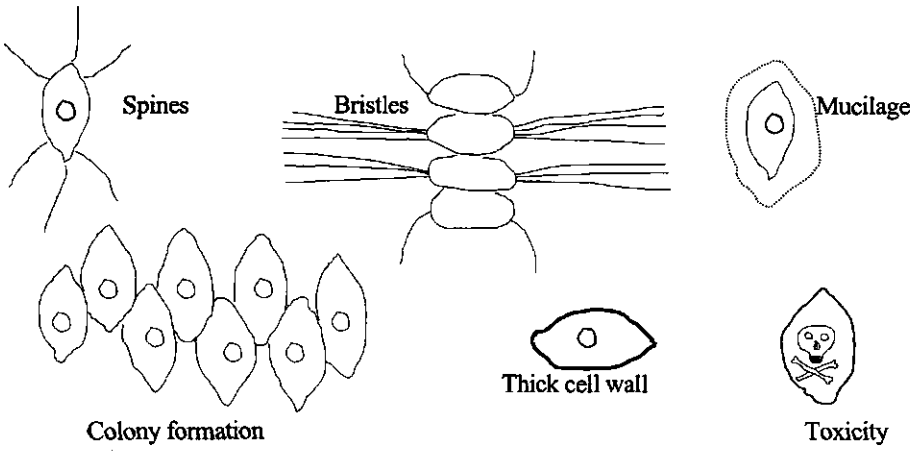


Figure 6.9: Potential defenses in *Scenedesmus*: Spines, Bristles, Mucilage, Colonies, Thick cell wall and Toxicity.

In surface waters, grazers are always present, but the abundance, activity and taxonomic composition may vary greatly both on spatial and temporal scale. *Scenedesmus* is exposed to an assemblage of grazers varying from small protozoan to large metazoan predators. *Daphnia* can easily ingest unicells and small *Scenedesmus* coenobia (Lampert *et al.*, 1994), but not large eight-celled coenobia (Hessen & Van Donk, 1993). Most coenobia will undoubtedly be too large to be grazed by protist grazers, such as the phagotrophic flagellate *Paraphysomonas* (Grover, 1989). A fixed defense, or a phenotypic stability with four- or eight-celled coenobia as the most dominant morphs, would still confront *Scenedesmus* with the problem of sinking. Although bristles but also spines reduce the sinking in *Scenedesmus*, colonies still experience higher sinking losses than unicells (Conway & Trainor, 1972). Since no metabolic costs were detected and based on the plastic nature of the defense, costs have to be attributed to sinking out of the euphotic zone. This could, however, also be interpreted as an escape in time since *Scenedesmus* is capable of surviving prolonged periods of darkness (Dehning & Tilzer, 1989), where coenobia disintegrate and unicells may serve as inocula for subsequent blooms (Dehning & Tilzer, 1989; Egan & Trainor, 1989a,b). However, one has to keep in mind that even in the absence of grazers *Scenedesmus* may express considerable morphological variability (Trainor, 1998). Grazing is only one of the selection pressures that has shaped *Scenedesmus* plasticity.

CHAPTER 7

Impact of grazer-induced colony formation in *Scenedesmus* on herbivorous zooplankton

Parts of this chapter are based on:
Lürling, M. & Van Donk, E. (1996). *Oecologia* **108**: 432-437
Lürling, M., De Lange, H.J. & Van Donk, E. (1997). *Freshwater Biology* **38**: 619-628

"Grazing pressure is not a constant force on the many diverse species that compose the phytoplankton communities, and it is unlikely that antiherbivore compromises are directed against single zooplankton species"

- J.T. Lehman 1988

7.1 INTRODUCTION

In aquatic ecosystems planktonic herbivores are confronted with a broad range of both edible and inedible algal taxa. Edible algae are considered easily encountered, ingested and digested by rotifers, copepodes, and cladocerans. Among the most successful species of zooplankton in freshwater systems are members of the suspension feeding genus *Daphnia*. It is commonly accepted that their success depends on the ability to feed efficiently on a wide size range of particles. Clear relationships between the grazers' body-size and the size of edible particles exist (Burns, 1968). The upper limit of edible particles depends on the grazers' body size, and generally this limit is lower for smaller daphnids. The dietary width is mainly determined by the upper limit since the lower size limit does not differ much among species (DeMott, 1982; 1986). This means that in situations where large algae, inedible for a small species A, but still edible for a larger species B, are more abundant, the species B will ultimately dominate the zooplankton community. Natural phytoplankton assemblages can be highly variable in species composition and there is broad consensus that dominance of inedible algal species is promoted when grazing pressure on edible algal taxa is high (Sommer *et al.*, 1986).

It is known from several studies that *Daphnia* can alter the morphological appearance of phytoplankters. *Aphanizomenon* shows a shift from flakes in the presence of *Daphnia* to single filaments or smaller flakes in its absence (Lynch, 1980; Holm *et al.*, 1983). *Synedra* occurs as colonies consisting of dozens to hundreds of cells in presence of *Daphnia*, but as single cells in its absence (Sterner, pers. comm). Also *Scenedesmus* can form large multicelled aggregates in the presence of *Daphnia* and eight-celled coenobia may be common when infochemicals released from grazing *Daphnia* are present (Hessen & Van Donk 1993, Lampert *et al.*, 1994; This thesis). The presence of large and heavily spined colonies of *Scenedesmus subspicatus* reduces grazing by *Daphnia magna* (Hessen & Van Donk, 1993), but when fed non-spiny colonies of *S. acutus* no depressed grazing by *D. magna* was observed (Lampert *et al.*, 1994). The colonies of the non-spiny *S. acutus* may, however, exceed the size of grazable particles for juveniles and small zooplankters. Therefore, in this chapter the effects of different *Scenedesmus* morphotypes on feeding and growth of herbivorous zooplankton are examined.

7.2 ROTIFERS

7.2.1. Animals

The rotifers *Brachionus calyciflorus* and *Keratella quadrata* were obtained from the culture collection at the Max-Planck-Institute for Limnology (Plön, Germany). Both rotifer species were cultured separately in a climate-controlled room at 20 ± 1°C on WC-medium with *S. acutus* as food. *B. calyciflorus* was cultured in a 350 ml chemostat system with a

dilution rate of 0.35 d^{-1}, while *K. quadrata* was cultured in a 350 ml batch system and fed every two days with *S. acutus* (1 mg $C \cdot l^{-1}$).

7.2.2. Ingestion experiments

Ingestion (IR) and clearance rates (CR) of *B. calyciflorus* and *K. quadrata* feeding on *Scenedesmus* were determined with ^{14}C-labelled algae analogous to the method described by Rothhaupt (1995). Algae (10 ml) were harvested from the culture vessels and added to 40-ml fresh WC-medium (non-induced) or WC medium with rotifer exudates (induced) in 100-ml cellulose-plug-stoppered Erlenmeyer flasks. The 50-ml algal suspensions were incubated with 2.5 MBq $NaH^{14}C0_3$ for 24 h at 95 $\mu mol \cdot m^{-2} \cdot s^{-1}$ continuous light provided from above by daylight fluorescent tubes (Osram L18W/19 5000 Deluxe daylight). Additional algal suspensions were not labeled but received 1 ml from a $NaHCO_3$ solution (12.6 $g \cdot l^{-1}$) to allow determination of algal morphological characteristics. Labeled algae were centrifuged twice and resuspended in WC medium. The algal concentrations in carbon equivalents (mg $C \cdot l^{-1}$) were determined using a calibration curve of extinction at 800 nm *vs.* carbon content. Aliquots of 50 μl labeled algae were pipetted into scintillation vials. Experimental animals were pipetted from the cultures into 10 ml unlabeled food in 50 ml bottles and allowed to adapt to the desired food concentration. After at least 1 h of adaptation 25 ml labeled food with a similar concentration was added, and 2 × 1 ml food suspension was pipetted from the bottles and inserted into scintillation vials. After 10 minutes the experimental animals were collected on a 52 μm sieve, rinsed with de-ionized water, narcotized in carbonated water and killed in a Petri-dish with a few drops of formaldehyde (37%). Groups of 12 (*Brachionus*) or 25 animals (*Keratella*) were pipetted into scintillation vials. An additional vial was filled with a similar aliquot of fluid from the Petri-dish, but without rotifers, to check for background radioactivity. The rotifers were dissolved overnight at 60°C in 300 μl of tissue solubilizer (Soluene 350, Packard). Rotifers and 50 μl samples of labeled food were counted with 5 ml water absorbing scintillation cocktail (Instant scint-gel II plus + 5% Carbo-sorb, Packard), 1 ml labeled food samples were counted with 3.5 ml scintillation cocktail. The activity of the samples was measured in a Tri-Carb 1900CA liquid scintillation analyzer (Packard). Clearance and ingestion rates were calculated according to Peters (1984).

7.2.3. Results rotifer grazing

Rotifers were fed with 3 size classes of *Scenedesmus*, unicellular ("small") and colonial *S. acutus* ("medium") and colonial *S. armatus* ("large"). The morphological characteristics, such as cell dimensions and colony size, of unlabeled algae differed between the algal size classes (Table 7.1).

Table 7.1: Morphological characteristics of *Scenedesmus* size classes used as food for the rotifers *Keratella quadrata* (*Kq*) and *Brachionus calyciflorus* (*Bc*) presented as mean particle volumes (MPV, μm^3), mean number of cells per colony, equivalent spherical diameter (ESD, μm) and cell dimensions (length × width, μm).

Scenedesmus size	MPV (μm^3)		Cells colony^{-1}		ESD (μm)		Cell dimensions (length × width, μm)		
	Kq	*Bc*	*Kq*	*Bc*	*Kq*	*Bc*	Unicells	4-celled	8-celled
Small *S. acutus*	126	219	1.2	1.6	5.7	6.9	15 × 5	17 × 12	---
Medium *S. acutus*	355	382	3.1	3.8	7.9	8.3	16 × 6	22 × 17	33 × 25
Large *S. armatus*	695	2130	4.2	5.5	9.6	14.4	18 × 7	23 × 23	42 × 26

Differently sized *Scenedesmus* affected the feeding of both *B. calyciflorus* (Fig. 7.1) and *K. quadrata* (Fig. 7.2). Clearance rates decreased gradually with increased food concentration. *B. calyciflorus* had the highest clearance rates with medium sized *S. acutus* (4.3 ± 0.5 $\mu l \cdot h^{-1}$), *K. quadrata* with small *S. acutus* (7.4 ± 1.4 $\mu l \cdot h^{-1}$). The feeding of the rotifers on differently sized algal food showed remarkable differences. In *K. quadrata* larger sized *Scenedesmus* resulted in reduced clearance and ingestion rates. However, *B. calyciflorus* had the highest clearance and ingestion rates on medium sized *Scenedesmus*.

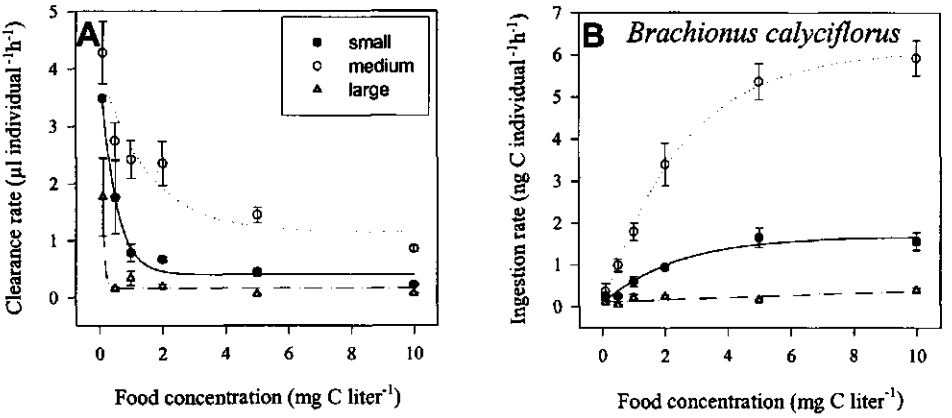


Figure 7.1: Clearance rates (ml·ind.$^{-1}$·h^{-1}: Panel A) and ingestion rates (ng C·ind^{-1}·h^{-1}: Panel B) for *Brachionus calyciflorus* feeding on ^{14}C-labeled small unicellular (●), medium sized (○) or large colonial *Scenedesmus* (▲) at different food concentrations (in mg C liter^{-1}). Error bars represent 1 SD (n = 8).

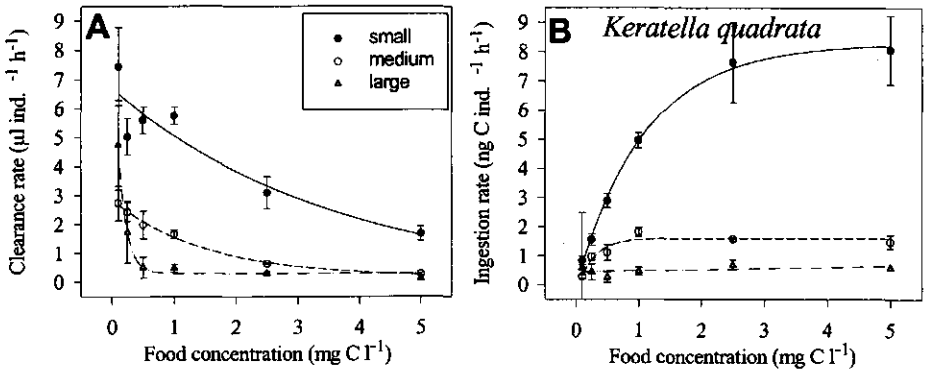


Figure 7.2: Clearance rates (ml· ind.$^{-1}$·h^{-1}: Panel A) and ingestion rates (ng C·ind^{-1}·h^{-1}: Panel B) for *Keratella quadrata* feeding on ^{14}C-labeled small unicellular (●), medium sized (○) or large colonial *Scenedesmus* (▲) at different food concentrations. Error bars represent 1 SD (n = 5).

To unravel whether the lowest CR and IR when feeding on *S. armatus* was due to algal size or the taste of an unknown food species, *B. calyciflorus* that had been cultured with *S. acutus* as food, was offered five different *Scenedesmus* species. Although all five strains had been cultured in the absence of a colony inducing zooplankton factor, they were not similarly sized (Table 7.2).

Table 7.2: Characteristics of five *Scenedesmus* species fed to *B. calyciflorus*.

Scenedesmus species	MPV (µm^3)	Cells colony^{-1} – dominant morphs	ESD (µm)
S. acutus	200	~2.0 - 1, 2 and 4 celled coenobia	6.7
S. armatus	730	~3.8 - 2 and 4 celled coenobia	9.9
S. falcatus	177	~1.5 - 1 and 4 celled coenobia	6.3
S.quadricauda	139	~1.2 - 1 and 2 celled coenobia	5.8
S. subspicatus	96	~1.2 - 1 and 4 celled coenobia	4.9

Differences in IR and CR of rotifers feeding on the various species were observed, but no major differences between animals feeding on *S. acutus* or *S. armatus* were detected (Fig. 7.3). At low food concentration, *B. calyciflorus* fed even better on *S. armatus* than on *S. acutus*. Hence, the lower IR and CR in the former experiment were probably due to a size effect rather than different algal taste.

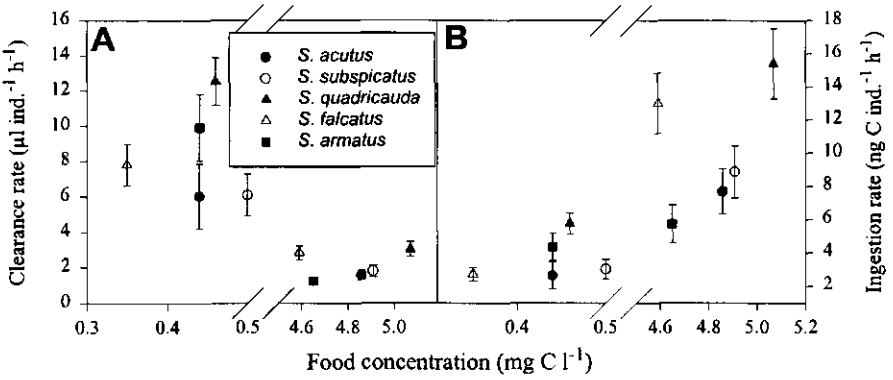


Figure 7.3: Clearance rates (ml·ind.$^{-1}$·h^{-1}: Panel A) and ingestion rates (ng C·ind^{-1}·h^{-1}: Panel B) for *Brachionus calyciflorus* feeding on ^{14}C-labeled *Scenedesmus acutus* (●), *S. subspicatus* (○), *S. quadricauda* (▲), *S. falcatus* (Δ) and *S. armatus* (■) at low (~0.5 mg C·l^{-1}) and high (~5 mg C·l^{-1}) food concentrations. Error bars represent 1 SD (n = 12).

7.3 Cladocerans

7.3.1 Animals

In a first experiment (§ *7.3.2*) the relatively small cladoceran *Ceriodaphnia reticulata* was used, isolated from lake Zwemlust (The Netherlands) and cultured in the laboratory on modified WC medium with *S. acutus* as food. In a radiotracer-experiment (§ *7.3.3*), *Bosmina longirostris*, isolated from lake Zwemlust, and the daphnids *Daphnia cucullata* TJ33 and *D. galeata* were used. The former daphnid was obtained from the culture collection at the Center for Limnology (NIE-CL, The Netherlands), the latter species was isolated from lake Zwemlust. All daphnids were cultured in the laboratory at 20°C in 1 liter jars on RT medium (Tollrian, 1993) with *S. acutus* as food.

7.3.2 Ceriodaphnia *grazing on* Scenedesmus

The non-spiny *S. acutus* was offered in three size classes to *Ceriodaphnia*: mainly unicells (~85 µm^3 per particle), mainly 2- and 4-celled coenobia (~250 µm^3) and mainly 4- and 8-celled coenobia (~600 µm^3). Spined *Scenedesmus* were offered in similar size classes, but as different species: *S. subspicatus*, unicellular (~60 µm^3), 2-4 celled *S. quadricauda* (~220 µm^3) and mainly 4-celled *S. protuberans* (~600 µm^3).

A five day old cohort of *Ceriodaphnia* was used in the experiment with animals having a body-length between 0.68 and 0.73 mm. Ten *Ceriodaphnia* were transferred in 50 ml *Scenedesmus* suspensions of similar biovolumes (5·10^6 µm^3·ml^{-1}) in 100 ml cellulose-plug stoppered Erlenmeyer flasks. The different *Scenedesmus* treatments were then incubated in quadruplicate for 18 h on a rotating shaking device in continuous light (100 µmol·m^{-2}·s^{-1}). Four incubations per *Scenedesmus* size class and species were incubated without *Ceriodaphnia* and served as controls. Algal biovolume and number of particles were

determined initially and after 18 h incubation in the size range 3.0 – 25.0 µm ESD using a Coulter Multisizer II (100 µm capillary). Clearance rates were calculated from Coulter data, corrected for growth in the controls, according the equation (7.1):

$$CR = \left(\frac{\left[\ln(C_{18} - C_0) - \ln(B_{18} - B_0) \right]}{\Delta t} \right) \times \left(\frac{V_E}{N} \right) \quad (7.1)$$

Where CR = clearance rate (ml·ind^{-1}·h^{-1}), C_{18} = algal concentration in control after 18 h, C_0 = initial algal concentration in control, B_{18} = algal concentration in treatment after 18 h, V_E = volume of medium in the experimental vessels (ml) en N = the number of *Ceriodaphnia* per Erlenmeyer.

The clearance rates of *Ceriodaphnia* were significantly affected by the different *Scenedesmus* size classes (Fig. 7.4). Two-way ANOVA indicated a significant size effect (F = 4.83; P = 0.021), but no significant difference between non-spiny and spined species (F = 0.42; P = 0.526) and no significant interaction effect (non-spiny/spined × size: F= 0.91; P = 0.421). Tukey's test revealed two homogeneous groups, whereby feeding on *S. quadricauda, S. protuberans* and the largest *S. acutus* size-class was significantly reduced compared to feeding on the smallest size-classes, the unicellular *S. subspicatus* and *S. acutus*.

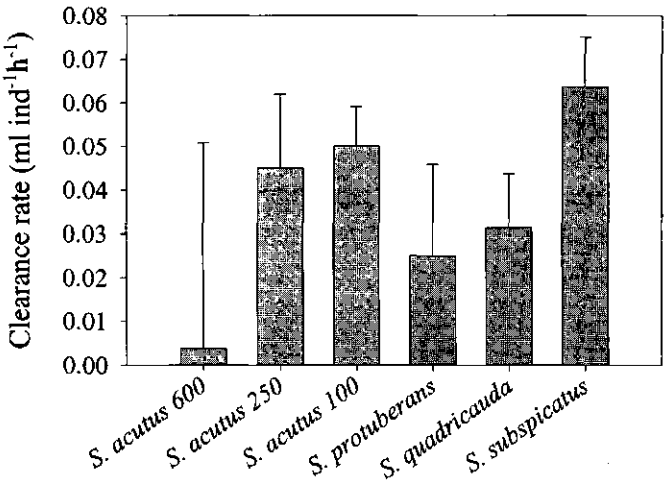


Figure 7.4: Clearance rates (ml·ind.$^{-1}$·h^{-1}) of *Ceriodaphnia reticulata* feeding on equal amounts of either non-spiny or spined *Scenedesmus* of different size classes (see text for more details). Error bars represent 1 SD (n = 4).

7.3.3 Grazing experiment with small cladocerans and ^{14}C-labelled Scenedesmus

Ingestion and clearance rates were determined in radio-tracer experiments for *Bosmina longirostris, Daphnia cucullata* and *D. galeata* feeding on unicellular or colonial

Scenedesmus. The labeled algae cultured as described in section *7.2.2* were centrifuged and resuspended in WC-medium. The food concentrations in carbon equivalents (mg $C \cdot l^{-1}$) were determined using a calibration curve of the extinction at 800 nm *vs.* carbon content. One concentration (0.6 mg $C \cdot l^{-1}$) was used for *B. longirostris*, a higher concentration (1.0 mg $C \cdot l^{-1}$) for *D. cucullata*, while three concentrations were used for *D. galeata*, low (0.1 mg $C \cdot l^{-1}$), intermediate (0.5 mg $C \cdot l^{-1}$) and high food (1.0 mg $C \cdot l^{-1}$). Experimental animals were pipetted from the cultures into 10 ml (*Bosmina*) or 25 ml (*Daphnia*) unlabeled food in 50 ml bottles and allowed to adapt to the desired food concentration. After 1 h adaptation, 25 ml labeled food with a similar concentration was added, and 2 × 1 ml of the suspension was immediately transferred into scintillation vials.

After 5 minutes for *Daphnia* or 10 minutes for *Bosmina* the experimental animals were collected on a 52 μm sieve, rinsed with de-ionized water, narcotized in carbonated water and killed in a Petri-dish with a few drops of formaldehyde (37%). Groups of 6 *Bosmina* were pipetted into scintillation vials, while *Daphnia*'s were pipetted individually into vials after their length had been measured. An additional vial was filled with a similar aliquot of fluid from the Petri-dish, but without animals, to check for background radioactivity. The animals were dissolved and the activity measured as described in section *7.2.2*. Clearance and ingestion rates were calculated according to Peters (1984).

7.3.3.1 Bosmina longirostris

Both clearance rate and ingestion rate of *Bosmina* were significantly reduced (*t*-test; $P < 0.001$) when the animals were offered large colonial *Scenedesmus* (Table 7.3).

Table 7.3: Mean clearance rates (± 1 SD; $ml \cdot ind.^{-1} \cdot h^{-1}$) and ingestion rates ($ng\ C \cdot ind.^{-1} \cdot h^{-1}$) of *Bosmina longirostris* feeding on similar concentrations (~0.6 mg $C \cdot l^{-1}$) of ^{14}C-labeled unicellular or colonial *Scenedesmus*.

Food type	Cells·colony^{-1}	Clearance rate ($ml \cdot ind.^{-1} h^{-1}$)	Ingestion rate ($ng\ C \cdot ind^{-1} \cdot h^{-1}$)	*N*
Unicells	1.55 (0.25)	0.041 (0.007)	25.88 (4.54)	8
Colonies	4.24 (0.11)	0.011 (0.002)	6.90 (1.22)	8

7.3.3.2 Daphnia cucullata

Clearance rates and ingestion rates of *D. cucullata* were lower when fed with colonial *Scenedesmus* than if a similar concentration unicells (1 mg $C \cdot l^{-1}$) was the food (Fig. 7.5). A *t*-test to check for differences between the regression lines revealed no significant differences for the clearance rates ($t = 1.50$; df =24; $P = 0.073$), however, ingestion rates were significantly different ($t = 5.98$ df = 24; $P < 0.001$).

7.3.3.3 Daphnia galeata

Two-way ANOVA on clearance rates (CR) of *D. galeata* indicated a significant food type effect (F = 35.2; P = 0.028) and a significant effect of food concentration (F = 54.2; P = 0.018). At low (0.1 mg C·l^{-1}), intermediate (0.5 mg C·l^{-1}) and high food levels (1.0 mg C·l^{-1}) clearance rates on colonies were significantly lower than rates on unicells (Table 7.4). However, two-way ANOVA on mean ingestion rates (IR) indicated no food type effect (F = 4.24; P = 0.176) and no effect of food concentration (F = 2.07; P = 0.326). Moreover, two-way ANOVA indicated no significant differences in body-length of the experimental animals, which were on average 0.95 (± 0.13) mm.

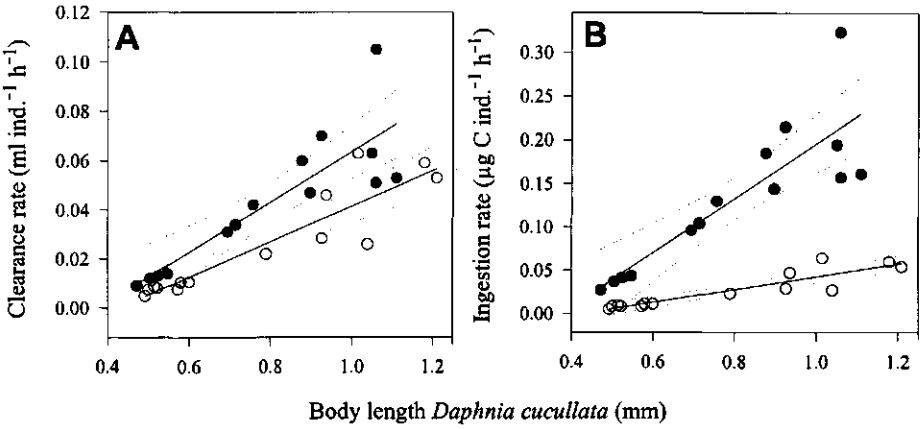


Figure 7.5: Clearance rates (ml·ind.$^{-1}$·h^{-1}: Panel A) and ingestion rates (µg C·ind^{-1}·h^{-1}: Panel B) of *Daphnia cucullata* feeding on ^{14}C-labeled unicellular (●) or colonial (○) *Scenedesmus* (~1 mg C·l^{-1}). Straight lines represent linear regressions, dotted lines the 95% confidence intervals.

Table 7.4: Mean clearance rates (CR ± 1 SD; ml·ind.$^{-1}$·h^{-1}) and mean ingestion rates (IR ± 1 SD; mg C·ind^{-1}·h^{-1}) of *Daphnia galeata* feeding on ^{14}C-labeled unicellular or colonial *Scenedesmus* at food concentrations of ~0.1, 0.5 and 1.0 mg C·l^{-1}, including mean (± 1 SD) body-lengths of animals per series.

Food type	mg C·l^{-1}	IR	CR	Body-length	*N*
Unicells	0.1	0.026 (0.010)	0.263 (0.100)	0.90 (0.10)	15
Colonies	0.1	0.014 (0.013)	0.144 (0.130)	0.92 (0.11)	18
Unicells	0.5	0.072 (0.038)	0.143 (0.058)	0.93 (0.12)	18
Colonies	0.5	0.032 (0.020)	0.064 (0.039)	0.97 (0.12)	20
Unicells	1.0	0.122 (0.053)	0.122 (0.053)	1.02 (0.17)	21
Colonies	1.0	0.039 (0.043)	0.039 (0.043)	0.98 (0.14)	13

7.4 GROWTH OF *DAPHNIA* ON UNICELLULAR AND COLONIAL *SCENEDESMUS*

Life-table experiments were performed to investigate the effect of unicellular or colonial *S. acutus* on the life history of two *Daphnia* species, *D. cucullata* and *D. pulex*.

7.4.1 The algal food and experimental animals

The food algae were cultured in two 1.0 liter chemostats on either 20% Z8 medium (Skulberg & Skulberg, 1990) or on water from lake Zwemlust (The Netherlands) enriched with Z8 nutrients and filtered through a 0.2 µm membrane filter. During the course of the experiment 81 ± 7% of *S. acutus* cultured in the chemostat on 20% Z8 medium occurred as unicells with mean cell dimensions (± 1 SD) of 10 (2) × 4 (1) µm. The average number of cells per particle was 1.30 (0.12) with a mean particle volume (± 1 SD) of 85.0 (6.6) μm^3. The lake water appeared suitable to induce colonies in *Scenedesmus* (Lürling & Van Donk, 1997b). In the chemostat with filtered lake water 30 ± 5% of the *S. acutus* remained unicellular, 21 ± 8 % was four-celled, 22 ± 10% occurred as eight-celled, while the remaining colony sizes (i.e. 2-, 3-, 5-, 6-, 7- and more than eight cells per colony) made up the rest of the population. The mean dimensions (± 1 SD) of an eight-celled coenobium were 27 (8) × 21 (3) µm. On average 4.03 (0.47) cells formed a colony with a mean particle volume (± 1 SD) 413.0 (79.9) μm^3. The chemostats were continuously illuminated by circular fluorescent tubes (Philips TLEM 40W/33RS) at an irradiance of 125 $\mu mol \cdot m^{-2} \cdot s^{-1}$ in a temperature controlled chamber at 20°C and with a dilution rate of 1.2 d^{-1}.

The cladoceran *Daphnia pulex* (adult female ~ 3 - 4 mm) was isolated from Lake Zwemlust (The Netherlands) and has been cultured for over a year in the laboratory. A clone (Tj33) of the smaller species *Daphnia cucullata* (adult female ~0.8 -1.1 mm) was obtained from the culture collection of the Centre for Limnology (Nieuwersluis, The Netherlands). Animals were cultured at 20°C in 1 liter jars containing a suspension of *S. acutus* in 0.45 µm filtered lake water.

7.4.2 Grazing by D. cucullata *and* D. pulex

Two size classes of *D. pulex* and *D. cucullata* were fed with either unicellular or colonial *S. acutus* resuspended in filtered lake water. A cohort of *D. pulex* and one of *D. cucullata* were transferred into separate 1 liter beakers containing lake water filtered through 0.2 µm. Then, 10 *D. pulex* and 20 *D. cucullata* were selected and transferred into separate 100 ml algal suspensions (unicellular algae ~1 mg $C \cdot l^{-1}$). The used algal biovolumes were similar for both treatments. The animals were fed with either unicellular algae from the 20% Z8 chemostat or colonial algae derived from the lake water chemostat.

The tests were run for 3 h in the dark at 20°C in quadruplicate. Algal suspensions without zooplankters served as referent. Total algal volumes between 3.0 and 20.0 µm ESD

were determined at the beginning of the experiments and after 3 h of grazing using the Coulter Multisizer II. Clearance rates (CR, ml·ind.$^{-1}$·h^{-1}) were computed from Coulter data.

Algae harvested from both chemostats and fed to two size classes of each *Daphnia* revealed great differences in clearance rate between the two zooplankters (Table 7.5). *D. pulex* showed no differences in clearance rate when fed unicellular or colonial *Scenedesmus*, while *D. cucullata* showed depressed clearance rates when fed colonies compared to unicells.

Table 7.5: Mean clearance rates (± 1 SD; ml·ind.$^{-1}$·h^{-1}) of *Daphnia cucullata* and *D. pulex* feeding on unicellular or colonial *Scenedesmus acutus*, including mean (± 1 SD) body-lengths of animals per series.

Body-size (mm)	Clearance rate (ml·ind.$^{-1}$·h^{-1})	
D. pulex	Unicellular food	Colonial food
1.02 (0.09)	0.24 (0.10)	0.19 (0.08)
2.48 (0.13)	0.97 (0.05)	0.87 (0.26)
D. cucullata	Unicellular food	Colonial food
0.60 (0.06)	0.32 (0.06)	0.03 (0.02)
1.09 (0.12)	0.61 (0.11)	0.19 (0.12)

Two-way ANOVA indicated a significant *Daphnia* species (F = 49.8; $P < 0.001$) and food type (F = 18.8; $P < 0.001$) effect, but no interaction effect (F = 3.1; P = 0.057) on clearance rates. Post-hoc comparisons showed that only *D. cucullata* had lower clearance rates when feeding on colonial *Scenedesmus*. Comparison between the smaller size class of *D. pulex* and the larger size class of *D. cucullata*, which in fact are similarly sized, reveals that both species had similar clearance rates when fed colonies, but that the adult *D. cucullata* had a significantly higher clearance rate than juvenile *D. pulex* when fed unicells.

Inasmuch in the presence of coenobial *Scenedesmus* the clearance rate of *D. cucullata* was reduced, it seems that those coenobia with dimensions of 30 × 20 µm exceeded the size range of easily ingestible particles.

7.4.3 Design Daphnia *life-history experiments*

Life-table experiments with both *Daphnia cucullata* and *D. pulex* were conducted to investigate the influence of altered algal morphology on growth and reproduction. Animals belonging to the same cohort were placed individually in 100 ml tubes containing log-phase *S. acutus* in 0.45 µm filtered lake water (Lake Maarsseveen, The Netherlands). Newborns from the third clutch were collected within 20 h of birth and put together in a 500 ml beaker. For

each series neonates of *D. pulex* and *D. cucullata* (15 respectively 14) were selected from this beaker and placed individually in 100 ml test tubes containing 60 ml of a unicellular or colonial *S. acutus* suspension in 0.45 µm filtered lake water. Both *Daphnia* species were fed with equivalent biovolumes of unicellular or colonial algae (i.e. $8 \cdot 10^6$ $\mu m^3 \cdot ml^{-1}$, yielding approximately 3 mg $C \cdot l^{-1}$). The tubes were incubated at 20°C in the dark to prevent algal growth. The animals were transferred daily into clean tubes containing fresh medium. Length was recorded and the animals were examined daily for molting. Time needed to reach maturity, survival and number of newborns were recorded. Newborns were removed from the tubes. Growth and reproduction were measured until the animals reached the fourth adult instar and consequently had released their third clutch, because the population growth rate is mainly determined by the first three clutches (Porter *et al.*,1983; Vanni & Lampert, 1992). The intrinsic rate of population increase (r) was estimated using the Euler equation (7.2):

$$1 = \sum_{x=0}^{N} e^{-rx} l_x m_x \qquad (7.2)$$

where r = rate of population increase (d^{-1}), x = age class (0...N), l_x = probability of surviving to age x, m_x = fecundity at age x. Because of the impossibility of computing standard errors of the population parameter r directly, a jackknifing method was used to calculate them (Meyer *et al.*, 1986). For both daphnids r values, age and length at maturity were compared by applying t-tests. Numbers of newborns were statistically compared applying two-way ANOVA.

7.4.4 Results life-history experiment

Growth of *D. pulex*, as increase in body length in time, shows no differences between animals fed unicellular or animals fed colonial *Scenedesmus* (Fig. 7.6; Panel A). This in contrast with *D. cucullata* in which a significant difference in increase in body length occurred (Fig. 7.6; Panel B). After 16 days, body size of *D. cuculllata* fed with colonies was significantly smaller than that of animals reared on unicells (t-test; $P < 0.001$), at 1.01 ± 0.04 and 1.13 ± 0.07 mm, respectively.

Age at maturity was not affected by the different food types in both *Daphnia* species (t-test; $P = 0.760$ and $P = 0.767$ for *D. pulex* and *D. cucullata*, respectively). Neither was the length at maturity in *D. pulex* ($P = 0.595$), however in *D. cucullata* length at maturity was significantly lower when fed colonies ($P = 0.008$; Table 7.6).

The number of live neonates released per mature female differed significantly between successive clutches in *D. pulex* ($F = 43.8$; $P < 0.001$). However, no significant effect of unicellular or colonial food ($F = 0.34$; $P = 0.565$) was found. *D. cucullata* had more offspring in all three clutches when fed unicells compared to clutch-size of animals grown on colonial *Scenedesmus* (Fig. 7.7). Two-way ANOVA indicated no significant clutch ($F = 2.2$; $p = 0.123$) and interaction effects ($F = 1.1$; $p = 0.361$), but a significant food type effect ($F = 21.5$;

$P < 0.001$) on reproduction. Tukey's post-hoc comparison revealed that there were significantly more *D. cucullata* offspring in the third clutch when they were fed unicells than for animals fed colonies.

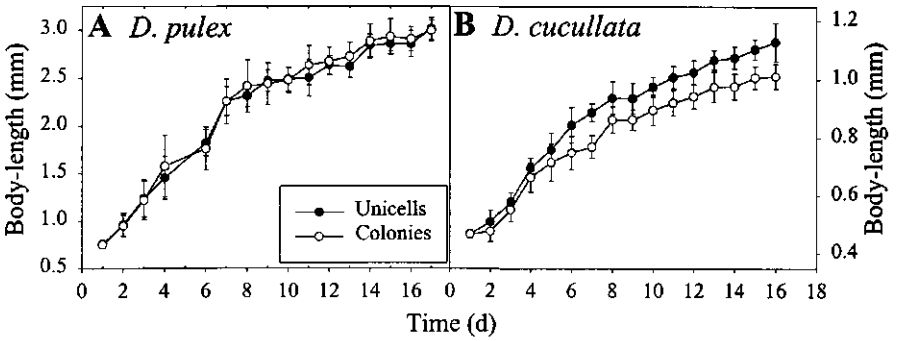


Figure 7.6: Relationship between age (d) and carapace length (mm) of *Daphnia pulex* (Panel A) and *D. cucullata* (Panel B) grown on unicellular (●) or colonial (O) *Scenedesmus acutus*. Error bars represent 1 SD.

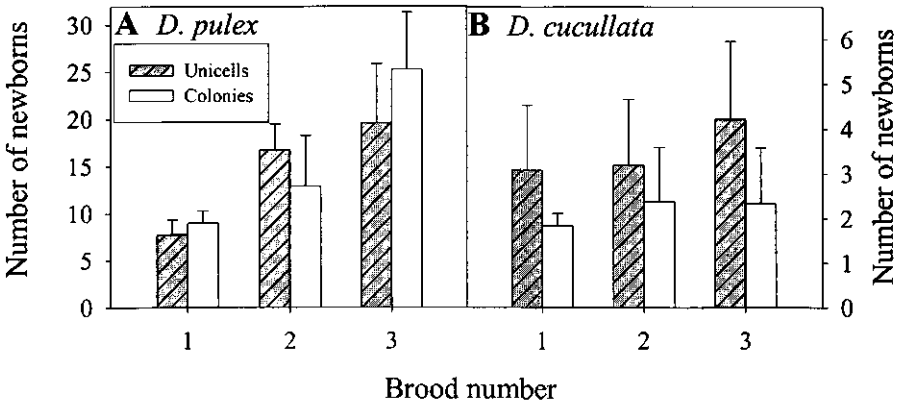


Figure 7.7: Mean clutch sizes (± 1 SD) of *D. pulex* (Panel A) and *D. cucullata* (Panel B) grown on either unicellular (gray bars) or colonial (open bars) *S. acutus*.

The *Daphnia* intrinsic rate of population increase (r) was similar for *D. pulex* on both food types ($P = 0.757$), but significantly different for *D. cucullata* ($P = 0.020$). Feeding on colonial *Scenedemus* resulted in a lower *D. cucullata* growth rate (Table 7.6).

Table 7.6: Population growth rate ($r \pm 1$ SD), age and length at maturity (± 1 SD), interclutch duration (± 1 SD) and survival to day 16 of *Daphnia pulex* and *D. cucullata* fed unicellular or colonial *Scenedesmus acutus*.

Scenedesmus Food type	Growth rate (r, d^{-1})	Age at maturity (d)	Length at maturity (mm)	Interclutch Duration (d)	Survival (%)
			D. pulex		
Unicells	0.44 (0.06)	5.4 (0.7)	2.29 (0.07)	2.82 (0.39)	67
Colonies	0.42 (0.07)	5.4 (1.4)	2.31 (0.09)	2.86 (0.64)	73
			D. cucullata		
Unicells	0.25 (0.05)	6.8 (1.9)	0.92 (0.05)	2.78 (1.00)	86
Colonies	0.18 (0.05)	6.6 (1.4)	0.86 (0.05)	2.77 (0.61)	78

7.5 BIOCHEMICAL COMPOSITION OF UNICELLS AND *DAPHNIA*-INDUCED COLONIES IN *SCENEDESMUS*

In the previous section (§ 7.4) an effect of colonial *Scenedesmus* on growth of *D. cucullata* was observed. However, as colonies have a different surface to volume ratio, their chemical composition may also differ from that of unicells. Besides morphological features, also mineral and biochemical composition of the algae may affect zooplankton growth. Several studies have shown that P-limited cells are poorer food for *Daphnia* than N-limited cells, which are of intermediate quality, while nutrient-sufficient cells are highest in quality (Groeger *et al.*, 1991; Mitchel *et al.*, 1992; Sterner *et al.* 1993). Recently, interest has focused on the role of fatty acid (FA) composition of phytoplankton in determining the food quality for zooplankton. Some studies suggest that polyunsaturated fatty acids (PUFA's) like EPA (eicosapentaenoic acid, 20:5ω3) and DHA (docosahexanaenoic acid, 22:6ω3) may improve the quality of algae as food for zooplankton (Ahlgren *et al.*, 1990; Müller-Navarra, 1995a). In this section, therefore, special attention will be paid to the biochemical composition of unicellular and colonial ecomorphs of *Scenedesmus* emphasized on fatty acid composition.

7.5.1 Species and cultures

The green algae *Scenedesmus obliquus* Kützing NIVA-CHL 6 and *Scenedesmus subspicatus* Chodat NIVA-CHL 55 were obtained from the culture collection of the Norwegian Institute for Water Research (NIVA, Norway). A subculture of *Scenedesmus acutus* Meyen was derived from a chemostat culture at the Max-Planck Institute for Limnology (Plön, Germany). The algae were cultured in 1.0 liter chemostats on COMBO-medium (Kilham *et al.*, 1999), continuously illuminated by circular fluorescent tubes (Philips

TLEM 40W/33RS) at an irradiance of 125 $\mu mol \cdot m^{-2} \cdot s^{-1}$ in a temperature controlled room at 20 ± 1°C and with a dilution rate of 1.0 d^{-1}.

The cladocerans *Daphnia magna* Straus and *D. cucullata* Sars were obtained from the Centre for Limnology (Netherlands Institute of Ecology, Nieuwersluis). Animals were cultured in 1 liter jars containing a suspension of *S. acutus* in COMBO medium in a temperature controlled chamber at 20°C under a 14:10 h L:D cycle.

7.5.2 Colony-induction:

For the production of colony inducing infochemicals, about 100 non-egg bearing *D. magna* were collected from culture vessels and transferred into a flask with 500 ml of COMBO and a suspension of *S. acutus* (~45.000 $cells \cdot ml^{-1}$; biovolume of $5.4 \cdot 10^6$ $\mu m^3 \cdot ml^{-1}$; ~2 mg $C \cdot l^{-1}$). Two replicate flasks were incubated for 24 h in the dark at 20°C. After this period water from both flasks was combined, filtered and used as test water in a colony induction experiment. This induction experiment was performed in 300 ml Erlenmeyer flasks containing 150 ml *Scenedesmus*-COMBO suspensions in triplicate. Each flask contained 115 ml of COMBO medium, a 10 ml inoculum of unicellular log-phase *Scenedesmus* from a chemostat and either 25 ml of additional COMBO medium (controls) or 25 ml of test water (treatments). In an additional treatment two live *D. magna* were added to 150 ml algal suspensions of *S. obliquus* and *S. subspicatus*. The flasks were incubated in triplicate on a shaking table at 20 °C, continuously illuminated from above by fluorescent cool-white tubes (Philips TL 36W/82) at ~125 $\mu mol \cdot m^{-2} \cdot s^{-1}$. After an incubation period of 3 days, subsamples were taken and algal densities and particle size distributions were determined in the range 3.0 - 25.0 µm equivalent spherical diameter (ESD) using a Coulter Multisizer II (100 µm capillary). For each replicate at least 100 aggregates (i.e. unicells and coenobia) of *Scenedesmus* were also counted in a subsample preserved in Lugols fixative using a Nikon light-microscope at 600× magnification. Both the mean particle volumes (μm^3) and the mean number of cells per colony were determined. The algae were concentrated by centrifugation, freeze-dried and stored at -20 °C until further biochemical analysis.

The three *Scenedesmus* species showed different responses to the *D. magna* infochemicals. Formation of coenobia was clearly promoted by infochemicals in the two non-spiny species, *S. acutus* (*t*-test; $P < 0.001$) and *S. obliquus* ($F_{2,6} = 2788$; $P < 0.001$) in the presence of medium from a *D. magna* culture. The one-way ANOVA was also highly significant for the spined *S. subspicatus* ($F_{2,6} = 3045$; $P < 0.001$: Table 7.7). A Tukey test revealed that significant colony formation was promoted in *S. subspicatus* exposed to live *Daphnia*, but not in *S. subspicatus* exposed to filtered water from *D. magna* culture. The one-way ANOVA for this species was highly significant because of the very small within group variations.

Table 7.7: Mean particle volumes (MPV in μm^3, ± 1 SD) and mean number of cells per colony (Cel/Col ± 1 SD) in *Scenedesmus* in response to *Daphnia magna* infochemicals. Algae were incubated without infochemicals (Control), with 17% (v/v) filtered water from a *D. magna* culture (Filtrate), and with 17% filtrate and two live *Daphnia* added (*Daphnia*).

	Scenedesmus acutus		*Scenedesmus obliquus*		*Scenedesmus subspicatus*	
Treatment	MPV	Cel/Col	MPV	Cel/Col	MPV	Cel/Col
Control	74 (5)	1.3 (0.1)	51 (0.4)	1.2 (0.1)	117 (10)	2.7 (0.3)
Filtrate	337 (5)	4.8 (0.6)	281 (4)	6.5 (0.3)	113 (11)	2.9 (0.2)
Daphnia	-----	-----	241 (4)	6.9 (0.4)	192 (14)	3.6 (0.1)

7.5.3 Biochemical analysis

Unicellular and colonial ecomorphs of *Scenedesmus* were analyzed for total protein, total carbohydrates, total lipid content and fatty acid composition. Total protein content was determined according the method as described by Lowry *et al.* (1951). Carbohydrates were measured using the anthrone method (Hassid & Abraham, 1957). Total lipid content was determined following the method described by Meyer & Walther (1988). For fatty acid analysis *ca.* 1 mg of freeze-dried algae was transferred into 10 ml tubes, and C21:0 (heneicosaenoic acid) was added as internal standard. Fatty acids were extracted with 2 ml 2:1 v/v chloroform/methanol (analytical grade), vortexed and centrifuged. After repeating this extraction twice, supernatants were joined and washed with demineralized water with 0.88% NaCl (according to Folch *et al.*, 1957). The lipid esters were transmethylated at 80°C during 4 hours in 1 ml of 3% H_2S0_4 in dry methanol, and extracted with hexane. The fatty acids were analyzed on a Hewlett Packard 5890A Gas Chromatograph, with a very polar 50 m silica column.

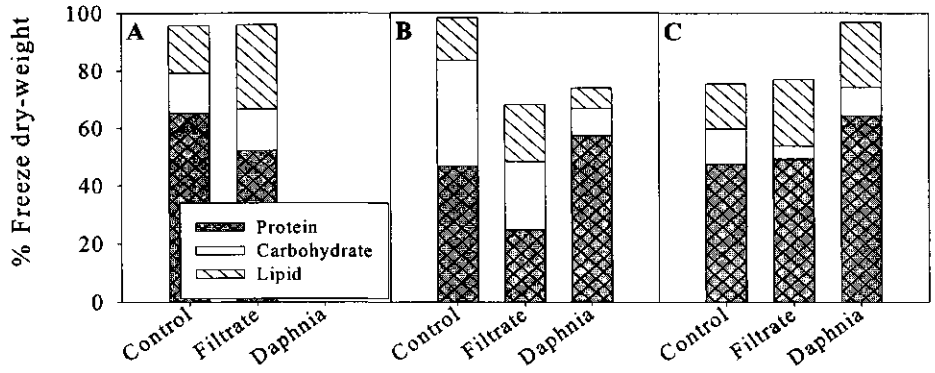


Figure 7.8: Biochemical composition (total protein, carbohydrate and lipid content) of *Scenedesmus* from control, filtrate, and *Daphnia* (as in Table 7.7) as a percentage of frozen dry-weight.

There was no clear and consistent change in total protein, carbohydrate and lipid content between the unicellular and colonial ecomorphs of the three *Scenedesmus* species (Fig. 7.8). However, fatty acid concentrations differed between ecomorphs (Table 7.8).

Table 7.8: Fatty acid composition (FA) as % of dry-weight for three *Scenedesmus* species, both in unicells and colonies. (Filtrate = *Scenedesmus* incubated with filtrate from 200 *Daphnia magna* l^{-1}, *Daphnia* = *Scenedesmus* incubated with filtrate + 2 live *D. magna* added, nd = not detected).

	Scenedesmus acutus		*Scenedesmus obliquus*			*Scenedesmus subspicatus*		
Fatty acid	Unicells	Filtrate	Unicells	Filtrate	*Daphnia*	Unicells	Filtrate	*Daphnia*
C16:0	0.511	4.732	1.442	2.352	2.188	3.375	1.647	4.965
C16:1ω7	0.314	nd	0.114	0.313	0.056	0.148	Nd	0.364
C16:3ω4	nd	nd	nd	nd	0.993	nd	Nd	nd
C16:3ω?	nd	nd	nd	nd	nd	0.113	Nd	nd
C16:4ω3	1.190	2.262	1.197	2.682	1.735	0.464	0.522	nd
C17:0	nd	nd	nd	nd	nd	nd	Nd	0.495
C18:0	0.509	1.340	0.354	0.396	0.270	0.731	0.197	0.247
C18:1ω9	nd	nd	0.522	0.941	0.775	0.836	0.491	2.664
C18:1ω7	nd	nd	0.065	0.082	0.074	0.077	0.044	0.085
C18:2ω6	1.537	nd	0.345	0.753	0.599	0.477	0.409	1.762
C18:3ω6	0.053	nd	nd	nd	nd	nd	Nd	nd
C18:3ω3	1.891	nd	2.136	4.039	2.790	1.337	1.271	3.420
C18:4ω3	0.543	12.439	0.292	0.475	0.367	0.314	0.246	1.021
Total Fa's:	6.548	20.772	6.467	12.033	8.954	7.872	4.819	14.578
SAFA's:	1.020	6.072	1.796	2.749	2.459	4.106	1.844	5.262
MUFA's:	0.314	0	0.701	1.335	0.904	1.061	0.536	3.134
PUFA's:	5.214	14.701	3.970	7.948	5.591	2.705	2.440	6.203
SAFA/UFA	0.098	0.207	0.226	0.173	0.220	0.759	0.378	0.424
ω3/ω6 FA's	2.278	-----	10.495	9.557	8.163	4.435	4.970	2.520

Total fatty acids as percentage of freeze dry-weight increased in colonies compared to unicells due to an increase in both UFA and SAFA (Fig. 7.9). In *S. acutus* and *S. obliquus* ratios of mono-unsaturated (MUFA) and polyunsaturated fatty acids (PUFA) was lower in colonies indicating a stronger relative share of PUFA. The saturated fatty acids (SAFA) 16:0 and 18:0, and the unsaturated (UFA) 18:4ω3 were present in all samples, while EPA (20:5ω3)

and DHA (22:6ω3) were absent from all samples. The ω3/ω6 fatty acid ratio showed no significant differences in colonies compared to unicells.

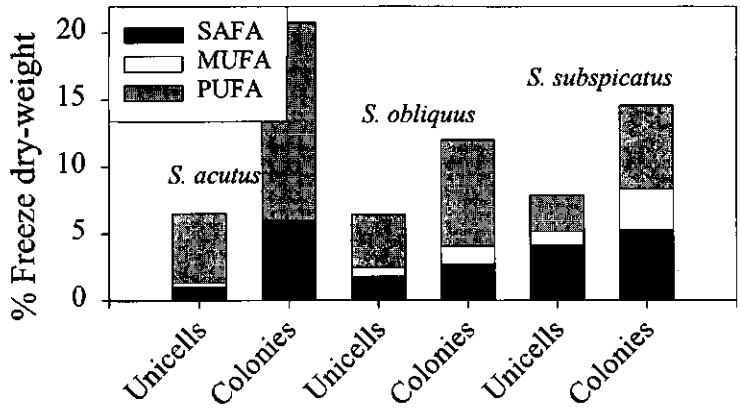


Figure 7.9: Fatty acid (FA) composition (SAFA = totally saturated FA, MUFA = monounsaturated FA, and PUFA = polyunsaturated FA) of unicellular and colonial *Scenedesmus* as a percentage of frozen dry-weight.

7.6 EFFECTS OF *SCENEDESMUS* MORPHOLOGY AND BIOCHEMICAL COMPOSITION ON *DAPHNIA* GROWTH

In this section the results of short-term grazing experiments and life-history experiments with *Daphnia magna* and *D. cucullata* will be related to the morphological and biochemical features of different *Scenedesmus* ecomorphs determined in section 7.5.

7.6.1 Grazing experiment

A grazing experiment was performed with 2 size classes of *D. magna* and *D. cucullata* to examine the effect of different *Scenedesmus* ecomorphs (unicells versus coenobia) on the grazing rates of daphnids.

Different ecomorphs were obtained by incubating *S. obliquus* in COMBO-medium, in COMBO medium with 10% (v/v) of 0.1 µm filtered water from a *Daphnia* culture and in COMBO with water from a *Daphnia* culture plus one live *D. magna*. After a 48 h incubation, 3 different algal size classes were obtained. Cell dimensions (length and width, µm) of *S. obliquus* unicells and eight-celled coenobia preserved in Lugol's fixative were measured using an image analysis system (SIS, Soft Imaging Software®) at 500 × magnification. Five animals were taken from two cohorts of *D. magna*, 5 adults and 10 juveniles from two *D. cucullata* cohorts and transferred separately into 50 ml *S. obliquus* suspensions. The algal biovolumes as

food amounts were similar for each treatment and on average (mean ± 1 SD) 4.7 ± 0.04·10^6 $\mu m^3 \cdot ml^{-1}$, yielding a carbon content of approx. 1.75 mg $C \cdot l^{-1}$. Three replicate bottles were used for each algal size class, while 2 bottles per algal class without daphnids served as controls. The bottles were incubated for 3 h in the dark at 20°C and manually shaken every 30 min. Initially and after 3 h of grazing, algal volumes were determined in the range 3.0 - 25.0 µm ESD using the Coulter Multiszer II and clearance rates ($ml \cdot ind^{-1} \cdot h^{-1}$) were computed. Clearance rates were compared applying two-way ANOVA, with the four different *Daphnia* classes and the three *Scenedesmus* size classes as the two factors, followed by a Tukey's-test.

The short term grazing experiment revealed clear effects of algal morphology on the clearance rates of the daphnids (Fig. 7.10). The three algal size classes had mean particle volumes of 89, 224 and 476 μm^3, and mean number of cells per colony of 1.8, 3.0 and 6.8, respectively.

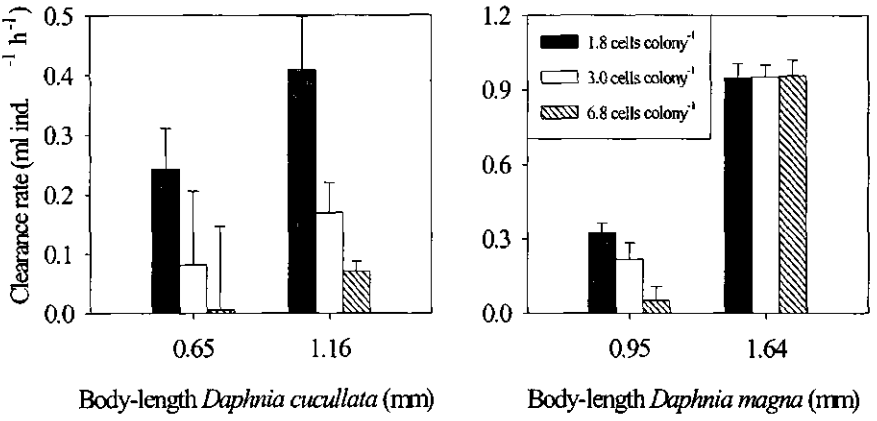


Figure 7.10: Effect of *Scenedesmus obliquus* with different percentages of eight-celled coenobia (1, 21 and 74%, i.e. 1.8, 3.0 and 6.8 cells per colony) on clearance rates (ml *Daphnia*$^{-1}$h^{-1}) of two size classes of *D. cucullata* (left panel) and *D. magna* (right panel). Error bars represent 1 SD (n = 3).

The smallest *S. obliquus* size class consisted of only 1% 8-celled coenobia, the intermediate class had 21% and the largest contained 74% large 8-celled coenobia with dimensions (length ± 1 SD × width ± 1 SD) of 34 ± 6 × 19 ± 3 µm (n = 30). Unicells had dimensions of 11 ± 2 × 7 ± 1 µm (n = 30). The *D. magna* cohorts had mean body-lengths (± 1 SD) of 0.95 ± 0.07 and 1.64 ± 0.08 mm, while the *D. cucullata* cohorts were 0.65 ± 0.07 and 1.16 ± 0.07 mm. Feeding on equal biovolumes of unicellular or colonial *S. obliquus* resulted in a statistically significant *Daphnia* species ($F = 81.1$; $P < 0.001$) and food type effects ($F = 8.9$; $P = 0.001$), but the interaction between these two factors was not significant ($F = 1.2$; $P = 0.339$). Tukey post-hoc comparison revealed that the clearance rate of large *D. magna* was

significantly higher than the rates of the other daphnids. The clearance rates of adult *D. cucullata* and small *D. magna* were significantly higher when feeding on unicells of *S. obliquus* than on large colonies of the same species.

7.6.2 Life table experiments

Cohorts of both *Daphnia* species had been reared in 1 liter jars on *S. acutus* in COMBO-medium. Life history experiments were conducted analogous to the procedure described in section (*§7.4.3*), but now with the freshwater medium COMBO instead of filtered lake-water. Both species were fed equal amounts of algae, i.e. equivalent biovolumes ($5 \cdot 10^6$ $\mu m^3 \cdot ml^{-1}$, yielding a carbon content of 1.9 mg $C \cdot l^{-1}$). On average, unicellular *S. acutus* had a volume (± 1 SD) of 87 ± 6 μm^3, while colonial ecomorphs were 489 ± 32 μm^3.

Size at maturity was not significantly influenced when daphnids were grown on either unicellular or colonial *S. acutus* (*t*-test: *D. magna*: $P = 0.143$; *D. cucullata*: $P = 0.219$). Feeding on colonies seemed to result in an older age at maturity in *D. cucullata* (Table 7.9), however, the difference was not significant ($P = 0.086$). Age at first reproduction also did not vary significantly with food type (*D. magna*: $P = 0.068$; *D. cucullata*: $P = 0.164$). Mean interclutch duration of the first three adult instars was significantly prolonged in *D. cucullata* when colonies were the food ($P = 0.024$), but not in *D. magna* ($P = 0.812$) (Table 7.9).

Table 7.9: Intrinsic rate of population increase (r ± 1 SD, d^{-1}), age at maturity (AM ± 1 SD, d), length at maturity (LM ± 1 SD, mm), age at first reproduction (AFR ± 1 SD, d), clutch sizes (± 1 SD), mean interclutch duration (ID ± 1 SD, d) and survival (% to day 18) of *Daphnia cucullata* and *Daphnia magna* fed either unicellular or colonial *Scenedesmus*.

Life-history	***Daphnia cucullata***		***Daphnia magna***	
Parameters	On unicells	On colonies	On unicells	On colonies
r	0.22 ± 0.04	0.15 ± 0.04	0.31 ± 0.02	0.30 ± 0.02
AM	6.0 ± 0.7	6.6 ± 1.1	7.0 ± 0.0	7.0 ± 0.0
LM	0.88 ± 0.03	0.86 ± 0.06	2.84 ± 0.08	2.81 ± 0.04
AFR	8.6 ± 1.4	9.9 ± 2.6	10.5 ± 0.5	10.8 ± 0.4
1st Clutch	2.3 ± 0.6	1.8 ± 0.6	10.3 ± 1.5	9.6 ± 1.2
2nd Clutch	3.7 ± 0.7	2.5 ± 1.2	12.5 ± 0.8	8.8 ± 1.2
3rd Clutch	5.0 ± 1.7	3.0 ± 1.1	8.2 ± 1.8	7.2 ± 1.8
ID	2.6 ± 0.5	3.3 ± 1.3	4.0 ± 1.6	4.1 ± 0.4
Survival	50	47	86	100

The number of newborns produced was lower in all clutches when animals were reared on colonial *S. acutus* compared to animals fed with unicells (Table 7.9). This resulted

for *D. magna* in a significant clutch (brood number)(F = 25.3; P < 0.001), food type (F = 24.1; P < 0.001) and interaction effect (F = 7.7; P = 0.001). These results imply a different response of the three consecutive clutches to unicellular or colonial food. Tukey's post-hoc comparison revealed significant differences in clutch size among successive instars of *D. magna* and also that the clutch size of the second brood was significantly different between animals fed either unicells or colonies. The two-way ANOVA for *D. cucullata* also indicated a significant clutch (F = 9.6; P = 0.001) and food type effect (F = 10.3; P = 0.004), but the interaction was not significant (F = 1.9; P = 0.169). Survival did not differ between treatments, but was higher for *D.* magna than for *D. cucullata.* The intrinsic rate of population increase r was significantly lower for daphnids feeding on colonial *Scenedesmus* than when they were fed with unicells (t-test: *D. magna*: P = 0.035; *D. cucullata*: P < 0.001; Table 7.9).

7.7 DISCUSSION

Considerable attention has been focused on the grazing activity of herbivorous zooplankton on phytoplankton. Extensive information exists on the highly different feeding success of zooplankton on various algal species. This is primarily owing to chemical and morphological properties of the various phytoplankton species. Clear relationships exist between the grazers' body size and the maximum size of spherical beads that can be ingested (Burns, 1968). Porter (1977) noted that spherical algae above ~ 45 µm could not be ingested by the largest *Daphnia* species. Also hardness of algae influences their ingestibility, flagellates are more readily ingested than diatoms (DeMott, 1995). Large gelatinous colonial chlorophytes may be ingested, but are hardly digested by zooplankters like *Daphnia* (Porter, 1973; 1976), resulting in depressed zooplankton growth rates (Vanni & Lampert, 1992; Stutzman, 1995). Zooplankton feeding on cyanobacteria is often limited because of the size and toxicity of blue-greens (Lampert, 1981; 1987; Fulton & Pearl, 1987; DeMott & Moxter, 1991). Extracellular substances released from cyanobacteria inhibit grazing activity of *Daphnia* (Ostrofsky *et al.*, 1983; Haney *et al.*, 1994), while mucous excretion by diatoms inhibits copepod grazing (Malej & Harris, 1993). Apparently, phytoplankters have evolved a set of defense mechanisms against grazing depending on parameters like size, cell-wall structure, hardness, mucous excretion and toxicity. In general, due to the selection pressure genotypes with better defenses will have gained an advantage above genotypes with worse or no defenses resulting in an adaptation to predation (Pianka, 1983). This advantageous form may be genetically fixed and therefore permanent or mobilized only when necessary (Dodson, 1989). Grazing pressure on algae varies temporally (Horn, 1981; examples in Sterner, 1989) and spatially in aquatic systems favoring the evolution of defenses to reduce mortality through grazing (Havel, 1987; Lehman, 1988). Often metabolic costs are associated with mobilizing defense mechanisms (Riessen, 1984; Dodson, 1989; Kusch & Kuhlmann, 1994), implying that

genotypes with fixed defenses have lower fitness than genotypes that mobilize defenses only when necessary (Havel, 1987; Larsson & Dodson, 1993). However, in *Scenedesmus* these allocation costs have not been detected and may even be absent. In spined *Scenedesmus*, fewer spines per cell are produced when cells are cemented together in a colony compared to unicells (Trainor, 1998). Thus, the material necessary to cement cells together may be derived from material otherwise used for the formation of spines. By contrast, in the non-spiny *Scenedesmus*, some additional cementing material is required to form colonies. Nevertheless, this does not seem to result in reduced growth suggesting that either allocation costs are too small to be detected or absent. One could claim that under these conditions the inducible defense will eventually become fixed, simply because no costs are associated with it. However, environmental costs (cf. Tollrian & Harvell, 1999) are most likely the major costs for the algal cells operating in the system of grazer-induced colony formation. As presented in CHAPTER 6, induced colonies experience higher sinking losses than unicells do. The unicell-colony transformation was triggered by an infochemical released from well-fed *Daphnia*. In the presence of large eight-celled coenobia of *S. subspicatus*, heavily armoured with spines, grazing by *D. magna* was reduced (Hessen & Van Donk, 1993). In contrast, Lampert *et al.* (1994) found no reduction in uptake of unicells or coenobia of *S. acutus* by *D. magna*. A similar result was obtained when colonial *S. acutus* was fed to *D. pulex* (*see* Table 7.5) and *D. magna* (*see* Fig. 7.10). However, *D. cucullata* feeding on colonial *S. acutus* instead of unicells showed a clearly depressed clearance rate (*see* Figs. 7.5 and 7.10; Table 7.5). Moreover, smaller zooplankters such as rotifers, *Ceriodaphnia* and *Bosmina* had clearly lower clearance rates on colonies than on unicells. Also protozoa may experience reduced grazing success when confronted with colonial *Scenedesmus* (e.g. Goulder, 1972; Grover, 1989) or *Chlorella* (Boraas *et al.*, 1998). Although it does seem that colonies are not protected from large metazoan grazers, such as large *Daphnia* and copepods, these organisms themselves are more vulnerable to predation by fish than smaller zooplankters. The reduced ability to ingest colonial *Scenedesmus* clearly indicates that grazer-induced coloniality is effective as a defense against numerous grazers, including several *Daphnia* species.

Morphology & biochemistry

The biochemical composition of the unicellular and colonial ecomorphs of *Scenedesmus* was similar. The colonies showed only a modest decrease in total protein and an increase in total lipid content. Interestingly, large size may confer higher sinking rates (Reynolds, 1984; *see* CHAPTER 6), but changes in biochemical composition (i.e., increased lipid content) may increase buoyancy. Total protein, carbohydrate and lipid contents are similar to values reported in literature for *Scenedesmus* (Piorreck *et al.*, 1984; Groeger *et al.*, 1991; Ahlgren *et al.*, 1992; Sterner, 1993). Total fatty acids (FA) as a percentage of dry-weight showed a clear increase in colonies, which may reflect an increase in the total lipid

content of colonial *Scenedesmus*. The percentage of FA in unicellular *S. acutus* (~7%) as a percentage of dry-weight resembled the value of 9% reported by Müller-Navarra (1995b) and the 12% found by Ahlgren *et al.* (1992). Some studies suggest that PUFA, especially EPA (20:5ω3) and DHA (22:6ω3), may improve the quality of algae as food for zooplankton (Ahlgren *et al.*, 1990; Müller-Navarra 1995a & b). However, EPA (20:5ω3) and DHA (22:6ω3) were not detected in our *Scenedesmus* species, in contrast with Ahlgren *et al.* (1992) and Müller-Navarra (1995) who found, respectively, traces of EPA and DHA in *S. acutus*. However, Ahlgren *et al.* (1990) did not detect any EPA or DHA in *S. acutus*, neither did De Lange & Van Donk (1997) nor Weers & Gulati (1997) suggesting that EPA and DHA are difficult to detect in *Scenedesmus*. If these PUFA were present in our *Scenedesmus*, the concentrations are probably too low to play an important part in determing the quality of the alga as food for *Daphnia*.

The short term grazing experiment revealed a marked decrease in the clearance rates of small *Daphnia* when feeding on colonial *S. obliquus* compared to the clearance rates when feeding on unicells. Apparently, colonial *S. obliquus* exceeded the maximum size of ingestible particles which is directly related to *Daphnia* body size (Burns, 1968). A similar observation was made when *D. cucullata* was fed colonial *S. armatus* and *S. acutus* (§7.3 and §7.4). McCauley & Downing (1985) and Bern (1990) also found depressed feeding of *D. cucullata* on particles larger than *ca.* 18 μm. Boersma & Vijverberg (1995) suggested that culture conditions of *S. obliquus* can make this alga toxic to *Daphnia*. The results presented here, however, indicate that this was probably not the case since large *D. magna* showed no differences in clearance rate when exposed to different ecomorphs of *S. obliquus*. The largest size class of *S. obliquus* was obtained by incubation in presence of one live *Daphnia*. Although *Daphnia* exudates have been reported to reduce the feeding of congeners (Matveev, 1993) and conspecifics (Helgen, 1987), those chemicals are obviously not involved in our experiment. The largest *D. magna* did not show depressed clearance rates when exposed to colonies, strongly suggesting that the morphological properties of *S. obliquus* rather than allelochemicals from *Daphnia* are responsible for the reduced feeding of the smaller daphnids.

Both species of *Daphnia* exhibited reduced rates of intrinsic population growth (*r*) when feeding on colonial *Scenedesmus* in comparison to unicells (Table 7.9). In *D. cucullata* the reduction in *r* was about 32%. However, in *D. magna* this reduction was only 5% and the difference was only significant because of small within group variation. The small clutch sizes of *D. magna* of the second and third brood indicate that the adult daphnids were food limited. Probably the 60 ml food suspensions were depleted by these large *Daphnia*. Although this food limitation could obscure any effects of food type on population growth, no large differences in *r* are expected for *D. magna* as clearance rates were similar for 1.6 mm *D. magna* feeding on unicellular or colonial *Scenedesmus*. The *r* value for *D. magna* is lower than values of ~0.38-0.40 d^{-1} reported in literature (Goulden *et al.*, 1982; Enserink, 1995). For

the much smaller cladoceran *D. cucullata* food depletion was probably not a problem. However, different sinking rates of unicellular (~0.15 $m \cdot d^{-1}$) and colonial *S. acutus* populations (~ 0.3 $m \cdot d^{-1}$) could have resulted in lower suspended food levels in the test tubes with colonial *S. acutus*.. For the large *D. magna* this may not have been a major problem as the animals were observed mainly at the bottom of the tubes. By contrast, *D. cucullata* was mainly observed in the center of the tube, about 7 cm from the bottom. The lower *r* value of *D. cucullata* feeding on colonies resembles the effect of resource depression (Boersma & Vijverberg, 1994) and could be the result of reduced ingestion because of less food particles in suspension and more difficulties in harvesting these food particles.

Results show that the fatty acid and biochemical compositions are similar in single cells and in infochemically induced colonies of *Scenedesmus*. However, clearance rates are reduced in small *Daphnia* when exposed to large colonial *Scenedesmus*. Therefore, the negative influence of colonial *Scenedesmus* on growth and feeding of small *Daphnia* can be attributed to the morphological features rather than the biochemical composition.

CHAPTER 8

Daphnia-induced colony formation in phytoplankton

Parts of this chapter are based on:

Lürling (1999). *Journal of Phycology* **35**: 19-23

Lürling, M. & Beekman, W. *Accepted for publication in Phycologia*

Van der Grinten, E., Lürling, M. & Burger-Wiersma, T. *Accepted for publication in Verhandlungen der internationalen Vereinigung für theoretische und angewandte Limnologie*

"The next step would appear to be a confirmation
of the predator-induced results with more...
algal species and genera...
is the genus Scenedesmus really unique?"

- F.R. Trainor 1998

8.1 INTRODUCTION

The colonial growth form is widespread among phytoplankton. For example, among the Cyanophyceae there are colonial and filamentous forms. All species belonging to the Bacillariophyceae are unicellular or colonial coccoid algae (Van den Hoek *et al.*, 1995). Most members of the class Chrysophyceae are unicellular or colonial flagellates (Sandgren, 1988) and members of the Chlorophyceae are unicellular or colonial, coccoid or palmelloid. This latter class is comprised of about 355 genera, encompassing 2650 species, and almost entirely restricted to freshwater habitats (Van den Hoek *et al.*, 1995). The order Volvocales consists of unicellular and colonial flagellates, while the non-flagellate Chlorophyceae are covered in the order of Chlorococcales.

A high degree of phenotypic plasticity is characteristic of the algal genus *Scenedesmus* (Trainor, 1991), one of the commonest genera of freshwater algae (Canter-Lund & Lund, 1995), and a representative of the "colonial" Chlorococcales (Trainor, 1998). The order of Chlorococcales, which contains about 215 genera with approximately 1000 species, is almost entirely restricted to freshwater habitats. The genus *Scenedesmus* contains more than 100 species (Uherkovicz, 1966; Hegewald, 1982). Although a member of the colonial Chlorococcales, in culture *Scenedesmus* often fails to form colonies but remain in the unicellular "Chodatella/Chlorella" stages (Fott, 1968; Van den Hoek *et al.*, 1995; Trainor, 1998).

The well-known fact that many clonal algal isolates loose their typical colonial or filamentous appearance after some generations in the laboratory suggests that some factor is absent in the culture media (Van Donk *et al.*, 1999). For *Scenedesmus subspicatus* (Hessen & Van Donk, 1993) and *S. acutus* (Lampert *et al.*, 1994) the factor triggering their "typical" appearance in the field may originate from grazing *Daphnia*. In contrast, no *Daphnia*-induced formation of colonies in the (unicellular) cyanobacterium *Microcystis aeruginosa* was found (Fulton III & Paerl, 1987; Hessen & Van Donk, 1993). These previous experiments did, however, not elucidate whether the phenomenon of *Daphnia*-induced colony formation is restricted to members of *Scenedesmus*, to the Chlorococcales, or perhaps more widespread among representatives of other algal classes or phyla.

In this chapter I report results of biotests performed with 9 representatives (comprising 23 strains) of the algal genus *Scenedesmus* (§ 8.2), of 9 representatives of other Chlorophyceae (§ 8.3), 2 species of Bacillariophyceae (§ 8.4) and 5 strains of Cyanobacteria (§ 8.5). In all tests, chemical information substances were produced by *Daphnia* which were incubated for 24 h at a density of ~300 animals·l^{-1} on *S. acutus* (ca. 4 mg C·l^{-1}) in WC medium. The biotest for representatives of the Chlorophyceae was identical to the test as described in the previous CHAPTER 3. However, biotests for diatoms and cyanobacteria were slightly different and were run at a 16:8 h light:dark cycle, for more than 48 h and at lower irradiance in the case of cyanobacteria.

8.2 *SCENEDESMUS* (CHLOROCOCCALES; CHLOROPHYCEAE)

Strains of the non-spiny *Scenedesmus acutus* (4), *S. falcatus* (1), *S. obliquus* (5), *S. obtusiusculus* (1), and of the spined *S. armatus* (1), *S. gutwinskii* (3), *S. protuberans* (1), *S. quadricauda* (3), and *S. subspicatus* (4) were examined for inter- and intraspecific variance in *Daphnia*-induced colony formation. The test algae were grown axenically in 300 ml batch cultures on WC medium in 100 $\mu mol \cdot m^{-2} \cdot s^{-1}$ continuous light (provided by Osram L 36W/21-840 cool-fluorescent white tubes) at 20°C on a rotating shaking table.

Prior to the biotests an inoculum of 5 ml per strain was transferred into 50 ml fresh and autoclaved WC medium. After 4 days incubation, exponentially growing algae were taken from these cultures and used as inocula in the biotests, which were run in triplicate or quadruplicate for 48 h.

8.2.1 Scenedesmus acutus Meyen

Four different strains of *S. acutus* were used in two replicate biotest-experiments; both were run in triplicate. *Scenedesmus acutus* MPI was obtained from the Max-Planck-Institute for Limnology (Plön, Germany). Strain UTEX 72 (abbreviated to U72) was obtained from the culture collection of the University of Texas (U.S.A.) and the strains UTCC 7 (T7) and 10 (T10) were obtained from the Saskatchewan Research Council (Canada), but originate from the University of Toronto Culture Collection (UTCC). *S. acutus* MPI was inoculated at $2.5 \cdot 10^4$ particles$\cdot ml^{-1}$ ($3.45 \cdot 10^5$ $\mu m^3 \cdot ml^{-1}$). The three other strains were inoculated at similar algal biovolumes, i.e. $3.45 \cdot 10^5$ $\mu m^3 \cdot ml^{-1}$.

The results of both biotests were joined and two-way ANOVA indicated no *Daphnia*-water effect on the mean particle volume ($F = 0.07$; $P = 0.799$), a significant strain effect ($F = 27.4$; $P < 0.001$) and a significant interaction ($F = 37.5$; $P < 0.001$). Separate t-tests revealed significant larger mean particle volumes in *Daphnia*-water treatments compared to controls in the strains MPI ($t = 5.05$; $P < 0.001$) and T10 ($t = 3.70$; $P = 0.002$), no difference for strain U72 ($t = 1.90$; $P = 0.086$) and a significantly smaller mean particle volume for *Daphnia*-water treatments in strain T7 ($t = 8.65$; $P < 0.001$).

The variability in response of *S. acutus* to *Daphnia*-water appeared considerable. Colony formation occurred in strain MPI, not in the treatment populations of strains U72 and T10, while colonies were observed in control populations of strain T7, but not in treatments (Fig. 8.1). However, variability in *Daphnia*-induced colony formation in strain MPI was low. In 10 separate biotests the average mean particle volume (μm^3) in control populations was 265.0 (± 21.1) μm^3 and in treatments 527.5 ± 53.4 μm^3. Although one-way ANOVA indicated significantly different mean particle volumes between controls ($F_{9,30} = 4.75$; $P < 0.001$) and between treatments ($F_{9,30} = 6.09$; $P < 0.001$), Tukey's test revealed that the ANOVAs were only significant because of one experiment and small within group variation. Therefore, strain MPI

was chosen as positive control in the following biotests, i.e. a treatment in which always colony formation should occur when exposed to *Daphnia* infochemicals.

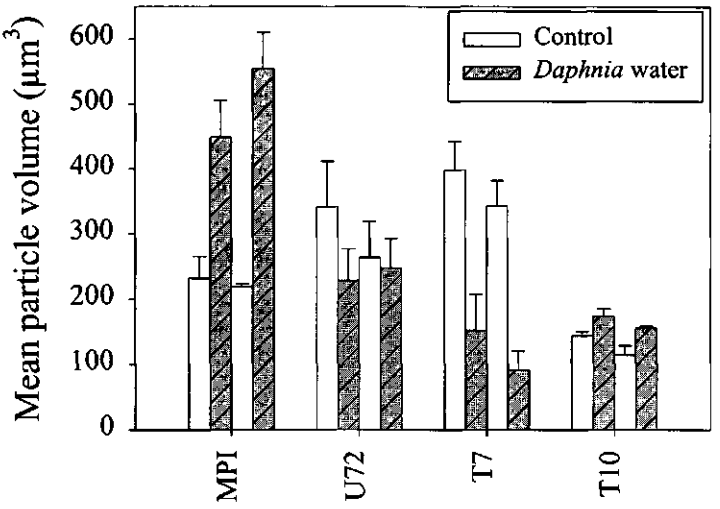


Figure 8.1: Colony sizes of 4 strains of *S. acutus*, expressed as mean particle volumes (µm³), after 48 h in control incubations and incubations with 10% (v/v) water from a *Daphnia* culture. Error bars indicate 1 SD (n = 3).

8.2.2 Scenedesmus obliquus (Turpin) Kützing

Five strains of *S. obliquus* were exposed to *Daphnia* water in a standard biotest (run in triplicate). Incubations with *S. acutus* MPI served as positive control. The strain *S. obliquus* NIVA-CHL6 was obtained from the Norwegian Institute for Water Research (NIVA, Norway). The strains UTEX 78, UTEX 1450 and UTEX 2630 were obtained from the culture collection of the University of Texas (U.S.A.) and strain SAG 276-1 was obtained from the culture collection of the University of Göttingen (Germany).

After 48 h, all control populations were strongly dominated by unicells, while coenobia occurred in the treated cultures. However, considerable variation in the response of *S. obliquus* strains to grazing-associated infochemicals was observed. In strain NIVA-CHL6, 20% of the treatment populations consisted of irregular aggregates with more than eight cells per colony. In the strains UTEX 78 and UTEX 2630, less than 5% of those aggregates were observed, while no colonies with more than eight cells were detected in the strains UTEX 1450 and SAG 276/1. Eight-celled coenobia were observed in the strains NIVA-CHL6 (12%), UTEX 78 (21%), UTEX 1450 (25%), and UTEX 2630 (26%), but were rare in SAG 276/1 (2%). The latter remained mainly unicellular with over 80% unicells in the treated cultures. In strain UTEX

1450, over 50% of the treated populations were comprised of four-celled coenobia. Colony formation was reflected in the mean particle volumes and mean number of cells per colony for each strain after 48 h incubation (Fig. 8.2).

Two-way ANOVA indicated a significant *Daphnia*-water (F = 141.6; P < 0.001), a significant strain (F = 141.6; P < 0.001) and a significant interaction (F = 42.5; P < 0.001) effect on the mean particle volume. The individual factors were compared with the MS for the interaction, which revealed no significant effects of the factors (F = 3.40 and F = 3.33). Two-way ANOVA on the mean number of cells per colony indicated also a significant *Daphnia*-water (F = 325.5; P < 0.001), a significant strain (F = 27.0; P < 0.001) and a significant interaction effect (F = 17.6; P < 0.001). Comparison of individual factors with the interaction MS indicated a significant *Daphnia*-water effect (F = 18.5), but no strain effect (F = 1.54). Therefore, t-tests were performed separately to evaluate for each strain the effect of *Daphnia* water on the mean particle volume and cells per colony (Fig. 8.2).

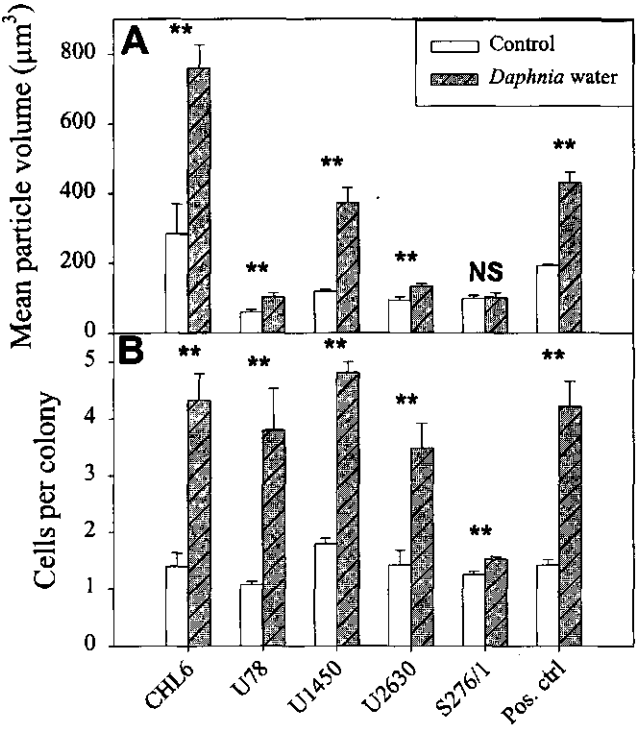


Figure 8.2: Mean particle volumes (µm³; panel A) and cells per colony (panelB) in 5 strains of *S. obliquus* cultured in the absence (Controls) and presence of water (10% v/v) from a *Daphnia* culture (*Daphnia* water). Incubations with *S. acutus* MPI served as positive control. Error bars indicate 1 SD (n = 3), ** a significance level of $P \leq 0.01$, and NS not significant.

In strain SAG 276/1, no significant difference in mean particle volumes between controls and treatments was found, and the *t*-test for cells per colony was only significant because of small within-group variation. In the other four strains, *t*-test showed that both the mean particle volumes and the number of cells per colony were significantly higher in treated than in the control populations (*see* Fig.8.2). Growth rates based on cell numbers and on algal volume differed among strains (Fig. 8.3A). Moreover, the presence of filtered medium from a *Daphnia* culture affected the growth rates in the strains NIVA-CHL6, UTEX 78, 1450 and 2630, but not in SAG 276/1.

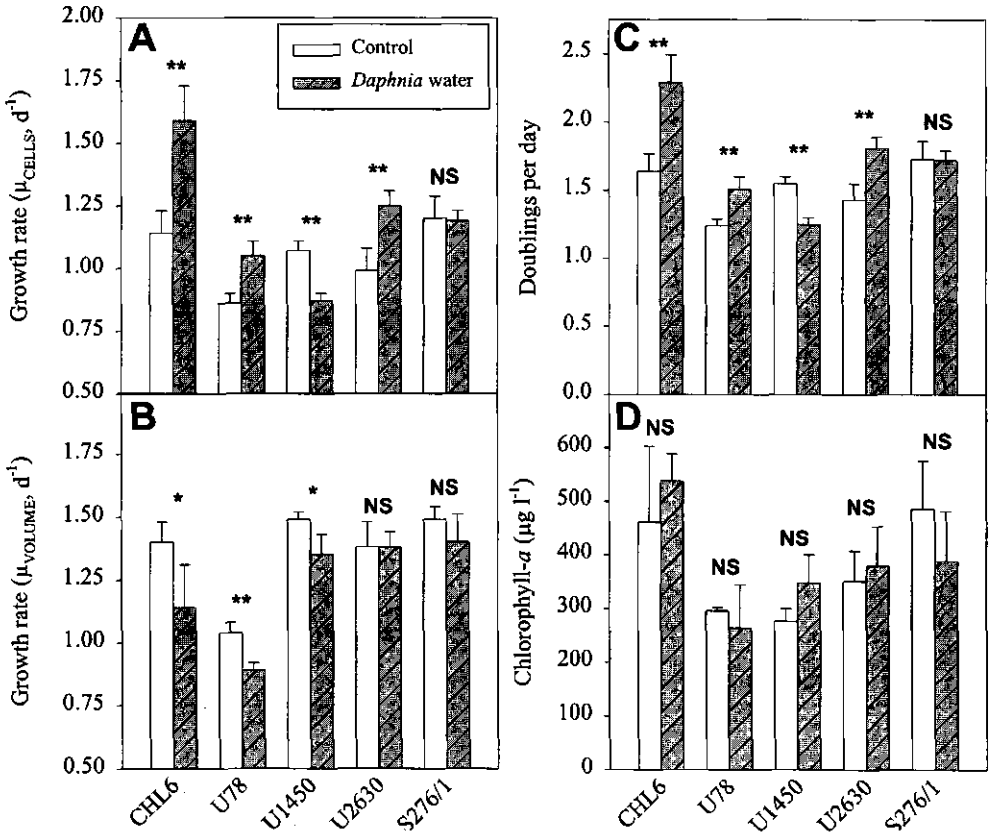
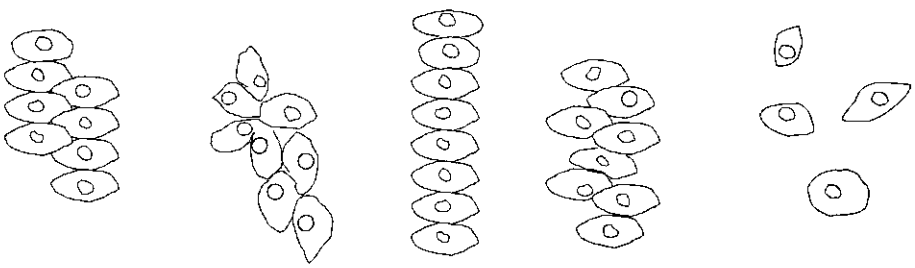


Figure 8.3: Growth rates (μ, d^{-1}) based on cell numbers (panel A) and biovolumes (panel B), number of doublings per day (panel C) and chlorophyll-*a* concentration (panel D) in five strains of *S. obliquus* cultured in the absence (Control, open bars) and presence of water (10% v/v) from a *Daphnia* culture (*Daphnia* water, filled bars). Error bars represent 1 SD (n = 3). ** indicates a significance level of $P \leq 0.01$, * of $P \leq 0.05$ and NS indicates not significant ($P > 0.05$).

Growth rates based on cell numbers tended to be slightly higher in the treatments than in the controls, while the opposite tendency was observed for growth rates on a volume basis (Fig.

8.3B). The number of doublings per day varied between 1.24 (± 0.05) and 2.29 (± 0.20) (Fig. 8.3C). The chlorophyll-*a* concentration differed significantly between strains (F = 8.43; P < 0.001), but was not affected by *Daphnia* chemicals (F = 0.12; P = 0.736) (Fig. 8.3D). Cell dimensions (length and width in µm) of unicells and eight-celled coenobia were measured using a Leica Quantimet 500 MC image analyzer coupled with a Nikon light microscope at 500 × magnification. Dimensions of both unicells and eight-celled coenobia differed significantly among *S. obliquus* strains (Table 8.1). Moreover, differences among strains in the most prominent eight-celled coenobial form were found. In NIVA-CHL6 costulatoid (*see* Fig. 8.4A) and irregular coenobia (Fig. 8.4B) were found, linear coenobia (Fig. 8.4C) were dominant in UTEX 78, costulatoid coenobia in UTEX 1450 and 2630, while alternating (Fig. 8.4D) and costulatoid coenobia were observed in SAG 276/1.



A: Costalutoid **B:** Irregular **C:** Linear **D:** Alternating **E:** Unicells

Figure 8.4: Eight-celled coenobia (A,B,C and D) and unicells (E) observed in *Scenedesmus obliquus*.

Table 8.1: Length and width dimensions (µm ± 1 SD) of unicells and eight-celled coenobia in five strains of *S. obliquus*, including *F*- and *P*-values of one-way ANOVAs. Similar symbols ^{a,b,c} within a column indicate homogeneous groups that are not significantly different at the 95% level (Tukey's test).

S. obliquus		Start experiment (t = 0)		End experiment (t = 48 h)			
Strain		Unicells (n = 15)		Unicells (n = 15)		8-celled coenobia (n = 15)	
NIVA-CHL6		13.8 (1.0)^{a} × 6.8 (0.5)^{a}		17.1 (2.3)^{a} × 9.0 (1.3)^{a}		41.8 (8.8)^{a} × 26.8 (10.4)^{a}	
UTEX 78		10.2 (1.1)^{b} × 5.1 (0.4)^{b}		13.3 (1.1)^{b} × 5.9 (0.8)^{b}		30.4 (2.5)^{b} × 17.4 (1.7)^{b}	
UTEX 1450		10.4 (0.9)^{b} × 4.9 (0.4)^{b}		15.1 (1.4)^{c} × 7.4 (1.0)^{c}		39.9 (6.0)^{a} × 24.2 (2.4)^{ac}	
UTEX 2630		10.1 (1.3)^{b} × 5.4 (0.7)^{b}		12.5 (1.6)^{b} × 6.3 (1.5)^{bc}		31.3 (2.8)^{b} × 21.1 (2.2)^{bc}	
SAG 276/1		9.9 (1.3)^{b} × 5.0 (0.6)^{b}		13.2 (2.0)^{b} × 5.8 (1.5)^{b}		30.5 (7.6)^{b} × 25.6 (6.2)^{ac}	
One-way ANOVA	F	32.2	31.3	18.3	17.9	11.6	8.37
	P	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001

8.2.3 *Scenedesmus quadricauda* (Turpin) Brébisson

S. quadricauda strain NIVA-CHL 7 was obtained from the Norwegian Institute for Water Research (NIVA, Norway), strain UTEX 76 was obtained from the culture collection of the University of Texas (U.S.A.), and strain F11 was obtained from the Saskatchewan Research Council (Canada). The organisms were transferred weekly into fresh medium and adapted to the environmental conditions for three months. The population composition of the inocula is presented in Table 8.2. The test was run in quadruplicate with an initial density of $1.3 \cdot 10^4$ particles·ml^{-1}.

Table 8.2: The mean number of cells per colony (c/c) and the composition of the inocula of *Scenedesmus* populations as percentage unicells, two-, four-, and eight-celled coenobia. Rest indicates three-, five-, six-, seven- and multicelled (>8) colonies.

Strain	1	2	4	8	rest	c/c
CHL7	39	27	29	----	5	2.26
F11	76	13	9	----	2	1.46
U76	4	68	24	----	4	2.49
MPI	74	10	12	1	3	1.64

In the three *S. quadricauda* strains tested, *Daphnia* water induced no colony formation (Fig. 8.5). Two-way ANOVA on the mean particle volume indicated a significant *Daphnia* water effect ($F = 53.0$; $P < 0.001$), a significant strain effect ($F = 3958$; $P < 0.001$) and a significant interaction ($F = 56.1$; $P < 0.001$). The individual factors were tested against the MS of the interaction that revealed a significant strain effect ($F_{3,3} = 70.6 > F_{crit} = 6.59$), but no *Daphnia* water effect ($F_{1,3} = 0.95 < F_{crit} = 10.1$). Because of this significant strain effect separate *t*-tests were performed to examine for each strain the effect of *Daphnia* water on the mean particle volume (Fig. 8.5A). Also for the number of cells per colony the two-way ANOVA indicated a significant *Daphnia* water effect ($F = 20.0$; $P < 0.001$), a significant strain effect ($F = 414$; $P < 0.001$) and a significant interaction ($F = 11.5$; $P < 0.001$). Hence, the same procedure as for mean particle volume was performed on the mean number of cells per colony (Fig. 8.5B). Both procedures clearly showed colony formation in the positive control, i.e. *S. acutus* MPI, but not in the *S. quadricauda* strains.

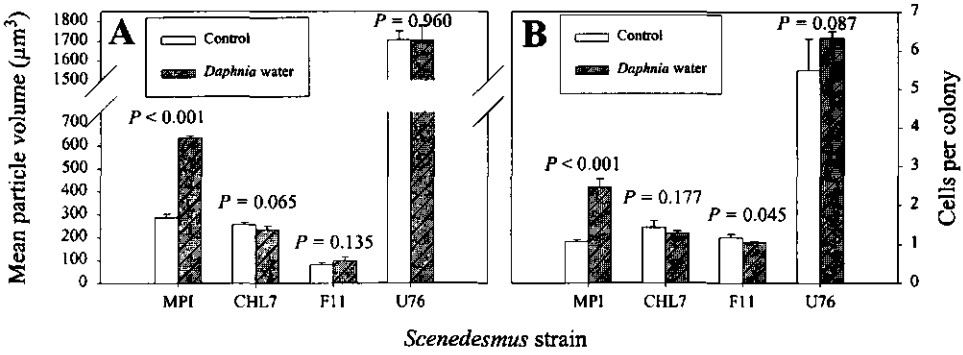


Figure 8.5: Effect of *Daphnia* water on the mean particle volume (Panel A) and the number of cells per colony (Panel B) in three strains of *S. quadricauda*, including *P*-values of *t*-tests. MPI represents positive controls with *S. acutus*. Error bars indicate 1 SD (n = 4).

The composition of the populations varied among the strains used (Table 8.3). *S. quadricauda* strains NIVA-CHL7 and F11 were dominated by unicells both in the absence and presence of *Daphnia* water, whereas strain UTEX 76 was dominated by eight-celled coenobia. However, at the beginning of the experiment no eight-celled coenobia were present in this strain (Table 8.2).

Table 8.3: Composition of *Scenedesmus* population after 48 hours in the absence (C) or presence (*DW*) of medium from a *Daphnia* culture (10% v/v) as percentage (± 1SD) unicells (1), two-, four- and eight-celled coenobia. The rest group represents three-, five-, six-, seven- and multicelled (>8) coenobia.

Strain→	NIVA-CHL7		F11		UTEX 76		*S. acutus* MPI	
cells↓	C	*DW*	C	*DW*	C	*DW*	C	*DW*
1	86 (4)	88 (1)	92 (3)	96 (1)	14 (7)	10 (4)	96 (3)	45 (3)
2	5 (2)	7 (0.6)	5 (2)	3 (1)	12 (5)	6 (2)	2 (1)	23 (6)
4	5 (2)	2 (0.8)	1 (0.5)	----	14 (4)	10 (4)	1 (0.8)	15 (2)
8	2 (0.5)	1 (0.4)	1 (0.4)	----	50 (13)	63 (2)	----	8 (3)
rest	2 (0.3)	2 (0.5)	1 (1)	1 (0.4)	10 (2)	11 (4)	1 (1)	9 (4)

Growth rates based on algal volume differed among strains (Table 8.4). Two-way ANOVA indicated a significant *Daphnia* water effect ($F = 4.83$; $P = 0.038$), a significant strain effect ($F = 192.3$; $P < 0.001$) and no interaction ($F = 1.96$; $P = 0.147$). Separate *t*-tests for each strain showed no differences in growth among control and treated cultures (Table 8.4). Thus, *Daphnia* water had no effect on the growth rates.

Table 8.4: Volume based growth rates (d^{-1} ± 1SD) of four strains of *Scenedesmus* cultured for 48 hours in the absence (Control) or presence of medium from a *Daphnia* culture (*Daphnia* water), including *t*- and *P*-values of *t*-tests.

Strain	Control	*Daphnia* water	*t*	*P*
CHL7	1.22 (0.04)	1.24 (0.09)	0.59	0.578
F11	1.15 (0.12	1.29 (0.03)	2.27	0.063
UTEX 76	0.89 (0.03)	0.94 (0.08)	0.96	0.374
MPI	1.68 (0.03)	1.67 (0.02)	0.63	0.549

Dimensions of unicells differed significantly among *S. quadricauda* strains (Table 8.5). Two-way ANOVA on cell length indicated no *Daphnia* water effect ($F = 0.24$; $P = 0.673$), but a significant strain effect ($F= 169$; $P = 0.006$). Also the two-way ANOVA on cell width indicated no *Daphnia* water effect ($F = 13.4$; $P = 0.067$) and a significant strain effect ($F= 1143$; $P < 0.001$). Thus, *Daphnia* water had no effect on the cell dimensions of *S. quadricauda* unicells. Unicells produced by strain NIVA-CHL7 always possessed two spines, unicells produced by strain UTEX 76 possessed two, or four spines, whereas strain F11 unicells were spineless (Fig. 8.6). One-way ANOVA indicated significant differences in spine length among treatments ($F_{3,64}= 9.9$; $P < 0.001$). However, Tukey's test revealed that this was caused by a significant difference between strain NIVA-CHL7 and UTEX 76. No *Daphnia* water effect on spine number and length was detected. The mean length (± 1 SD) of the spines was 6.4 (1.5) µm in UTEX 76 and 8.9 (1.5) µm in NIVA-CHL7. Eight-celled coenobia were only prominent in strain UTEX 76 and the coenobia always possessed four spines. Separate *t*-tests on the length, width and spine length were performed to evaluate differences between control and treated populations. However, as for unicells no effect of *Daphnia* water on length, width and spine length was observed (Table 8.5).

Table 8.5: Length and width dimensions (± 1 SD) of unicells (1) and eight-celled (8) coenobia, including the length of spines (µm) of *Scenedesmus* cultured in the absence (Control) and presence of medium (10% v/v) from a *Daphnia* culture (*Daphnia* water).

	Control		Spines	*Daphnia* water		Spines
Strain	Length	Width	Length	Length	Width	Length
CHL7 (1)	10.0 (0.9)	7.2 (1.2)	6.3 (1.4)	9.7 (1.5)	7.1 (1.2)	6.6 (1.8)
F11 (1)	6.9 (1.5)	5.1 (1.6)	----	6.9 (1.5)	4.9 (1.3)	----
UTEX 76 (1)	15.3 (4.0)	8.5 (2.0)	9.5 (1.3)	16.3 (4.5)	8.9 (2.8)	8.4 (1.5)
MPI (1)	18.5 (2.2)	7.7 (1.5)	----	----	----	----
UTEX 76 (8)	42.0 (6.6)	13.2 (1.8)	7.8 (1.5)	43.1 (6.2)	13.6 (1.3)	7.7 (1.2)

Morphological forms

Differences among strains in the most prominent morphological forms were found. In *S. quadricauda* NIVA-CHL7 unicells had two spines and two-and four-celled coenobia were linear in shape bearing four spines (Fig. 8.6A). In strain F11, unicells were spineless (Fig. 8.6B). Strain UTEX 76 contained a few unicellular forms, including both unicells and pseudo-unicells, and linear eight-celled coenobia (Fig. 8.6C).

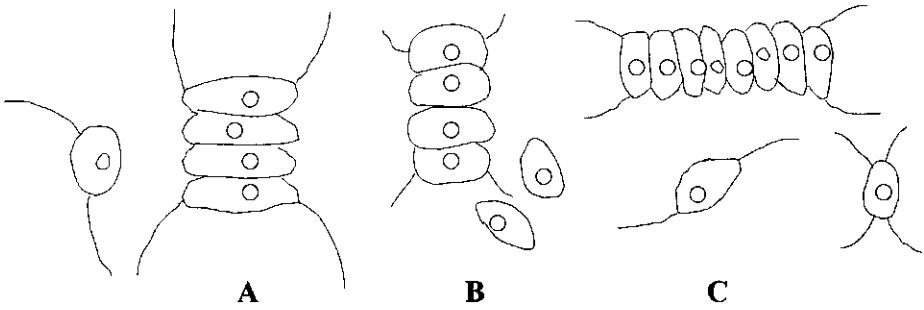


Figure 8.6: Unicells and four-celled coenobia of *S. quadricauda* strain NIVA-CHL7 (**A**), F11 (**B**) and UTEX 76 (**C**).

8.2.4 Scenedesmus subspicatus Chodat

Four strains of the spined *S. subspicatus* were examined on *Daphnia*-induced colony formation. The strain NIVA-CHL 55 was obtained from the Norwegian Institute for Water Research (NIVA, Norway). Strain RWTH was obtained from the University of Aachen (Germany). Strains UTEX 2532 and UTEX 2594 were obtained from the University of Texas (U.S.A.).

In a first biotest, the mean particle volume hardly differed between control and treatment populations (Fig. 8.7). Two-way ANOVA showed no *Daphnia*-water effect ($F = 2.76$; $P = 0.116$), a significant strain effect ($F = 8.81$; $P = 0.001$) and no interaction ($F = 1.28$; $P = 0.314$). NIVA-CHL 55 was the first strain in which *Daphnia*-induced colony formation was observed (Hessen & Van Donk, 1993), but here hardly showed a response. Therefore, the biotest was repeated (Fig. 8.7A). The two-way ANOVA of this second biotest indicated a significant *Daphnia* water effect on the mean particle volume ($F = 6.29$; $P = 0.019$). Separate *t*-tests were performed and showed a significant difference between controls and treatments in strain NIVA-CHL 55 ($t = 3.79$; $P = 0.010$), but not in the other strains RWTH ($t = 0.34$; $P = 0.748$), UTEX 2532 ($t = 1.08$; $P = 0.322$) and UTEX 2594 ($t = 0.94$; $P = 0.382$). Although the MPV in strain NIVA-CHL55 was significantly larger in the latter biotest, hardly any colony formation had been induced and no eight-celled coenobia were detected. Microscopic analysis of subsamples taken from the second biotest revealed that grazer-induced colony formation had only occurred in the positive control with the non-spiny *S. acutus* MPI (Fig. 8.7B). The population

composition varied among strains (Table 8.6). Unicells were abundant in all strains, but in strain UTEX 2532 also a considerable proportion of the population consisted of four-celled coenobia.

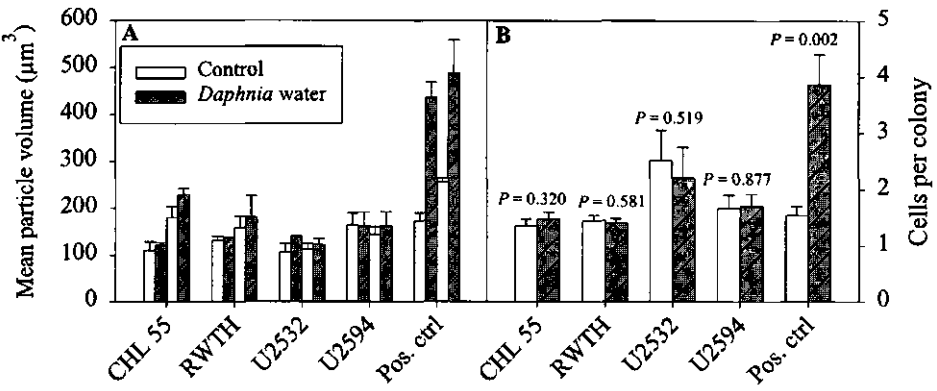


Figure 8.7: Effect of medium from a *Daphnia* culture (*Daphnia* water) on the mean particle volumes (Panel A) and the mean number of cells per colony (Panel B; including *P*-values of *t*-tests) in four strains of *Scenedesmus subspicatus*. Pos. ctrl indicates a positive control with *S. acutus* MPI.

Table 8.6: Composition of *Scenedesmus* populations after 48 hours in the absence (C) or presence (*DW*) of medium from a *Daphnia* culture (10% v/v) as percentage (± 1SD) unicells (1), two-, four- and eight-celled coenobia. The rest group represents three-, five-, six-, seven- and multicelled (>8) coenobia.

Strain→	MPI		NIVA-CHL55		RWTH		UTEX 2532		UTEX 2594	
cells↓	C	*DW*	C	*DW*	C	*DW*	C	*DW*	C	*DW*
1	78 (6)	21 (10)	84 (5)	80 (3)	78 (4)	79 (2)	48 (15)	60 (13)	77 (2)	69 (3)
2	9 (3)	16 (2)	7 (3)	11 (2)	14 (2)	12 (1)	7 (4)	9 (3)	9 (4)	17 (3)
4	5 (3)	30 (3)	2 (1)	3 (1)	2 (1)	3 (1)	39 (15)	18 (9)	3 (1)	4 (1)
8	2 (1)	16 (3)	----	1 (1)	1 (1)	1 (1)	1 (1)	4 (3)	1 (1)	----
rest	6 (4)	17 (10)	7 (4)	6 (3)	5 (4)	5 (4)	5 (3)	9 (7)	10 (4)	10 (7)

The experiment with this strain was repeated in 20% Z8 medium and performed as described by Hessen & Van Donk (1993). Again hardly any colony formation occurred. The MPV (± 1 SD) of control and treated cultures were 78.1 (6.7) and 104.4 (4.9) μm^3, respectively, while the mean number of cells per colony were 1.47 (0.18) and 1.80 (0.20), respectively. Growth rates were similar and 0.69 (0.13) and 0.67 (0.12) d^{-1}, respectively.

In their study, Hessen & Van Donk (1993) added 1 ml filtered water from a *Daphnia* culture to 50 ml algal suspension and used heavy inocula of $5.4 \cdot 10^5$ cells·ml^{-1}, but also longer incubation times (68 h). After 44 h already 41% of the treated populations consisted of eight-celled coenobia, while this proportion had further increased to 50% after 68 h (Hessen & Van

Donk, 1993). Therefore, incubations with *S. subspicatus* NIVA-CHL55 cultured in the absence and presence of filtered water from a *Daphnia* culture were followed for a period of 96 h. However, again no increase in mean particle volumes was detected (Fig. 8.8). Volume based growth rates over the four day period were similar (t = 0.22; P = 0.840) and on average 0.90 ± 0.03 d^{-1}. Maximal growth was measured over the first two days with mean rates (± 1 SD) of 1.16 (0.13) and 1.20 (0.04) for control and treated cultures, respectively.

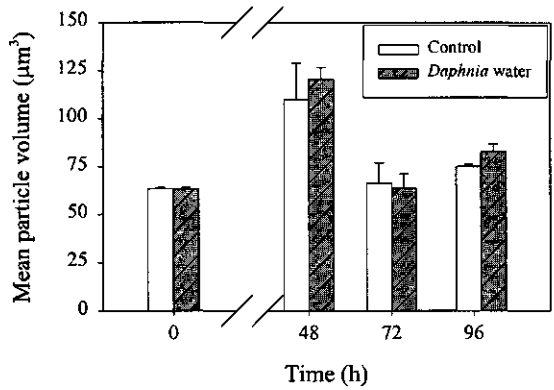


Figure 8.8: Mean particle volume (µm^3) in *S. subspicatus* NIVA-CHL55 cultured for 4 days in the absence (Control) and presence of water (10% v/v) from a *Daphnia* culture (*Daphnia* water). Error bars represent 1 SD (n = 3).

8.2.5 Scenedesmus gutwinskii Chodat

The three strains of *S. gutwinskii* tested on *Daphnia*-induced colony formation, i.e. strains B3-15, B8-7 and B8-27, were isolated by Dr. Fumie Kasai. After 48h, no colony formation had occurred in *S. gutwinskii* (Fig. 8.9).

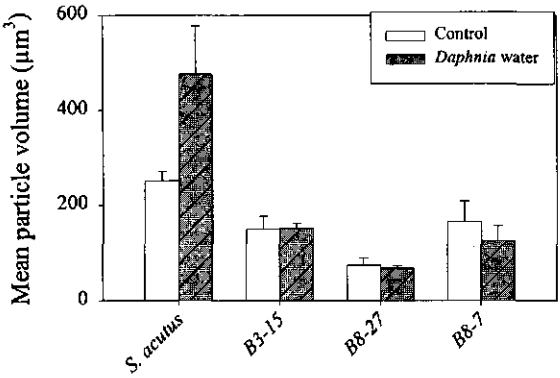


Figure 8.9: Mean particle volumes (µm^3) in three strains of *S. gutwinskii* cultured in the absence (Control) and presence of water from a *Daphnia* culture (*Daphnia* water). Error bars represent 1 SD (n = 4).

Two-way ANOVA indicated no *Daphnia* water effect ($F = 1.52$; $P = 0.233$), a significant strain effect ($F = 23.3$; $P < 0.001$) and no interaction ($F = 1.05$; $P = 0.372$). Also for biovolume based growth rates (μ, d^{-1}) no *Daphnia* water effect ($F = 3.58$; $P = 0.075$), a significant strain effect ($F = 55.4$; $P < 0.001$) and no interaction ($F = 0.30$; $P = 0.747$) was found.

8.2.6 *Other Scenedesmus strains*

The species *Scenedesmus armatus* Chodat, *S. falcatus* Chodat, *S. obtusiusculus* Chodat and *S. protuberans* Fritsch were exposed to *Daphnia* water in a standard biotest. *S. armatus* and *S. falcatus* were obtained from the Max-Planck-Institute for Limnology (Plön, Germany). *S. obtusiusculus* was obtained from the University of Turku (Finland), while *S. protuberans* was derived from the University of Amsterdam (The Netherlands). The different species were incubated in quadruplicates in the absence (Control) and presence of water (10% v/v) from a *Daphnia* culture (*Daphnia* water). One series was run with *S. armatus, S. obtusiusculus* and *S. protuberans*, whereas incubations with *S. acutus* MPI served as positive control. An additional series was run with *S. acutus* MPI, T10, UTEX 72 and *S. falcatus* Chodat.

Separate *t*-tests (two-tailed) showed a significant effect of *Daphnia* water on the mean particle volumes in *S. acutus* MPI, *S. armatus*, *S. obtusiusculus* and in *S. protuberans* (Fig. 8.10).

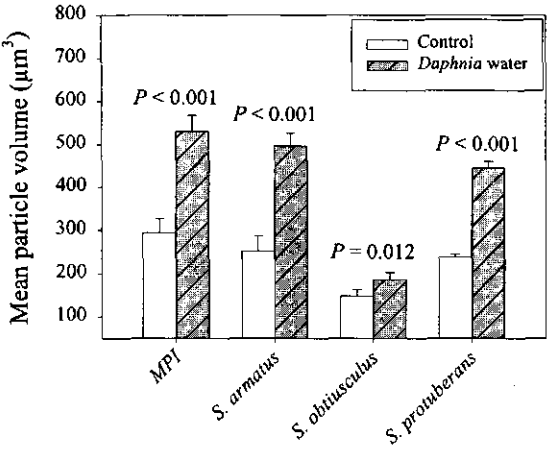


Figure 8.10: Mean particle volumes (μm^3) for four strains of *Scenedesmus* cultured in the absence (Control) and presence of water (10% v/v) from a *Daphnia* culture (*Daphnia* water), including *P*-values of *t*-tests. Error bars represent 1 SD (n = 4).

Microscopy subsamples from all incubations but *S. armatus* were lost. Analysis of the *S. armatus* samples revealed no difference (*t*-test; $P = 0.308$) between the mean number of cells per colony (± 1 SD) of control populations (4.63 ± 0.20) and treated populations (4.82 ± 0.19). The *S. armatus* stock culture appeared contaminated with *S. acutus* that caused the significantly

increased particle volumes in treated populations. Whether *S. obtusiusculus* and *S. protuberans* were contaminated remains unsolved, but based on the low mean particle volumes of *S. obtusiusculus* it seems safe to assume no contamination in this strain. The majority of *S. armatus* appeared as four-celled coenobia, very low numbers of pseudo-unicells were observed (Table 8.7).

Table 8.7: Composition of *S. armatus* populations, as percentage of unicells and two-, four, eight-celled coenobia, cultured for 48 h in the absence (Control) and presence of medium from a *Daphnia* culture (*Daphnia* water).

S. armatus	1	2	4	8	rest
Control	1 (1)	6 (1)	70 (1)	19 (3)	4 (1)
Daphnia water	2 (0.5)	5 (0.5)	68 (5)	20 (3)	5 (4)

In the second series, two-way ANOVA on the mean particle volume (MPV) indicated a significant *Daphnia* water effect ($F = 66.7$; $P < 0.001$), a significant strain effect ($F = 47.2$; $P < 0.001$) and a significant interaction ($F = 12.7$; $P < 0.001$). Separate *t*-tests showed significantly larger MPV in treatments of *S. acutus* MPI and *S. falcatus*, but no difference in the strains *S. acutus* T10 and UTEX 72 (Fig. 8.11A). Also for the number of cells per colony the two-way ANOVA indicated a significant *Daphnia* water effect ($F = 67.1$; $P < 0.001$), a significant strain effect ($F = 22.5$; $P < 0.001$) and a significant interaction ($F = 8.97$; $P < 0.001$). Separate *t*-tests showed not only significantly larger number of cells per colony in treatments of *S. acutus* MPI and *S. falcatus*, but also in *S. acutus* UTEX 72 (Fig. 8.11B). Thus, although the MPV was not significantly larger in treated populations of UTEX 72, colony formation had occurred after exposure to *Daphnia* water. This is also reflected in the population composition (Table 8.8). After 48 h in the presence of *Daphnia* water, the proportion of unicells in UTEX 72 had dropped, whereas the proportion of eight-celled coenobia had increased. Control populations remained dominated by unicells in all four strains, but dropped in three strains after 48 h exposure to *Daphnia* water. Only in strain *S. acutus* T10 no colony formation was observed (Table 8.8).

Dimensions of unicells and eight-celled coenobia of the different *S. acutus* strains were measured. Two-way ANOVA on unicell length indicated no *Daphnia* water effect ($F = 0.57$; $P = 0.504$), but a significant strain effect ($F = 84.0$; $P = 0.002$). Also the two-way ANOVA on unicell width indicated no *Daphnia* water effect ($F = 0.52$; $P = 0.523$), but a significant strain effect ($F = 30.4$; $P = 0.010$). Thus, *Daphnia* water had no effect on the cell dimensions of *S. acutus* unicells. However, unicells had significantly different dimensions among strains (Table 8.9).

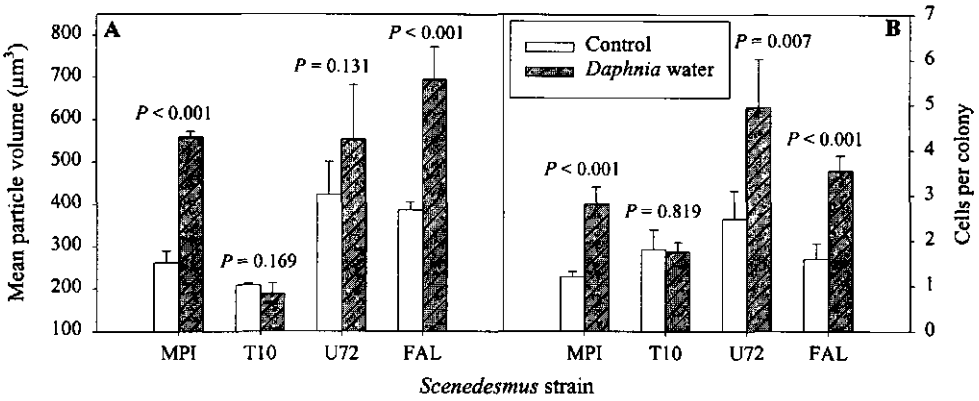


Figure 8.11: Effect of medium from a *Daphnia* culture (*Daphnia* water) on the mean particle volumes (Panel A) and the mean number of cells per colony (Panel B) in four non-spiny strains of *Scenedesmus*. Error bars represent 1 SD, also given *P*-values of *t*-tests.

In strain T10 a small and a large unicell-type was distinguished, that were significantly different sized (Fig. 8.12; Table 8.9). Eight-celled coenobia were observed in strain MPI and UTEX 72. In the latter strain, in the presence of *Daphnia* water two significantly different sized coenobia types were observed (Fig. 8.12; Table 8.9).

Table 8.8: Composition of *Scenedesmus* populations after 48 hours in the absence (C) or presence (*DW*) of medium from a *Daphnia* culture (10% v/v) as percentage (± 1SD) unicells (1), two-, four- and eight-celled coenobia. The rest group represents three-, five-, six-, seven- and multicelled (>8) coenobia.

Strain→	MPI		UTEX 72		UTCC 10		*S. falcutus*	
cells↓	C	*DW*	C	*DW*	C	*DW*	C	*DW*
1	90 (4)	42 (3)	56 (5)	29 (11)	67 (14)	68 (8)	79 (8)	32 (4)
2	6 (2)	14 (5)	14 (8)	4 (3)	12 (3)	12 (3)	9 (2)	11 (3)
4	2 (0.5)	24 (8)	9 (7)	13 (5)	13 (8)	10 (5)	3 (2)	27 (1)
8	----	7 (5)	11 (8)	47 (17)	1 (3)	2 (2)	2 (2)	17 (5)
rest	2 (2)	13 (4)	10 (5)	7 (1)	7 (5)	8 (2)	7 (4)	13 (3)

Different morphotypes were observed. In *S. armatus* the populations were dominated by four- and eight-celled coenobia, both morphotypes were bearing four spines (Fig. 8.12). In *S. acutus* MPI, unicells were spineless and eight-celled coenobia were always alternating. In strain T10 small and large unicells were observed and coenobia appeared irregular (Fig. 8.12). Strain

UTEX 72 contained spineless unicells and besides small and large eight-celled coenobia also costulatoid and alternating coenobia (Fig. 8.12).

Table 8.9: Length and width dimensions (± 1 SD) of unicells and eight-celled coenobia of three strains of *Scenedesmus acutus* cultured in the absence (Control) and presence of medium (10% v/v) from a *Daphnia* culture (*Daphnia* water).

	Control		*Daphnia* water	
Strain	Length	Width	Length	Width
MPI unicell	18.1 (1.5)	7.2 (1.3)	18.5 (1.5)	8.0 (1.7)
MPI 8 celled	----	----	36.9 (8.3)	25.3 (3.1)
U72 unicell	15.3 (2.3)	5.9 (0.9)	14.4 (1.5)	6.0 (0.8)
U72 small 8	----	----	25.1 (3.3)	19.1 (2.1)
U72 large 8	34.7 (5.9)	23.9 (3.7)	36.6 (5.1)	25.5 (3.8)
T10 small unicell	12.2 (1.8)	7.7 (1.2)	12.8 (1.4)	7.4 (1.1)
T10 large unicell	7.4 (1.2)	4.9 (1.2)	8.7 (0.9)	4.9 (1.0)

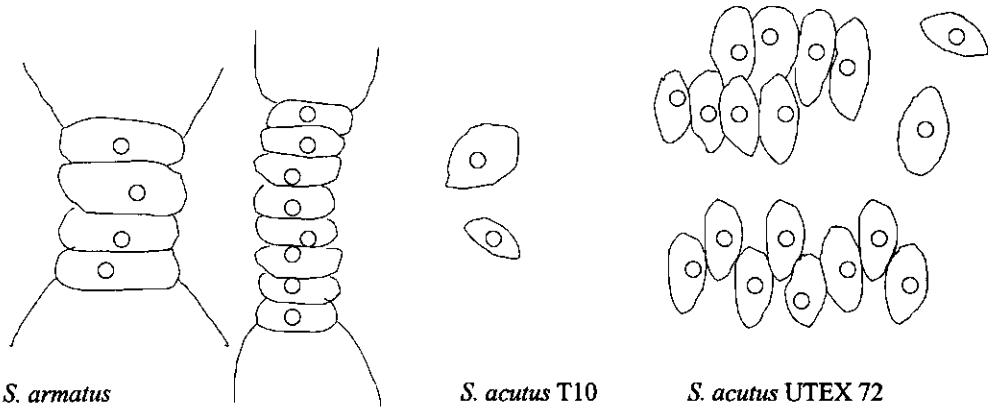


Figure 8.12: Different morphotypes of *S. armatus*, *S. acutus* UTCC 10 and *S. acutus* UTEX 72.

8.2.7 Scenedesmus in the presence of live Daphnia

In a first experiment, *S. acutus* MPI and *S. obliquus* SAG 267/1 were cultured in the absence and presence of 1 *D. galeata* (2.4 ± 0.2 mm) in 100 ml Erlenmeyer flasks with 50 ml 20% Z8 medium. In a second experiment, *S. subspicatus* NIVA-CHL55 and *S. obliquus* NIVA-CHL6 were cultured in 300 ml Erlenmeyer flasks with 150 ml COMBO medium (Kilham *et al.*, 1999) in the absence or presence of two *D. magna*. The former experiment was incubated for 48 h, the latter for 69 h. In a third experiment, *S. acutus* MPI was incubated for four days in the absence and presence of *Daphnia* and water from a *Daphnia* culture. This experiment was run in

50 ml WC medium in 100 ml cellulose plug stoppered Erlenmeyer flasks. All three experiments were run in triplicate.

In experiment I and II, based on both mean particle volumes and microscopy colony formation had occurred in the presence of live *Daphnia* (Fig. 8.13). Coulter analysis indicated the presence of numerous small particles in medium from the cultures with live *Daphnia*, probably faeces, bacteria and algal debris. Moreover, in the presence of *Daphnia* and already visual by eye were giant aggregates of algae and debris (Fig. 8.14). These particles were by sure too large to be measured with the electronic particle counter. For example in *S. obliquus*, image analysis showed mean aggregate dimensions (length × width, ± 1 SD) of 69 (33) × 55 (31) µm.

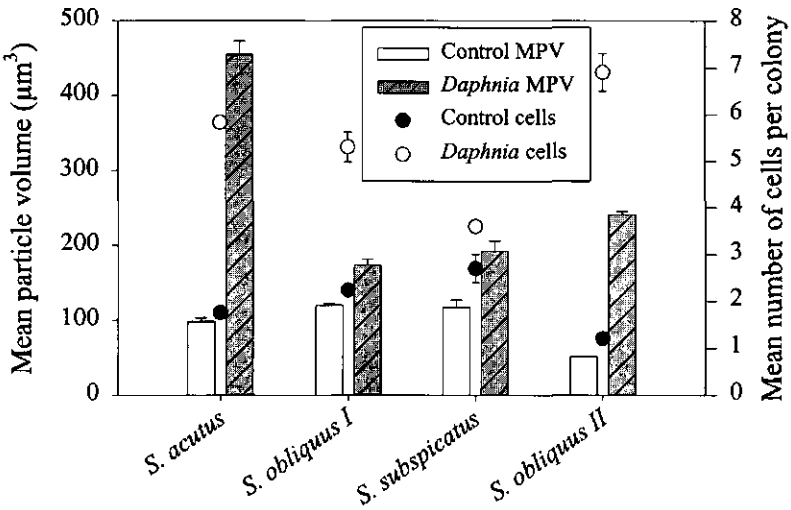


Figure 8.13: Mean particle volumes (bars) and mean cells per aggregate (symbols) in four strains of *Scenedesmus* cultured in the absence and presence of live *Daphnia*. Error bars represent 1 SD (n = 3).

Also in the third experiment, colony formation was significantly promoted in the presence of either water from a *Daphnia* culture or live *Daphnia* (Fig. 8.15). Despite the presence of giant aggregates, the mean particle volume in the presence of live *Daphnia* are lower than those of *S. acutus* cultured in the presence of filtered water from a *Daphnia* culture, because of the high proportion of small particles in the medium.

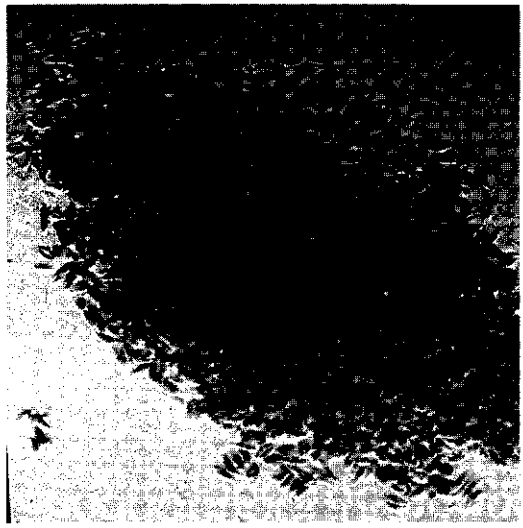


Figure 8.14: Giant *Scenedesmus acutus* aggregate in the presence of live *Daphnia*.

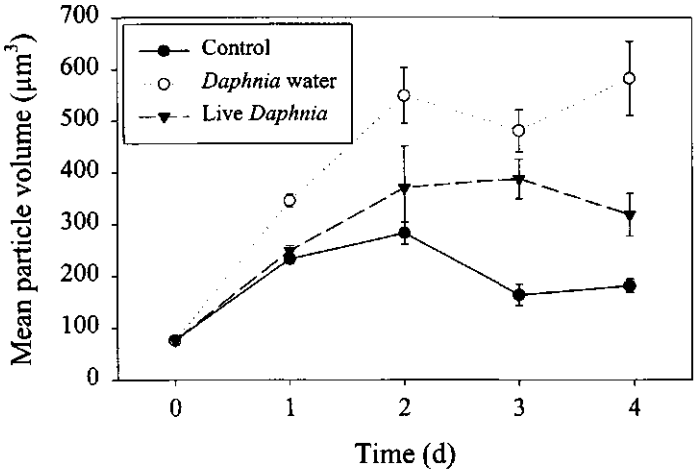


Figure 8.15: Effect of water from a *Daphnia* culture (open symbols) and a live *Daphnia* (filled triangles) on the mean particle volumes in *S. acutus* during a four days incubation period. Error bars represent 1 SD (n = 3).

The formation of these aggregates had already occurred after 21 h of incubation (Fig. 8.16). Microscopic analysis revealed that 44 ± 10% (± 1SD) of the algae were aggregated in these particles with on average 19.8 ± 12.6 cells per aggregate (± 1SD; n = 20). The cells were probably not aggregated as a result of viable gut passage, because faeces analysis showed empty cells (Fig. 8.16).

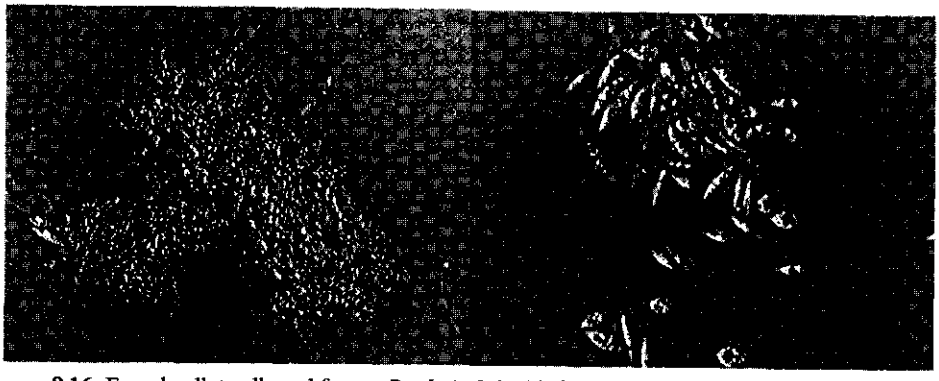


Figure 8.16: Faecal pellet collected from a *Daphnia* fed with *Scenedesmus acutus* comprised of numerous empty cells (left panel) and subsequent clogging of viable cells on such pellets (right panel).

8.2.8 *Daphnia-induced colony formation in Scenedesmus subspicatus*

The negative results with all spined strains do seem puzzling, because the phenomenon of *Daphnia*-induced colony formation has been reported first in a spined strain (Hessen & Van Donk, 1993). Moreover, the result has been confirmed for a spined *Scenedesmus* at the Dept. of Biological Sciences, University of Wisconsin (P. VanderPuy, pers. comment). Then in the presence of live *Daphnia* after 69 h a slight increase in the number of cells per colony has been observed (*§8.2.7*). This suggests that the biotest developed for the non-spiny *S. acutus*, may be applicable only to the non-spiny members of *Scenedesmus*. Therefore, a different experimental setup was chosen for testing the spined *Scenedesmus* on *Daphnia*-induced colony formation.

The spined *S. subspicatus* UTEX 2594 was incubated in fresh WC medium in the absence (controls) or presence of one live *D. pulex* (treatments). The incubations were placed on a rotating shaking device (80 rpm) in a climate-controlled cabinet at 20°C and illuminated using a 16:8 h light/dark-cycle. Since the number of cells per colony of *S. subspicatus,* in the presence of one *Daphnia* was slightly higher after 69 h (*see* § *8.2.7*), the incubation period was prolonged to 14 days. Moreover, the daphnid was removed after 72 h to overcome selective feeding on unicells and small coenobia.

Exponential growth was observed in both controls and treatments during the first three days. The growth rates were 0.976 (± 0.023) d^{-1} for controls and 0.885 (± 0.088) d^{-1} for treatments. Despite the grazing activity by the daphnid these growth rates were not significantly different (t = 1.73; P = 0.159). After three days colony formation was induced as the mean number of cells per colony (t = 4.29; P = 0.013) was significantly higher in the treatments than in control populations (Fig. 8.17). The colony formation was not just a result of selective removal of smaller algal particles since the number of cells per colony further increased for four more days after the daphnid had been removed.

In conclusion, colony formation in a spined *Scenedesmus* could be induced by *Daphnia*, but the time needed for the expression of an altered morphology was considerably longer than

for non-spined *Scenedesmus*. In the presence of live *Daphnia*, algae, debris and bacteria will be excreted from the animal thereby affecting the sterility of the algal suspension. This could affect colony formation in *Scenedesmus* (Trainor, 1998). The apparent ineffectiveness of filtered *Daphnia* water (*see* Fig. 8.8) could also be a result of bacterial activity needed for modification of the infochemical. However, in *S. acutus* the addition of antibiotics had no effect on the *Daphnia*-induced colony formation (*see* CHAPTER 3, page 55).

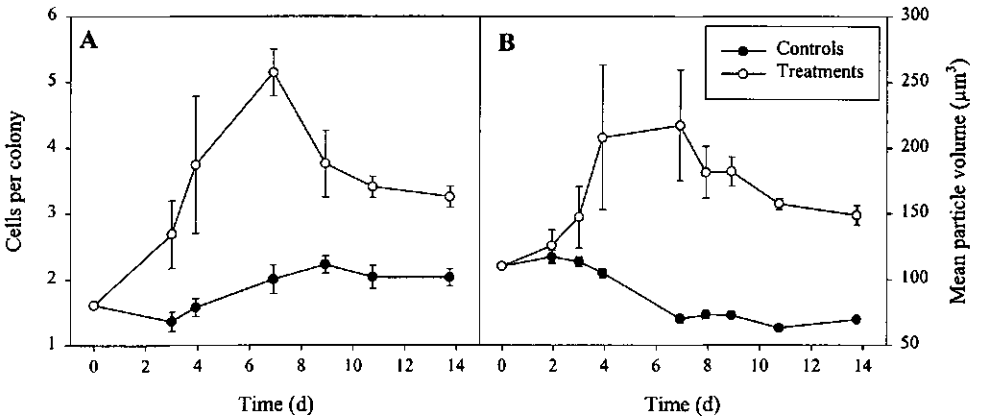


Figure 8.17: Morphological response expressed as the mean number of cells per colony (panel A) and as the mean particle volume (in μm^3; panel B) of *Scenedesmus subspicatus* UTEX 2594 cultured in the absence of one live *Daphnia* (Controls) or for three days in the presence of one *Daphnia* (treatments). Error bars represent 1 SD (n = 3).

8.3 OTHER CHLOROPHYCEAE

The effect of *Daphnia* water on growth and morphology of 9 different chlorophytes was examined in standard biotests (Fig. 8.18; Table 8.10). Besides in the positive control, in six of the tested Chlorophyceae the addition of *Daphnia* water had a significant effect on the mean particle volume (Table 8.10). Microscopy revealed that only in the two *Coelastrum* species the increase in the mean particle volume was caused by colony formation. In those two species the proportion of large multicelled aggregates (i.e. >16 cells) was increased, but unicells remained the most prominent morph.

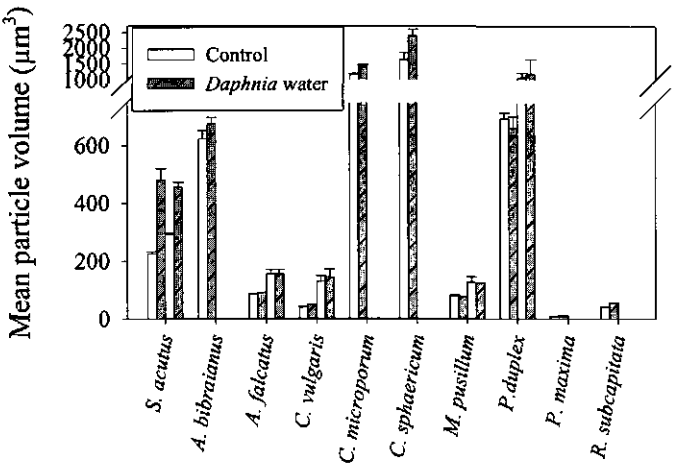


Figure 8.18: Mean particle volumes (µm³ ± 1 SD) for nine species of Chlorophyceae cultured in the absence (Control) and presence of water (10% v/v) from a *Daphnia* culture (*Daphnia* water). As positive control served incubations with *S. acutus*.

Table 8.10: Chlorophyte strains examined on *Daphnia*-induced colony formation, including *t*-and *P*-values of *t*-tests of mean particle volume (MPV) and cells per colony of control and treatment populations.

Strain	MPV (µm³)		Cells/colony	
	t-value	*P*-value	*t*-value	*P*-value
1) *Ankistrodesmus bibraianus* Korshikov SAG 278-1	2.40	0.074	---	---
2) *Ankistrodesmus falcatus* Ralfs NIVA-CHL 8	7.72	0.002	---	---
3) *Chlorella vulgaris* Beijerinck NIVA-CHL 19	7.54	0.002	1.03	0.361
4) *Coelastrum microporum* Nägeli SAG 217-1a	8.01	0.001	9.47	<0.001
5) *Coelastrum sphaericum* Nägeli SAG 32.81	4.45	0.011	6.47	0.003
6) *Micractimium pusillum* Fresenius CCAP 248/1	1.11	0.346	---	---
7) *Pediastrum duplex* Meyen SAG 261-3a	1.29	0.266	---	---
8) *Planktosphaeria maxima* Bischoff/Bold CCAP 65/1	21.4	<0.001	1.55	0.196
9) *Selenastum capricornutum* Printz NIVA-CHL 1	11.8	<0.001	1.41	0.232

The mean number of cells per colony (± 1 SD) was 2.44 (0.23) and 5.01 (0.41) in control and treated *C. microporum* cultures, respectively, while in *C. sphaericum* these numbers were 2.84 (0.97) and 7.12 (0.61), respectively. Spherical unicells were 10 ± 1 µm in size in *C. microporum* (including the mucous layer 18 ± 3 µm) and 21 ± 5 µm in *C. sphaericum*. The large aggregates had mean dimensions (length × width, ± 1 SD) of 82 (11) × 77 (12) µm and 88 (17) × 60 (12) µm in *C. microporum* and *C. sphaericum*, respectively.

8.4 BACILLARIOPHYCEAE

The diatoms *Asterionella formosa* CCAP 1005/9 and *Synedra tenuis* CCAP 1080/2 were obtained from the Culture Collection of Algae and Protozoa (CCAP, Windemere, UK). The algae were cultured for 3 days in the absence (control) and presence of *Daphnia* water (10% v/v), and in the presence of one live *Daphnia*. The algae were cultured in Z8 medium enriched with silicate at 20°C in 175 $\mu mol \cdot m^{-2} \cdot s^{-1}$ at a 16:8 h light:dark-cycle. The inoculum cultures were mainly uni- and bicellular with mean number of cells per aggregate of 1.9 and 1.5 for *A. formosa* and *S. tenuis*, respectively. The initial density differed slightly among the two species and was $6 \cdot 10^6$ $\mu m^3 \cdot ml^{-1}$ for *S. tenuis* and $9.8 \cdot 10^6$ $\mu m^3 \cdot ml^{-1}$ for *A. formosa*.

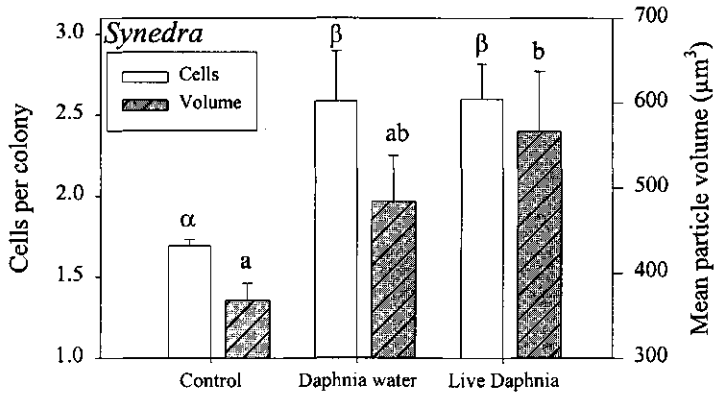


Figure 8.19: Mean number of cells per aggregate and the mean particle volumes (μm^3) for *S. tenuis* cultured in the absence (Control) and presence of water from a *Daphnia* culture (Daphnia water), and in the presence of one live *Daphnia* (Live Daphnia). Error bars represent 1 SD, similar symbols α,β,a, b homogeneous groups that are not different at a 95%-level (Tukey test).

After three days, variation among the three replicates per treatment in *A. formosa* was considerable. One-way ANOVA indicated no differences in the mean particle volumes ($F_{2,6}$ = 0.32; P = 0.741). The mean particle volume (± 1 SD) was 368.9 (60.2) μm^3. However, in *S. tenuis*, one-way ANOVA indicated significant differences in MPV among treatments ($F_{2,6}$ = 8.30; P = 0.019). Microscopic analysis revealed that the proportion of unicells had slightly decreased from 70% in the controls to 60% in the treatments and that some large aggregates with more than 10 cells were formed. This caused a small but significant increase in the number of cells per colony ($F_{2,6}$ = 17.0; P = 0.003). The mean number of cells per aggregate (± 1 SD) and the mean particle volumes (± 1 SD) for *S. tenuis* are presented in Fig. 8.19.

8.5 CYANOPHYCEAE

8.5.1 Oscillatoria and *Aphanizomenon*

Filamentous cyanobacteria have been reported to appear as flakes in the presence of *Daphnia* but as single filaments in their absence (Lynch, 1980; Holm *et al.*, 1983). Moreover, unicellular cyanobacteria may often occur as large numbers held within a common mucilage envelope, but fail to do so in laboratory cultures. As representatives of filamentous cyanobacteria *Oscillatoria agardhii* NIVA-CYA 29 (order Oscillatoriales) and *Aphanizomenon flos-aqua* NIVA-CYA 142 (order Nostocales) were selected. The former cyanobacterium is a representative of a genus in which the trichomes are never united into colonies and no mucilage sheat is present, whereas the latter may occur as flakes (Van den Hoek *et al.*, 1995). Stock populations were cultured in WC-medium at 20°C in 45 $\mu mol \cdot m^{-2} \cdot s^{-1}$ at a 16:8 h light:dark-cycle. The cyanobacteria were transferred into 300 ml Erlenmeyer flasks containing 150 ml of fresh WC-medium (control), medium with 10% (v/v) medium from a *Daphnia* culture (300 animals per liter), or with one live *Daphnia* added (treatments). After two and seven days, the incubations were microscopically examined for the presence of flakes and the filament density was determined using a Bürker-Turk counting chamber with a volume of 0.9 mm^3. However, no flakes were formed and all cyanobacteria remained present as single filaments even in the presence of live *D. pulex*. The growth rates (± 1 SD) were 0.27 (0.08) and 0.22 (0.08) day^{-1} for *Oscillatoria* and *Aphanizomenon*, respectively.

8.5.2 Microcystis

In a second experiment three strains of the unicellular cyanobacterium *Microcystis aeruginosa* (NIVA-CYA 43, 140 and 228/1; order Chroococcales) were selected. The colonial morphology of *Microcystis* may clearly reduce feeding in zooplankters like *Daphnia* (Fulton III & Paerl, 1987a), but chemicals released from *Daphnia ambigua* (Fulton III & Paerl, 1987b) or *D. magna* (Hessen & Van Donk, 1993) appeared ineffective as colony-inducing agents. On the other hand, several strains of *Microcystis* are among toxin producing phytoplankton. Numerous variants of the *Microcystis* toxin, microcystin, have been isolated and purified (Codd *et al.*, 1989). Especially the variant microcystin-LR may exert strong toxic effects on herbivorous zooplankton (e.g. DeMott *et al.*, 1991; Haney *et al.*, 1994; Reinikainen *et al.*, 1994; 1995). Abiotic factors, such as light-intensity (Watanabe & Oishi, 1985), and biotic factors, such as grazing (Benndorf & Henning, 1989) may cause variability in *Microcystis* toxicity. Hence, besides the possible effects of medium from a *Daphnia* culture on growth and morphology also the amount of microcystins were examined.

Induction experiment

Prior to the experiment 400 adult *Daphnia magna* were transferred into 1 liter WC-medium with a 1:1 mixture of *S. acutus* and *M. aeruginosa* CYA 43. After 48 h of grazing,

water was filtered through a glass-fiber filter and used as test-water in a biotest to examine the inducibility of MC (-LR) by *Daphnia* chemicals. This biotest was run in 300 ml cellulose-plug stoppered Erlenmeyer flasks containing 150 ml of medium. Controls contained *Microcystis* in fresh WC-medium, while treatments contained 135 ml fresh medium + 15 ml test-water. Both were run in quadruplicate. The batches were incubated in a climate controlled chamber at 20°C in 45 $\mu mol \cdot m^{-2} \cdot s^{-1}$ with a 16:8 h light:dark cycle. Each day the algal size distributions and densities were determined in the range 2-20 µm using a Coulter Multisizer II (100 µm capillary). A subsample was preserved in Lugol's fixative upon microscopic analysis. After 14 days, the exponentially growing cells were harvested by filtration and analyzed on their microcystin content. Microcystins (MC) were extracted by solid phase extraction and determined by reversed phase HPLC using MC-LR as external standard according to the method as described by Lawton *et al.* (1994).

Microcystin (MC) analysis revealed that both CYA 140 and CYA 228/1 contained MC (and MC-LR), but not CYA 43. The total MC-content per cell in strain CYA 228/1 was significantly higher than in CYA 140 ($F = 46.5$, $P < 0.001$). However, two-way ANOVA indicated no significant difference in MC-contents between controls without and treatments that had been cultured with 10% (v/v) water from a *Daphnia* culture ($F = 1.59$, $P = 0.232$). The MC-LR contents per cell were similar between CYA 140 and CYA 228/1 ($F = 4.11$, $P = 0.065$) and also between controls and treatments ($F = 2.03$, $P = 0.180$). Because the mean particle volume in CYA 228 is considerably larger than in CYA 140, the microcystin-content was standardized per unit biovolume (Fig. 8.20).

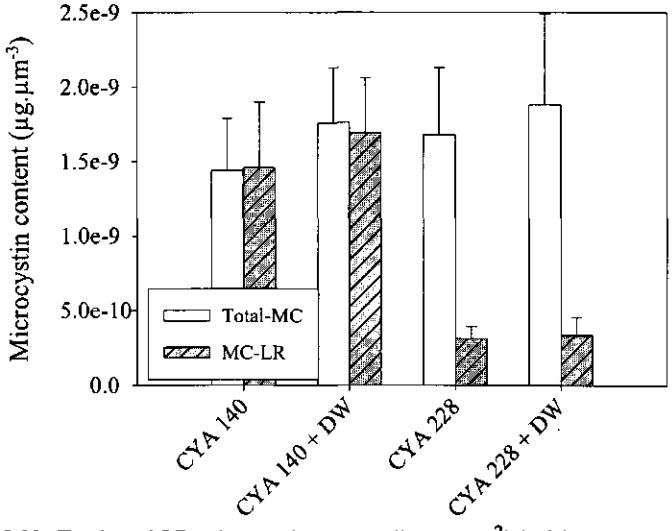


Figure 8.20: Total- and LR-microcystin content (in $\mu g \cdot \mu m^{-3}$) in *Microcystis aeruginosa* CYA 140 and CYA 228 cultured for two weeks in the absence or presence of filtered medium from a *Daphnia* culture (+ DW). Error bars indicate 1 SD (n = 4).

Now two-way ANOVA indicated no significant differences in total-MC content between CYA 228 and CYA 140 ($F = 0.77$; $P = 0.396$) and between controls and treatments ($F = 1.61$; $P = 0.229$). The MC-LR contents were significantly lower in CYA 228 ($F = 71.5$; $P < 0.001$), but no differences were observed between controls and treatments ($F = 0.83$; $P = 0.381$). Hence, neither total-MC nor MC-LR contents were influenced by *Daphnia* infochemicals.

Although the growth rates were significantly different between strains ($F = 32.9$; $P = 0.029$), no different growth rates between controls and treatments within one strain ($F = 0.24$; $P = 0.670$) were observed (Table 8.11). Moreover, growth rates based on either number of particles or on biovolumes were similar (*t*-test: $P = 0.622$). The mean particle volume (μm^3) was somewhat larger in *Daphnia* water treatments in strain CYA 140 and CYA 228, but the increase was marginal and microscopic analysis showed no colony formation (Fig. 8.21).

Table 8.11: Growth rates (μ, d^{-1}), based on total algal volume or on number of cells, for three *Microcystis aeruginosa* strains cultured in the absence (Control) or presence (*Daphnia* water) of medium from a *Daphnia* culture.

	μ-cell numbers			μ-volume		
	CYA 43	CYA 140	CYA 228	CYA 43	CYA 140	CYA 228
Controls	0.291	0.281	0.221	0.272	0.288	0.259
Daphnia water	0.277	0.293	0.211	0.266	0.295	0.245

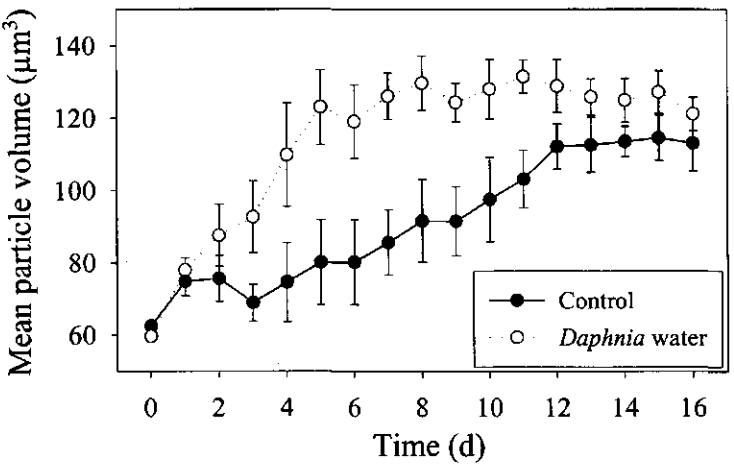


Figure 8.21: Course of the mean particle volume (μm^3) for *Microcystis aeruginosa* CYA 228 cultured for 16 days in the absence (Control) or presence of medium from a *Daphnia* culture (*Daphnia* water). Error bars represent 1 SD (n = 4).

8.6 DISCUSSION

The 23 *Scenedesmus* strains showed a considerable variation in colony formation when exposed to filtered water (10% v/v) from a *Daphnia* culture. In these experiments 8 of 23 strains were responsive on this trait, i.e. ~35% of the tested strains. Most of the responding strains were non-spiny *Scenedesmus*: 64% of the tested non-spiny strains were responsive, while only 4% of the spined strains showed *Daphnia*-induced colony formation. Colony formation was one morphological trait examined in this study. *Daphnia*-induced spine formation was examined in three strains, but the spine number and length was not affected. Other potential defensive traits such as cell wall thickness, mucous production (Van Donk *et al.*, 1998), toxicity (Boersma & Vijverberg, 1995), and the presence of bristles (Trainor & Burg, 1965; Massalski *et al.*, 1971) which all could hamper ingesting and digestion by zooplankters were not examined.

In biotests with *S. subspicatus*, *S. quadricauda,* and *S. gutwinskii* none of the tested strains showed formation of eight-celled coenobia in the presence of *Daphnia* infochemicals. Although *S. subspicatus* NIVA-CHL55 has been reported to respond to *Daphnia* infochemicals by formation of numerous 4-and 8-celled coenobia (Hessen & Van Donk, 1993), no infochemically induced colony formation was observed in this study. Among experiments after 44h of incubation, Hessen & Van Donk (1993) found considerable variation in the proportion of infochemically induced eight-celled coenobia with ~6% and 41% in the experiments II and III, respectively. This difference may have been the result of the use of a shaking device in the latter experiment. However, in this study always a shaking device has been used, but without any effect on colony formation. Also a longer incubation time appeared without any effect (*see* Fig. 8.8). By contrast, in the presence of live *Daphnia* a somewhat higher proportion of colonies was found in *S. subspicatus*. The susceptibility of *Scenedesmus* to *Daphnia* infochemicals may be affected by other factors such as the physiological state of the algal cells or other traits may be involved in the defensive strategy. Examination of figure 1 in Hessen & Van Donk (1993) suggests an increase in spine length in connection with colony formation. The unicellular *S. subspicatus* has spines with a length of ~2 μm, the eight-celled morph bears spines of ~3.5 μm. This increase in spine length could also occur in unicells exposed to *Daphnia* infochemicals, but has not been examined in this study. However, in the spiny *S. quadricauda* no effect of *Daphnia* exudates on the formation of spines was detected. In *§8.2.8*, a different setup was chosen to examine *Daphnia*-induced colony formation in a spined *Scenedesmus*. A biotest was run for 14 days in which the treatments were run with a live *Daphnia*. After three days the live *Daphnia* was removed and the incubations were followed. Formation of eight-celled coenobia was observed in these treatments, but not in the controls. Thus, the biotest used in this chapter to examine grazer-induced colony formation in *Scenedesmus*, is only useful for the non-spiny strains.

Eight-celled coenobia were also formed in both control and treated cultures in strain *S. quadricauda* UTEX 76. Thus, in this strain eight-celled coenobia were formed independent of

Daphnia water. The strain UTEX 76 was considered *S. quadricauda,* but has been redefined as *S. communis* (Hegewald 1977). In the study by Egan & Trainor (1990), UTEX 76 produced always unicells with 5-7 spines, but here this alga produced larger unicells with 2-4 spines. The length of the spines is, however, comparable among both studies. Moreover, in both studies eight-celled coenobia were easily formed in UTEX 76, when growth rates were high. Several factors have been reported to affect the coenobial cell number in *S. quadricauda*, such as light-rhythm (Steenbergen, 1975), nutrients (Steenbergen, 1978) and growth rate (Gavis *et al.*, 1979). The number of cells per colony in *Scenedesmus* seems closely related to the amount of energy stored or protoplasm produced in the parent cell (Šetlík *et al.,* 1972) and may be directly proportional to growth rates (Siver & Freeda, 1982). However, all studies were performed with only one strain. By contrast, in this study a considerable within-'species' variation in growth and morphology was observed. Light field and nutrients were similar among cultures, whereas growth rates varied among strains. Gavis *et al.* (1979) reported 90% eight-celled coenobia at growth rates around 1.0 day^{-1}, but here at a growth rate of ~0.9 day^{-1} 50-60% eight-celled coenobia occurred in strain UTEX 76, whereas at a growth rate of ~1.2 day^{-1} in strain NIVA-CHL7 only 2% eight-celled coenobia were observed. A difference between the studies is that Gavis *et al.* (1979) run chemostats, whereas in this study short-term batch experiments were run. At low inoculum size and the relatively short incubation time of 48 h employed in this study, cultures will approximate steady state conditions. The unicell-colony transformation might occur independent of growth rate by altering the chemical environment (Siver & Trainor, 1983), such as the addition of *Daphnia* water.

In the non-spiny strains considerable within-species variation in grazer-induced coenobia formation was observed. In *S. acutus* and *S. obliquus*, two of four and four of five strains showed induced colony formation, respectively. A different physiological state could be involved, but no major differences in physiological states were evident as cultures exhibited excellent growth and environmental conditions were similar. Inactivation of the infochemicals may occur by adsorption to the cell surface or metabolic activity (*see* §3.2.3). However, this may not be the reason for the different responses observed here. For example, in the experiment with *S. obliquus* different cell numbers per strain were inoculated, as a result of normalization to algal volume, but the cell numbers of the non-responsive strain were intermediate. Moreover, resource limitation was unlikely at the low inoculum size and relative short-term incubation period (48 h) employed in this study.

Different culture conditions did not affect the morphological appearance of *S. obliquus* strain SAG 276/1, populations which consisted of over 90% of unicells (Hegewald, 1982; Holtmann & Hegewald, 1986). Nevertheless, SAG 276/1 is able to produce coenobia, as they were always present in very low numbers. Even if the strain SAG 276/1 responded to *Daphnia* chemicals, the eight-celled coenobia of this strain with dimensions of 30 × 25 μm will hardly be protected against *Daphnia* larger than 1.1–1.2 mm (Burns, 1968), which is a common size for

animals in nature (Lürling & Van Donk, 1997). The same holds true for eight-celled coenobia of *S. obliquus* strains UTEX 78 and 2630 and the four-celled coenobia of UTEX 1450 and NIVA-CHL6.

In *S. acutus*, based on the mean particle volumes from the first two biotests, it was suggested that *Daphnia*-induced colony formation had only occurred in strain MPI. When this test was repeated including a treatment with the non-spiny *S. falcatus*, microscopy revealed that *Daphnia*-induced coenobia formation not only had occurred in strain MPI, but also in UTEX 72 and in *S. falcatus*. Moreover, in UTEX 72 control populations consisted of 10% of eight-celled coenobia, whereas only 1% eight-celled were found in strain UTCC 10.

The non-spiny "species" examined in this study were morphologically difficult to identify as separate taxa. For example, *S. acutus* MPI had morphological characteristics more identical to *S. obliquus* UTEX 1450 than the latter had to *S. obliquus* SAG 276/1. Chodat (1926) used in his description of plasticity in *Scenedesmus* the name *S. obliquus* for numerous non-spiny *Scenedesmus* including *S. acutus*. Also distinguishing *S. acutus* from *S. falcatus* could give some difficulties, as more or less identical unicells may be present in the cultures. Some authors could explain this by contamination, but more likely the species concept in non-spiny *Scenedesmus* needs reconsideration. Analogous to Trainor & Egan's (1990) criticism on the *Scenedesmus* catalogue of Hegewald & Silva (1988) with approximately 1330 taxa, based on phenotypic plasticity this species number will be definitely much lower. On the trait colony formation, *S. acutus* MPI seems closer to several *S. obliquus* strains than to the UTCC *S. acutus* strains used in this study. Since morphological identification of *Scenedesmus* is extremely difficult, and similar morphotypes may be genetically different, analysis of ribosomal and ITS (internal transcribed spacer region) DNA (Rausch *et al.*, 1989) may be very useful in unraveling phylogenetic relationships among *Scenedesmus*. In a recent study, sequence analyses of 18S rDNA from 16 *Scenedesmus* species suggested a division in two subgenera, the non-spiny species in Scenedesmus and the spined species in Desmodesmus (Kessler *et al.*, 1997). Also among different genotypes undoubtedly a variation in response to grazer chemicals will be demonstrated.

Gradually, more information is gathered on *Scenedesmus* plasticity. It is evident that not only abiotic factors such as nutrients, temperature and salinity (e.g. Trainor, 1992a, b; 1993; 1995; Mur, 1971; Wasmund, 1992), but also biotic factors such as the presence of grazers affect the morphological development in some *Scenedesmus*. The phenomenon of grazer-induced coenobia formation seems widespread, but not universal among *Scenedesmus*. Since about 43% of the tested strains were responsive on this trait, one can not claim that grazing-associated chemicals evoke a universal physiological response in *Scenedesmus*.

The phenomenon of *Daphnia*-induced colony formation seems not restricted to the genus *Scenedesmus* as colonies were formed in *Coelastrum* (Table 8.12 and *see* Fig. 8.22). In two-third

of the strains the addition of *Daphnia* water resulted in a significantly increased cell volume. This increase was also observed in the cyanobacterium *Microcystis aeruginosa*.

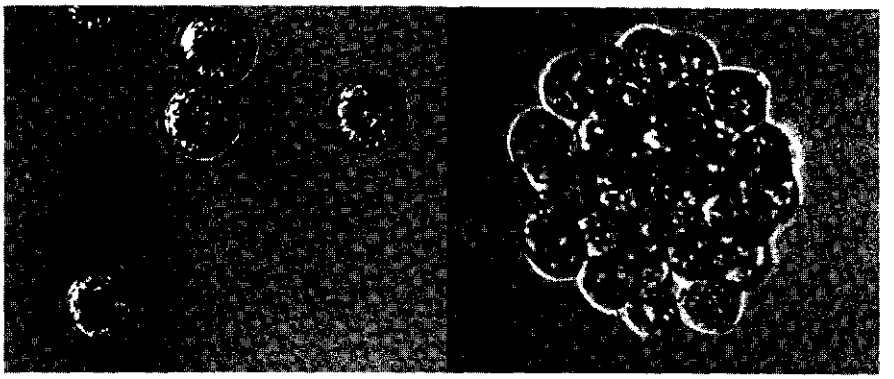


Figure 8.22: Unicellular *Coelastrum sphaericum* (left panel) and a colonial aggregate (right panel).

Moreover, grazer-induced morphological changes seem not restricted to the Chlorophyceae, but may also occur in diatoms, as *Synedra* showed a somewhat enlarged particle volume in the presence of *Daphnia* water and live *Daphnia* because of the presence of large aggregates. These large aggregates were commonly observed in the presence of live *Daphnia*. This process of cell aggregation seems caused by clogging of live cells onto faecal pellets. These aggregates were already visible within one day and thus aggregation seems a more rapid process than the chemically induced colony formation. Also Hessen & Van Donk (1993) reported the occurrence of large multicelled aggregates with mean diameters of 50 µm in the presence of live *Daphnia*. They referred to it as a culture phenomenon, but there is no reason to assume that the formation of multicelled aggregates will be restricted to the laboratory. From the marine world, flocculation of algae, "marine snow", is a well-known phenomenon that may be caused by heterotrophic flagellates (Nygaard & Hessen, 1994) and that inhibits copepod grazing (Malej & Harris, 1993). In freshwater systems, grazing *Daphnia* may release 10-17% of the ingested carbon as DOC (Lampert, 1978), that could be involved in clogging of algae. Especially during high zooplankton abundancy enhanced sedimentation of algae because of formation of large aggregates may contribute significantly to the clear-water phase. Perhaps the higher *Scenedesmus* sedimentation losses in the presence of zooplankton than in the absence (Visser *et al.*, 1996) were a result of both colony formation and formation of multicelled aggregates. By contrast, these large aggregates may be colonized by protozoa, such as vorticellid ciliates (Fig. 8.23), a phenomenon not uncommon in nature (f.e. figure 407 in Canter-Lund & Lund, 1995). In the presence of *Daphnia*, both may benefit from this interaction. The ciliates may have a refuge from *Daphnia*, the *Scenedesmus* could have a refuge from both *Daphnia* and ciliates, but may also be kept in suspension for longer period because of the water movements caused by the ciliates acting as an engine.

Table 8.12: Green algal strains tested for *Daphnia*-induced colony formation in a standard biotest

Algal taxon	Colony formation
Ankistrodesmus falcatus NIVA-CHL 8	NO
Ankistrodesmus bibraianus SAG 278/1	NO
Chlorella vulgaris NIVA CHL 19	NO
Coelastrum microporum SAG 217/1a	YES
Coelastrum sphaericum SAG 32.81	YES
Micractinium pussilum CCAP248/1	NO
Pediastrum duplex SAG 261/3a	NO
Planktosphaeria maxima CCAP 65/1	NO
Raphidocelis subcapitata NIVA-CHL 1	NO
Scenedesmus acutus MPI	YES
Scenedesmus acutus f. *alternans* UTEX 72	YES
Scenedesmus acutus UTCC 7	NO
Scenedesmus acutus UTCC 10	NO
Scenedesmus armatus MPI	??
Scenedesmus falcatus MPI	YES
Scenedesmus gutwinskii B3-15	NO
Scenedesmus gutwinskii B8-7	NO
Scenedesmus gutwinskii B8-27	NO
Scenedesmus obliquus NIVA-CHL 6	YES
Scenedesmus obliquus UTEX 78	YES
Scenedesmus obliquus UTEX 1450	YES
Scenedesmus obliquus UTEX 2630	YES
Scenedesmus obliquus SAG 276/1	NO
Scenedesmus obtusiusculus Univ. Turku	??
Scenedesmus protuberans Univ. A'dam	??
Scenedesmus quadricauda NIVA-CHL 7	NO
Scenedesmus quadricauda F11	NO
Scenedesmus quadricauda UTEX 76	NO
Scenedesmus subspicatus NIVA-CHL55	NO - YES♠
Scenedesmus subspicatus RWTH	NO
Scenedesmus subspicatus UTEX 2532	NO
Scenedesmus subspicatus UTEX 2594	NO – YES♣

♠ Hessen & Van Donk (1993), ♣ 14 day-biotest (*see* § *8.2.8*)

Since several strains seem not to gain an advantage by colony formation, this trait may reflect a response to grazing in general rather than to *Daphnia* in particular. In numerous waters, *Scenedesmus* is not exposed to large *Daphnia*, but is confronted with several much smaller grazers such as rotifers, ciliates, and phagotrophic flagellates. Unicells will probably not survive

an encounter with one of these grazers, and may use dissolved chemicals to detect these grazers prior to encounter.

Figure 8.23: A vorticellid ciliate has settled on a large aggregate of *Scenedesmus acutus* in the presence of live *Daphnia*.

Stanley (1973) suggested that multicellularity might have evolved as a defense to phagocytosis. Recent evidence has revealed that protozoan grazers caused a unicellular *Chlorella* culture to change into one with numerous colonies (resulting in a culture dominated by eight-celled colonies!), which were protected from protozoan grazing (Boraas *et al.*, 1998). These authors suggest mutations as the driving force, but this mechanism seems very unlikely for the responsive strains in this study. Moreover, it seems unlikely that for example SAG 276/1 and UTCC 10 have lost their ability to respond to grazers by mutation, especially since eight-celled coenobia were observed. Nevertheless, the dogma of random genetic mutation is firmly in place, but seems an assumption rather than scientific fact (Sheldrake, 1991). Certain bacteria have reported to employ directed mutations in the stress conditions where such a mutation was necessary to survive (Cairns *et al.*, 1988; Hall, 1988). Boraas *et al.* (1998) provide clear evidence that a multicellular form, that was a rare mutant in unicellular culture, was selected over unicells by phagotrophic predation. This *"rare mutant"* could imply the presence of two genotypes in their culture and thus they may have observed a phenomenon of clonal replacement. The colonial form breeded true and remained colonial for years even at low flagellate densities, but *"active photosynthesis and continued interaction with the predator are essential to maintain the colonial algae"* (Boraas *et al.*, 1998).

CHAPTER 9

The smell of algae and competitors

Parts of this chapter are based on:
Lürling, M. & Van Donk, E. *submitted to Aquatic Ecology*
Roozen, F., Lürling, M. & Plath, K. *submitted to Journal of Plankton Research*

"Humans have a complete set of organs which
are traditionally described as non-fucntional,
but which, if seen in any other mammal,
would be recognized as part of a pheromone system"

- A. Comfort 1971

9.1 INTRODUCTION

Chemical substances play an important role in plankton interactions. Theoretically, infochemicals may originate from every chemical process and may be involved in every interaction, simply because all organisms produce "odors" and thus potentially information (Dicke, 1988). The energy transfer from algae to herbivores may be influenced by chemicals acting between algal species, between algae and zooplankters, between zooplankton themselves and between zooplankton and their predators. In the previous chapters, research effort has been focused on the infochemicals flowing from zooplankton to algae and more specifically to *Scenedesmus*. The unidentified infochemical has to be defined as a kairomone, since the response in the receiving organism is beneficial to the receiver (*Scenedesmus*) but not to the emitter (a zooplankter). The induced anti-grazer response in *Scenedesmus* affects the energy-flow from primary producers to consumers. However, from all reports on induced defenses and chemical information transfer in aquatic systems (*see* Tollrian & Harvell, 1999; this thesis CHAPTER 1), it may be hypothesized that numerous infochemicals may affect this energy flow. Growth inhibiting substances among algae, such as an allelochemical from the cyanobacterium *Fischerella* that inhibits growth in several *Scenedesmus* strains (Gross *et al.*, 1991), the capability of *Daphnia* to respond to algal odors (Van Gool & Ringelberg, 1996) and the negative effects of grazers on each other (Folt & Goldman, 1981; Goser & Ratte, 1994; Goser, 1997) may affect the energy flow from algae to consumers. In this chapter, no attention will be paid to natural algicides and interactions among phytoplankters, but some exploration into the possible role of chemical information transfer 1) from algae to zooplankters and 2) among zooplankters will be undertaken (Fig. 9.1).

Chemicals that convey information from algae to zooplankton may be of major importance as they may influence the energy transfer between algae and their consumers (Larsson & Dodson, 1993). Infochemicals from algae should inform *Daphnia* about the location, quantity and quality of the algal food. In fact, Van Gool & Ringelberg (1996) demonstrated that *Daphnia* is attracted by odors associated with edible algal food. *Daphnia* has been shown to distribute themselves horizontally to a larger extent in a chamber with high algal concentrations (Jakobsen & Johnson, 1987) or with algal extracts (Laurén-Määttä *et al.*, 1997).

In food gradient experiments *Daphnia* showed the strongest aggregation response at intermediate food levels, and avoided high food levels (Neary *et al.*, 1994). *Daphnia* may perceive signals from toxic cyanobacteria as information associated with danger that may result in immediately reduced food intake (Haney *et al.*, 1995) and altered swimming behavior (Haney, 1993). The potential ability of *Daphnia* to locate high quality algal patches could have strong effects on population dynamics and may be one of the factors involved in swarm formation. In that respect, it would be interesting to examine whether *Daphnia* is capable of distinguishing algae belonging to the same species but differing in palatability and quality.

However, an alternative hypothesis is that *Daphnia* may not use infochemicals from algae, but the concentration of algal cells as mechanism to locate these regions (Cuddington & McCauley, 1994; Neary *et al.*, 1994). Also in the study of Porter *et al.* (1982), *Daphnia* appeared unable to locate and detect algal patches and diaptomid copepods have been reported not to respond to algal odors too (DeMott & Watson, 1991). Therefore, in the first part of this chapter (§9.2 and §9.3) an Y-tube olfactometer (Takabayashi & Dicke, 1992) was used to examine the effects of algal odors, algal cells and algal color on swimming behavior of individual *Daphnia*.

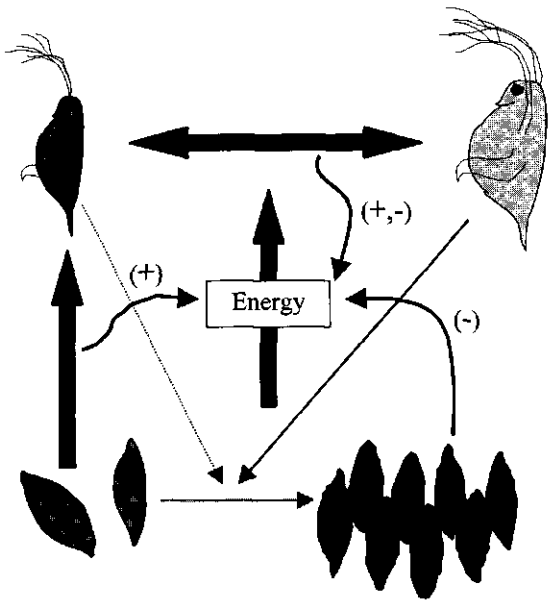


Figure 9.1: Potential infochemical flows (➡) examined in this chapter and the effect they could have (+,-) on the energy flow from the pelagic primary producers (green) to herbivorous zooplankton ( ).

Daphnia may not only respond to 'odor' from its food, but also to odors from competitors and predators (e.g. Larsson & Dodson, 1993; Pijanowska, 1994; Kleiven *et al.*, 1996). This response could result in altered horizontal distributions and the formation of swarms (e.g. Davies, 1985; Larsson & Dodson, 1993; Pijanowska, 1994; Kvam & Kleiven, 1995; Kleiven *et al.*, 1996). *Daphnia* may often form dense swarms (e.g. Colebrook, 1960; Ratzlaff, 1974; Malone & McQueen, 1983) at the cost of an increase in exploitative competition (Folt, 1987; Jakobson & Johnson, 1988b; Tessier, 1983; DeMott, 1989 *for review*). Swarms or aggregates may be the result of abiotic factors, such as wind (George & Edwards, 1976; Malone & McQueen, 1983) or water currents like Langmuir circulations (George & Edwards, 1973), but swarms may also be easily broken by wind-induced water currents (Irvine, 1989). The observation of Irvine that *Daphnia* aggregated more on calm days

suggests a behavioral activity (Irvine, 1989). Thus, swarms could also be the result of biological interactions between organisms belonging to similar or different species in similar or different trophic levels. However, swarming strongly depends on food availability (Jakobson & Johnson, 1987; Cuddington & McCauley, 1994; Neary *et al.*, 1994; Kleiven *et al.*, 1996). Inside a swarm, animals compete for resources and infochemicals released from crowding competitors may strongly affect the receiving animals thereby influencing competition (e.g. Folt & Goldman, 1981).

Zooplankton infochemicals may influence zooplankters directly by inducing various behavioral and physiological responses or indirectly by altering the algal food.
For example, Stirling (1995) reported that *D. galeata mendotae* performed vertical migration only in response to fish that had been fed with conspecifics, while *D. magna* responded on freshly crushed conspecifics (Pijanowska, 1997). Several studies have reported feeding or reproduction changes in zooplankton by chemicals released from potential competitors. Halbach (1969) reported that survival and fecundity of the competing rotifers *Brachionus calyciflorus* and *B. rubens* were reduced when animals were reared in preconditioned water. The filtering rate of the copepod *Diaptomus tyrrelli* was reduced by a high-molecular weight chemical released from its competitor and predator *Epischura nevadensis* (Folt & Goldman, 1981). Feeding in *Daphnia* is reduced by infochemicals released from congeners (Matveev, 1993) and conspecifics (Helgen, 1987). Chemicals released from crowded *D. cucullata* (Seitz, 1984) and *Daphnia magna* (Hobæk & Larsson, 1990; Burns, 1995) improved the reproduction in conspecifics. However, reductions in reproduction by intraspecific substances were reported for *Simocephalus vetulus* (Perrin, 1989), *Daphnia magna* (Guisande, 1993; Goser & Ratte, 1994; Cleuvers *et al.*, 1997), *Daphnia carinata* (Matveev, 1993) and *Daphnia hyalina* (Burns, 1995). Moreover, intraspecific chemicals resulted in less but larger and heavier neonates in *D. magna* that contained more lipids and survived longer starvation periods (Cleuvers *et al.*, 1997). Crowded congeners had either no effect or reduced the fecundity in *Daphnia cucullata, D. hyalina* and *D. magna* (Seitz, 1984; Hobæk & Larsson, 1990).

Thus, chemicals released under crowding conditions may influence life-history parameters of *Daphnia*. In the second part of this chapter the effects of chemicals released from crowded *D. cucullata* and *D. pulex* on life-history parameters of congeners and conspecifics are examined. The crowding conditions used in the experiments were based on previous field observations of the shallow Lake Zwemlust (The Netherlands), where *Daphnia* densities can go up to 300-600 animals·l^{-1} (own observations; Van Donk *et al.*, 1990a & b) and literature data. Davies (1985) reported swarms with more than 1000 *Daphnia* per liter, while Kvam & Kleiven (1995) measured up to 4000 animals per liter during the day and still 1/10 during the night. In the laboratory, a situation was mimicked when *Daphnia* migration (horizontal as well as vertical swarm disintegration) was impossible and consequently when animals were continuously exposed to infochemicals released from competitors. In order to

investigate whether the effects of crowded conditions can be explained by the mere presence of simple excretion products such as urea or ammonia the effect of these substances on life-history characteristics of *Daphnia pulex* was tested.

9.2 Y-TUBE OLFACTOMETER

The Y-tube olfactometer is an Y-shaped glass tube, with an internal diameter of 3.5 cm, one side with two arms 12 cm in length (at an angle of 75°) and one basal leg with a length of 13 cm (Fig. 9.2). The two arms served as inflows for the two test-media that were pumped into the tube by two peristaltic pumps (Gilson Minipuls 3), each with a rate of 7.2 $ml \cdot min^{-1}$. Blue colored water added to one of the inflows resulted in a laminar pattern, what indicated that the two media did not mix during the experiments, but remained separated in the outflow-leg. The experimental set-up was based on the one given by Van Gool & Ringelberg (1996).

The Y-tube was placed horizontally in a white bath containing the artificial freshwater COMBO-medium (Kilham *et al.*, 1995). The position was marked so that the tube could be placed in the same position every experiment. About 60 cm above the bath a lamp caused an illumination of 45 $\mu mol.m^{-2}.s^{-1}$. At the start of each experiment, the Y-tube olfactometer was connected with the two inflows. One *Daphnia* was placed in the leg, 4 cm from the end, and the leg was connected with the outflow.

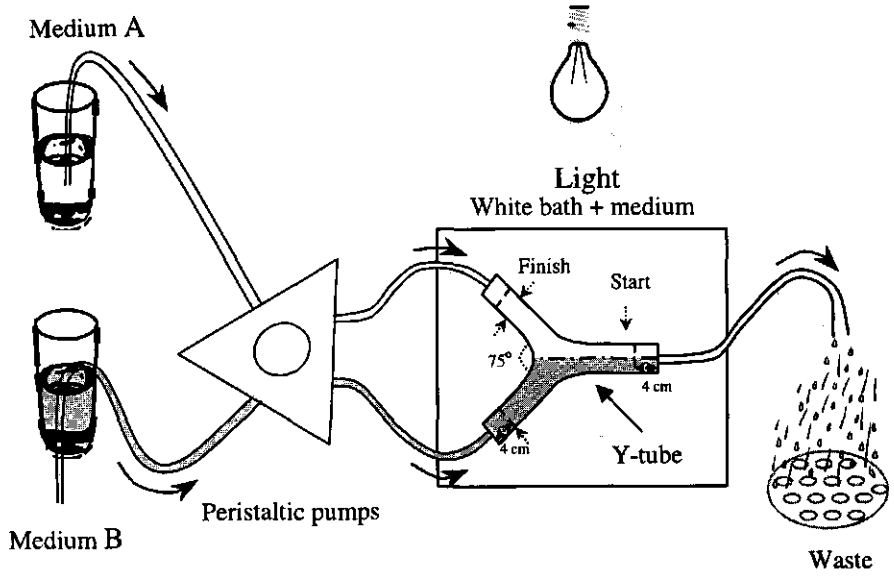


Figure 9.2: Experimental set-up and the Y-tube olfactometer.

A cover was put over the bath and only let the light pass through a small window above the place where the *Daphnia* was inserted in the Y-tube. The window served as an optical trap to ensure that each *Daphnia* started at the same position, and covered the full width of the tube, so that the *Daphnia* was able to contact both media during the incubation time of five minutes. The incubation of five minutes is too small to get one half of the Y-tube completely filled with the inflowing medium. Theoretically this should take about 25 minutes at the rate of 7.2 ml·min^{-1}. However, the experiments with coloured water showed, that after five minutes the colour already reached the end of the tube. This indicates that test-water should also reach the end of the tube after five minutes, and that the *Daphnia* should be able to come in contact with it.

The *Daphnia* was released by lifting the cover and was expected to swim against the current of the inflows. Daphnids that reached a distance of 9 cm into one of the inflow-arms were scored and marked, those that did not score after another five minutes were also marked. Each *Daphnia* was only used once and in one experiment about 40 animals were used.

After each run, the Y-tube was cleaned with hot water and a brush. After five runs the two inflows were changed to prevent possible position effects. The light field was checked after every ten runs with a LICOR LI-185B photometer, and the inflow rates were checked with a 10 ml pipette.

The binomial test was used to test whether the *Daphnia* had made a significant choice. The null hypothesis belonging to the experiments with the Y-tube olfactometer is given by: $P(odour) = P(clean) = 0.5$, which means that there is no preference for one of the two media.

In some experiments (Exp. II and III *see below*) the swimming-time of *Daphnia* was measured. This measurement started when the test organism crossed a line, marked 2 cm from the starting position (away from the end of the outflow-leg), and stopped when *Daphnia* had scored, or after five minutes. To test the differences in swimming-time belonging to the choices for both media, the t-test for unequal variances and the Wilcoxon Rank Sum test were used.

9.2.1 Plankton organisms

Daphnia magna was obtained from the Centre for Limnology (Nieuwersluis, the Netherlands) and *Daphnia pulex* G-clone originated from the culture collection of the Max-Planck Institute for Limnology (Plön, Germany). The two *Daphnia* species were cultured separately in artificial RT-medium (Tollrian, 1993), and were fed with the green alga *Scenedesmus acutus*. All animal cultures were maintained in a temperature-controlled room at 20°C in low light with a day-night interval of respectively 16 and 8 hours. The green-alga *Scenedesmus acutus* or its odor, was used in the test-media. These algae were cultured in a 1 liter chemostat with the artificial COMBO-medium (Kilham *et al.*, 1999), with a turnover time of one day. The chemostat was placed in a temperature-controlled room at 20°C with

constant light of about 100 $\mu mol \cdot m^{-2} \cdot s^{-1}$. Nitrogen-limited algae were obtained by placing algae from the chemostat in a batch culture with COMBO-medium that contained NaCl instead of $NaNO_3$. After six days the algae were strongly N-limited and could be used for the experiments.

9.2.2 Experiments

The different experiments needed different preconditioned test-media and test-organisms.

I. In the first series of experiments, the effect of odor from non-limited and N-limited *Scenedesmus* on the swimming behaviour of *Daphnia magna* and *D. pulex* was examined. Algal-odor of non- and N- limited algae was obtained by inoculation of *Scenedesmus* in clean COMBO-medium (N-limited in medium with NaCl instead of $NaNO_3$) at a concentration of $1 \cdot 10^4$ parts$\cdot ml^{-1}$. This density is about 0.25 $mgC \cdot l^{-1}$ and was based on the Incipient Limiting Level (ILL) for *Daphnia magna* grazing on *Scenedesmus acutus* (Muck & Lampert, 1984). After one day of incubation, the medium was filtered through 0.45 µm and the filtrate was collected in dark flasks and used in the experiments against clean 0.45 µm filtered COMBO-medium. The clean medium and the medium with algal odor were compared in pH, conductivity and color. The latter was done by spectrophotometric analysis. One day before the experiments, the daphnids were put in clean COMBO-medium with enough *Scenedesmus acutus* as food.

II. The second series of experiments comprised a first experiment (IIa) in which well fed *Daphnia magna* was offered the choice between clean medium and medium with live *S. acutus* cells. In a second experiment (IIb) well fed *D. magna* was offered the choice between a low and a high food level. The third and fourth experiments were replicates of the first (IIa), but now with starved *D. magna* (IIc) and with *D. magna* cultured in the presence of fish (IId). The algal concentrations used in the experiments IIa, IIc and IId were $1 \cdot 10^4$ parts$\cdot ml^{-1}$. This medium was tested against the non-filtered clean COMBO-medium. In the experiment IIb the algal concentrations were $5 \cdot 10^3$ parts$\cdot ml^{-1}$ in the low food inflow and $2 \cdot 10^4$ parts$\cdot ml^{-1}$ in the high food inflow. Moreover, in these experiments the swimming-time of *Daphnia* was measured. This measurement started when the test organism crossed a line, marked 2 cm from the starting position (away from the end of the outflow-leg), and stopped when *Daphnia* had scored, or after five minutes.

The experimental *Daphnia* were cultured in three different ways to obtain animals that were well fed, starved and had been exposed to fish. Well-fed *Daphnia* were put in clean COMBO-medium with enough *Scenedesmus acutus* as food a day before the experiment. Starved animals were obtained by transferring them two-days before the experiment into clean

COMBO-medium without food. Fish exposed animals were placed in an aquarium with medium, food and two fish (*Leuciscus idus*), but separated by a plankton-net.

III. The third series of experiments was designed to evaluate whether fed (IIIa) and starved (IIIb) *Daphnia* are capable to discriminate between clean medium and medium with a green color and perhaps a 'chlorophyll-odor' The color of the *Scenedesmus acutus* was simulated by the chlorophyll-a of the alga *Anacystis ridulans* (Sigma). The amount of chlorophyll-a used in the test-medium was comparable to the amount of this pigment in a concentration of $1 \cdot 10^4$ parts(*S. acutus*)$\cdot ml^{-1}$. Again swimming-speeds were determined.

9.3 RESPONSE OF *DAPHNIA* TO ALGAL ODOURS, CELLS AND COLOUR

The experiments with clean odourless medium in both arms of the Y-tube showed that the null hypothesis could not be rejected. *Daphnia magna* and *Daphnia pulex* showed comparable numbers of individuals scoring for either arm with 50-50% and 45-55%, respectively (N=20). This indicates that no environmentally induced position effect was present during the experiments.

I. In the first series of experiments the null hypothesis could not be rejected. *Daphnia pulex* as well as *Daphnia magna* had no preference for the medium with infochemicals of (N-limited) *Scenedesmus acutus* or for the clean medium. When *Daphnia* could chose between two different qualities of *S. acutus*, i.e. between medium that had contained non-limited and nitrogen-limited cells, the null hypothesis could not be rejected as well (Fig. 9.3). Although no significant effect of the differently odored media was observed, the response to similar treatments was significantly larger ($P = 0.023$) for *D. magna* than for *D. pulex* according to the Wilcoxon Rank Sum test.

II. *Daphnia magna* had no preference for clean medium or medium containing algae (IIa) (Fig. 9.4). The swimming speeds for animals with respect to the choice made were not significantly different (Fig 9.5; $P = 0.68$). Also when two different food levels were offered to *Daphnia* (IIb) the null hypothesis could not be rejected, although 63% (n = 38) of the *D. magna* scored the higher concentration (Fig. 9.4). However, the swimming speed was significantly (Fig. 9.5; $P = 0.03$) lower for animals in the high food concentration compared with the low algal concentration. *Daphnia* that had been in an aquarium with fish (IId) had no preference for either clean medium or medium with algae (Fig. 9.4). Thus again the null hypothesis could not be rejected.

The swimming speeds were not significantly different (Fig. 9.5; $P = 0.25$). Starved *Daphnia*, however, (IIc) showed a different response. Of a total of 30 animals 25 (i.e. 83%) did not score, but wanted to swim with the water current. From the five individuals that did score, four (80%) chose the medium with *S. acutus* cells.

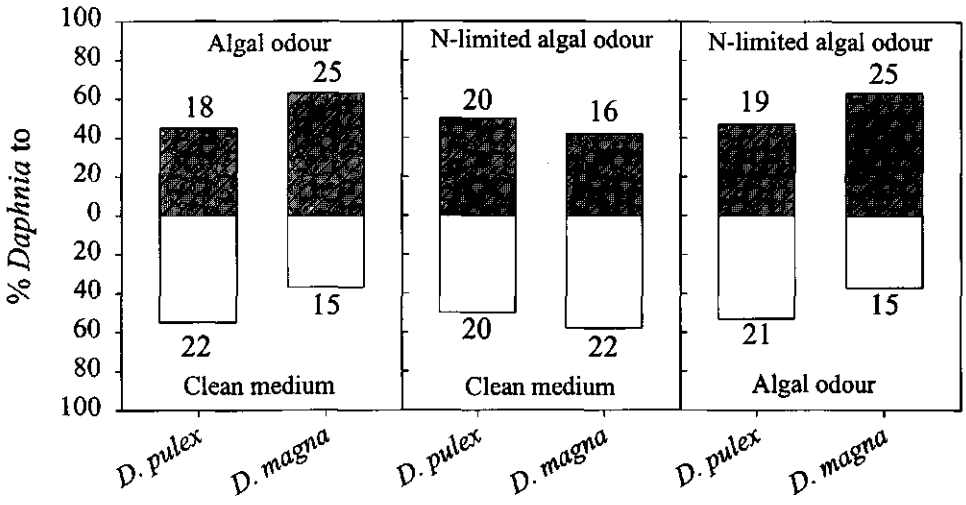


Figure 9.3: Response in Y-tube olfactometer of *Daphnia pulex* and *D. magna* toward clean medium or odored medium from non-limited and N-limited green alga *Scenedesmus acutus*. The numbers indicate the number of animals that made a specific choice.

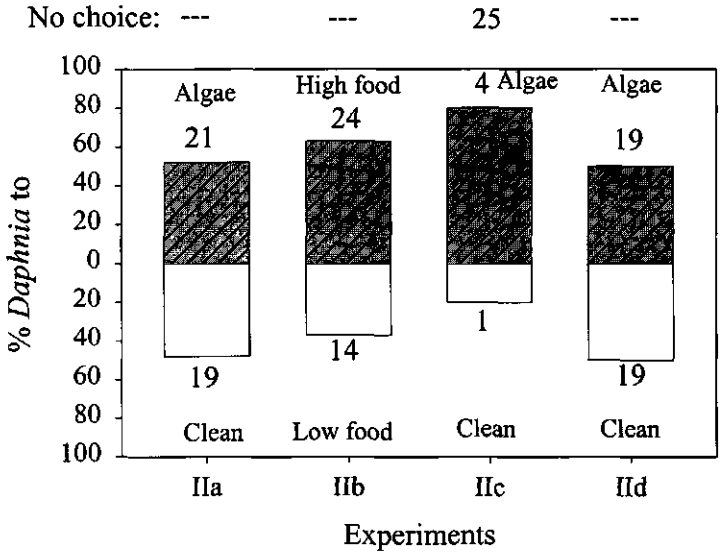


Figure 9.4: Response of *D. magna* in Y-tube olfactometer toward clean medium or medium with algal cells (IIa, IIc & IId) or toward medium with a low ($5 \cdot 10^3$ particles·ml^{-1}) or high algal concentration ($2 \cdot 10^4$ particles·ml^{-1}). *D. magna* was well fed (IIa & IIb)-, starved (IIc)-, or cultured in the presence of fish-odor (IId).

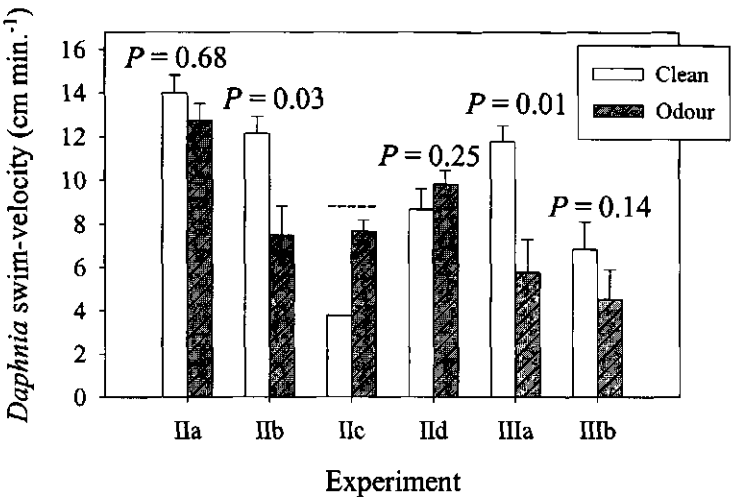


Figure 9.5: Swim velocities (cm min^{-1}) of *D. magna* related to the specific choice for a medium in the Y-tube olfactometer, including *P*- values of *t*-tests (*see* text).

III. Both experiments (IIIa and b) showed that the null hypothesis could not be rejected (Table 9.1). In experiment IIIa, *Daphnia* that scored the medium with chlorophyll-*a* swam significantly slower than those who preferred clean medium (Fig. 9.5; $P = 0.01$). In experiment IIIb starved *Daphnia* showed the same response as the starved ones in exp. IIb. A majority did not score, but wanted to swim along the current. The ones that did score were evenly distributed between the clean medium and the medium containing chlorophyll-a. However, no significant differences in swimming-speeds were detected (Fig. 9.5; $P = 0.14$).

Table 9.1: Numbers and precentages of *D. magna* that choose in Y-tube olfactometer clean or chlorophyll-*a* containing media, including χ^2-values.

Experiment	Clean *vs.* chl-*a*	%	χ^2	No choice
IIIa well fed *D. magna*	23 – 17	58 – 42	0.47	---
IIIb Starved *D. magna*	5 – 4	55 – 45	0.06	11

The data obtained in the previous experiments do not support the results obtained by Van Gool & Ringelberg (1996). However, several factors differed between the experiments. To unravel this discrepancy an additional experiment was performed with *Daphnia galeata* × *hyalina* (clone O2) obtained from the culture collection of the University of Amsterdam (kindly provided by Dr. T. Reede). This animal was cultured in aged and filtered lake Maarsseveen (MV) water and fed with *S. acutus*. The *D. galeata* × *hyalina* was offered the choice between filtered MV water and MV water with algal odor. *S. acutus* was harvested

from the chemostat, centrifuged at 2000 rpm and resuspended with MV water. This procedure was repeated twice. The resuspended algae were then transferred into 5 liter 0.45 µm filtered MV water at a final concentration of $2 \cdot 10^4$ particles·ml^{-1}. The 5 liter Erlenmeyer flask was incubated for 24 h at 20°C in continuous light of 100 µmol·m^{-2}·s^{-1} followed by a filtration to separate the algae from the MV water. This filtered "algal-odored" MV water was tested against clean filtered MV water. Prior to the experiment an experiment was conducted with only clean filtered MV water. This test revealed that no environmentally induced position effect was present (Fig. 9.6). The experiment showed that again the null hypothesis could not be rejected (Fig. 9.6).

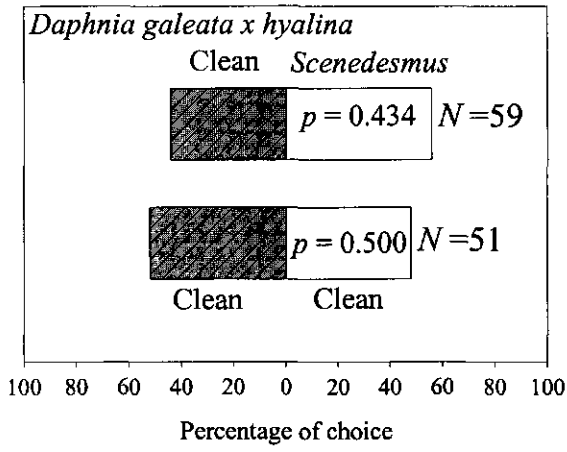


Figure 9.6: Response of *D. galeata* × *hyalina* in Y-tube olfactometer in clean MV water or when MV water was odoured with the green alga *S. acutus*, including *P*-values.

9.4 *DAPHNIA – DAPHNIA*

The behavioural response of *Daphnia magna* and *D. pulex* to intra- and inter-specific infochemicals was investigated in a Y-tube olfactometer. The *Daphnia* were investigated on their ability to distinguish between clean RT-medium and RT-medium that had contained conspecifics or congeners. The latter was produced by adding 600 well-fed *Daphnia* to 3 liter clean RT-medium without any food. After 24 h the animals were separated from the medium that was filtered through a 0.45 µm filter and used in the bioassay.

Both *Daphnia* species showed no preference for either the clean medium or medium that had contained conspecifics, but prefered the clean medium above medium previously inhabitated by congeners (Table 9.2).

Table 9.2: Numbers and precentages of *D. magna* and *D. pulex* that choose in Y-tube olfactometer clean or odored medium, including χ^2-values (*: $P < 0.05$).

Test species	Odor	Clean *vs.* odor	%	χ^2
D. magna	*D. magna*	18 - 22	45 - 55	0.20
"	*D. pulex*	29 - 11	73 - 27	4.05*
D. pulex	*D. pulex*	14 - 20	41 -59	0.53
"	*D. magna*	25 - 15	63 -37	1.25

These experiments clearly revealed that *Daphnia* is able to alter its distribution in response to chemicals from other daphnids. However, they only responded to medium that had contained congeners, the interspecific signaling causing them to choose the clean medium. Interestingly, both species showed a preference for the clean medium when medium from congeners was the alternative, but not in the case of an alternative with medium from conspecifics. The swim-away response is often related to low food conditions (Kleiven *et al.*, 1996), but in these experiments the animals were well fed and the exposure time in a run of maximally 10 minutes seems to short to starve the animals. The experimental animals did not encounter any food in the Y-tube runs that could influence their behaviour and their response. Nevertheless, *Daphnia* seems capable of responding to chemicals released from congeners.

9.5 CROWDING CHEMICALS: COSTS OF LIVING TOGETHER

9.4.1 *Plankton organisms*

Daphnia pulex De Geer (adult female *ca.* 3-4 mm) was isolated from Lake Zwemlust (The Netherlands) and has been cultured for several years in the laboratory. A clone (Tj33) of the smaller species *Daphnia cucullata* Sars (adult female *ca.* 0.8-1.1 mm) was obtained from the culture collection of the Centre for Limnology (Nieuwersluis, The Netherlands). Animals were cultured at 20°C in 1 litre jars containing a suspension of *Scenedesmus acutus* in COMBO medium (Kilham *et al.*, 1995.). The green alga *Scenedesmus acutus* Meyen was cultured in a 1.0 litre chemostat on COMBO medium at an irradiance of 125 µmol quanta m^{-2} s^{-1} in a temperature controlled chamber at 20°C with a dilution rate of 1.2 d^{-1}. In the chemostat *S. acutus* was mainly unicellular with dimensions of 14 × 4 µm.

9.4.2 *Life history experiments*

Life history experiments were conducted for *D. cucullata* and *D. pulex*. Newborns from cohorts reared in 1 liter jars on *S. acutus* in COMBO were collected within 24 h of birth and joined in a 500 ml beaker containing only COMBO. For each series 15 neonates were selected from this beaker and transferred individually into 125 ml test tubes containing 90 ml of *S. acutus* in standard COMBO or in "crowded" COMBO. The latter was filtered (0.45 µm) medium from culture vessels crowded with either *D. cucullata* or *D. pulex*, further referred to

as "Cucullata" or "Pulex" water, respectively. Three 1 liter jars per *Daphnia* species were used and crowding conditions at the end of the experiment were 4.89 ± 0.67 mg $DW \cdot l^{-1}$ for *D. cucullata* and 10.50 ± 1.63 mg $DW \cdot l^{-1}$ for *D. pulex*. Every day the animals were transferred into clean vessels containing 1 liter of fresh food suspension, while the "crowded" vessel water was filtered (0.45 µm) and used as test water in the life table experiments. The 125 ml test tubes, each with one experimental animal, were incubated in a temperature controlled room at 18°C in low light intensity of *ca.* 4 $\mu mol \cdot m^{-2} \cdot s^{-1}$ using a 16:8 h light-dark cycle. Both *Daphnia* species were fed equal amounts of algae, i.e. equivalent biovolumes of 1.01 ± 0.01 × 10^7 $\mu m^3 \cdot ml^{-1}$ (i.e. ~80 000 cells·ml^{-1} yielding a carbon concentration of ~3.9 mg $C \cdot l^{-1}$). The animals were transferred daily into clean tubes containing fresh medium, were inspected for molting and length was measured. The experiment was run as described in section *7.4.3.*

The standard-medium and both crowded media were analyzed for their total (in)organic-C, inorganic-N and inorganic-P content. Total (in)organic carbon was determined using a TOC-analyzer (model 700, OI-Analytical). NH_4^+-N, NO_2^-/NO_3^--N and PO_4^{3-}-P were determined using a SKALAR autoanalyzer. Moreover, pH and conductivity of the different media were measured routinely.

A second experiment was conducted with *D. pulex* in order to examine the effect of elevated ammonia/urea levels in crowded water on life history parameters. The experiment was performed analogous to the former experiment, only with one species (*D. pulex*) and 10 experimental animals per treatment. The highest ammonia concentration measured was ~1 $mg \cdot l^{-1}$ ("Pulex"-water). (In Lake Zwemlust the highest ammonia concentration measured in 1996 was 0.92 mg NH_4^+-$N \cdot l^{-1}$, in crowded medium the highest level was 0.83 mg NH_4^+-$N \cdot l^{-1}$). Therefore in two series daily either 1.0 $mg \cdot l^{-1}$ ammonia or urea was added to the (uncrowded) medium. Additionally, length of neonates of the third clutch was recorded and their dry-weight determined using a microbalance (Mettler ME 130).

9.4.3 *Results*

The pH (8.14 ± 0.54) and conductivity (330 ± 21 $\mu S \cdot cm^{-1}$) of the control medium and crowded water types were similar throughout the entire experiment. Crowded water had affected the life history parameters of *Daphnia*. In *D. cucullata* animals seemed to mature at older age in crowded water (Table 9.3), but differences were not significant. Also length at maturity was not significantly influenced. However, in *D. pulex* age at maturity was significantly different between animals reared in the three water types. One-way ANOVA indicated significant differences in length at maturity of *D. pulex* grown in different water types. Tukey's test revealed that animals reared in control medium were significantly smaller at maturity than animals in "Cucullata" water (Table 9.3). This is also reflected in the body length at successive instars (Fig. 9.7A), where *D. pulex* instars 4 and 5 in „Cucullata" water are significantly larger than animals in the other water types. In *D. cucullata* the adult instars

reared in crowded water were smaller compared to animals grown in control medium (Fig. 9.7B). The repeated measure ANOVAs with instar as repeated measurements revealed significant water type effects for body-length of *D. pulex* (F = 4.45; P = 0.032) and *D. cucullata* (F = 11.7; P = 0.001).

Reproductive rates were highest for animals reared in control medium (Fig. 9.8). In both *Daphnia* species a reduction in number of new-borns was observed when animals were cultured in crowded water. In *D. pulex* the reduction in offspring was most pronounced in animals grown in "Cucullata" water (Fig. 9.8A), while *D. cucullata* clutch sizes were most reduced in animals reared in "Pulex" water (Fig. 9.8B).

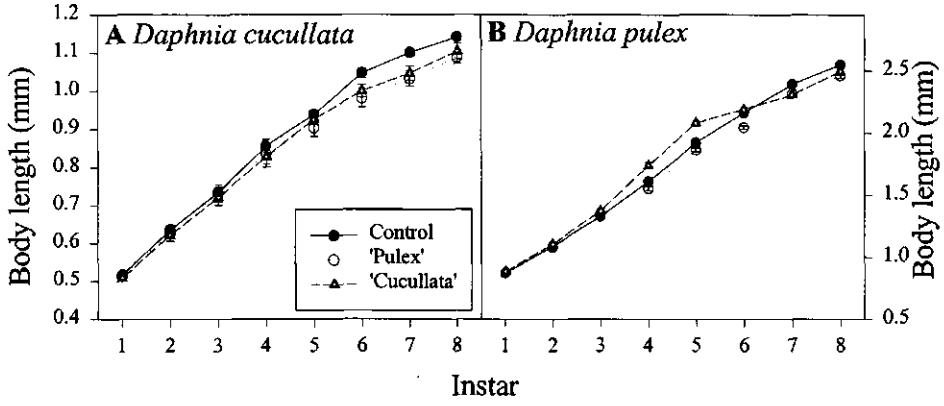


Figure 9.7: Body-length (mm) of *Daphnia cucullata* (Panel A) and *Daphnia pulex* (Panel B) of successive instars in standard medium (Control) and in medium crowded with either *D. pulex* ('Pulex') or *D. cucullata* ('Cucullata'). Error bars represent 1 SD.

Mainly in the second brood, *D. pulex* produced parthenogenetic ephippia. 23% of the animals developed ephippia in "Pulex" water, 64% in "Cucullata" water, while no ephippia occurred in the control group in control medium. Ephippial broods were in all cases followed by an empty brood pouch in the successive adult instar. Both were omitted from reproduction analysis, rather than scored as zero or as two. In *D. cucullata* no ephippia were produced in the three clutches, but egg degeneration occurred (only in "Pulex" water) in the first, second and third clutch, in 22%, 33% and 83% of the broods, respectively. No eggs were developed completely in these broods. The repeated measure ANOVA for number of newborns revealed no significant instar (F = 6.77; P = 0.052), water-type (F = 2.18; P = 0.229) and interaction effects (F = 2.03; P = 0.183) for *D. pulex*. For *D. cucullata* only a significant instar effect (F = 22.3; P = 0.043) was detected, but no water-type (F = 1.56; P = 0.390) and interaction effects (F = 5.67, P = 0.061).

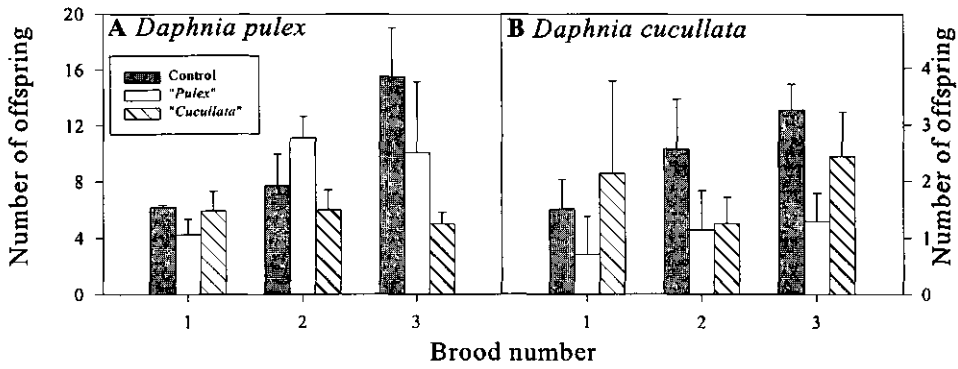


Figure 9.8: Clutch sizes of the first three broods of *Daphnia pulex* (Panel A) and *Daphnia cucullata* (Panel B) grown in standard medium (Control) or in medium crowded with either *D. pulex* ('Pulex') or *D. cucullata* ('Cucullata'). Error bars indicate 1 SD.

The body-length, however, of the new-born *D. pulex* was significantly different (F = 145.2; $P < 0.001$) in the three medium-types (Fig. 9.9A), while no differences ($F = 1.24$; P = 0.380) were observed in *D. cucullata* (Fig. 9.9B). *D. pulex* neonates born in crowded water were significantly larger than animals born in control medium.

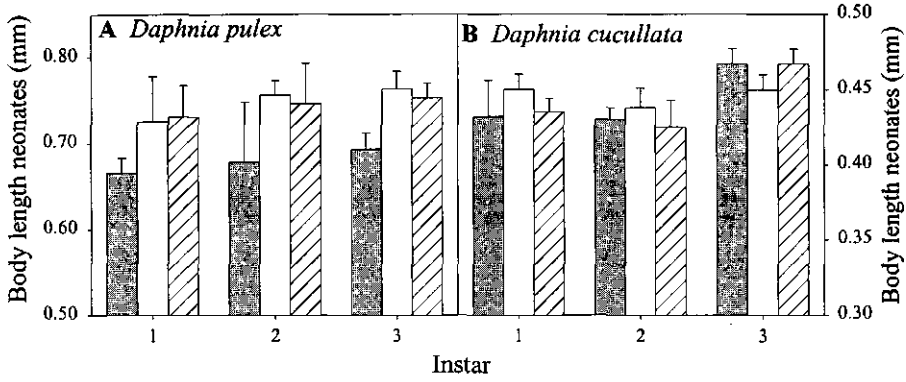


Figure 9.9: Body-length of new-born *Daphnia pulex* (Panel A) and *D. cucullata* (Panel B) released from the first three broods from mothers grown in either standard medium (Control) or in medium crowded with either *D. pulex* ('Pulex') or *D. cucullata* ('Cucullata'). Error bars represent 1 SD.

Survival was defined as percentage of animals that had survived until the end of the experiment. Survival differed between the experiments and was always highest in the control group (Table 9.4). The observed mortality was not caused by senescence since the experiments were stopped when the animals had reached the fourth adult instar. The higher mortality in *D. cucullata* probably reflects a higher sensitivity to handling. The observed

differences in life history parameters resulted in different population growth rates (r) for both species (Table 9.4). Two way ANOVA indicated a significant differences between the two *Daphnia* species ($F = 265.2$; $P = 0.004$) and a significant difference in r among treatments ($F = 21.2$; $P = 0.045$). In both species r was significantly lower for *Daphnia* grown in "Cucullata" water compared to control water, while r of animals cultured in "Pulex" water was significantly lower compared to "Cucullata" water.

Table 9.4: Rates of population increase (r, d^{-1}; means ± 1 SE), age at maturity (AM, d; means ± 1 SD), size at maturity (SM, mm; means ± 1 SD) and survival (S, %) until the end of the experiment of *Daphnia pulex* and *Daphnia cucullata* grown on *Scenedesmus acutus* in non crowded (Control) and crowded ("Pulex" or "Cucullata") medium, including F-and P-values of one-way-ANOVAs. Different symbols per vertical column (^{a,b,c}) indicate statistically significant differences at a 95% level (Tukey's-test).

Daphnia cucullata	r (d^{-1})	SM (mm)	AM (d)	S (%)
Control	0.122 (0.003)^{a}	0.90 (0.05)	7.3 (2.6)	60
"Pulex"	0.005 (0.007)^{b}	0.93 (0.04)	9.1 (2.0)	38
"Cucullata"	0.068 (0.003)^{c}	0.92 (0.07)	8.4 (0.8)	43
		$F_{2,26} = 1.83$	$F_{2,26} = 2.39$	
		$P = 0.180$	$P = 0.111$	
Daphnia pulex	r (d^{-1})	SM (mm)	AM (d)	S (%)
Control	0.301 (0.001)^{a}	1.92 (0.08)^{a}	7.8 (0.4)^{a}	100
"Pulex"	0.225 (0.002)^{b}	2.03 (0.05)^{b}	9.8 (0.7)^{b}	86
"Cucullata"	0.260 (0.001)^{c}	2.08 (0.06)^{b}	7.2 (0.6)^{c}	93
		$F_{2,39} = 21.6$	$F_{2,39} = 74.3$	
		$P < 0.001$	$P < 0.001$	

The NO_2^-/NO_3^- concentrations in the standard WC medium and in both crowded media were similar (Table 9.5). The PO_4^{3-} concentration was lower in both crowded media, while the NH_4^+ concentrations were considerably higher in both crowded media compared to standard medium. Both total inorganic (TIC) and organic carbon (TOC) were similar in standard medium and medium crowded with *D. cucullata*, but somewhat higher in the 'Pulex' medium (Table 9.5).

Table 9.5: Total inorganic carbon (TIC), organic carbon (TOC), NH_4^+-N, NO_2^-/NO_3^--N and PO_4^{3-}-P concentrations ($mg \cdot l^{-1}$) in standard, non-crowded WC medium (Control) and in crowded medium ('Cucullata' and 'Pulex').

Medium	TIC	TOC	PO_4^{3-}-P	NO_2^-/NO_3^--N	NH_4^+-N
Control	16.7	2.8	4.2	12.2	0.02
'Cucullata'	16.3	2.9	0.7	10.3	0.45
'Pulex'	20.2	4.2	3.2	11.6	0.83

Also in the second life-history experiment the population growth rate of *Daphnia* grown in crowded "Pulex" water was lowest. Some distinct differences were observed between experimental *D. pulex* reared in crowded water or in ammonia or urea enriched water. In ammonia enriched water, size at maturity was significantly reduced (Table 9.6).

Table 9.6: Rates of population increase (r, d^{-1}; means ± 1 SE), age at maturity (AM, d; means ± 1 SD), size at maturity (SM, mm; means ± 1 SD), survival (S, %) until the end of the experiment and body length (JL, mm; means ± 1 SD) and dry-weight (W, µg ind^{-1}; means ± 1SD) of juveniles from the 3rd broods of *Daphnia pulex* grown on *Scenedesmus acutus* in non crowded (Control), crowded ("Pulex") medium, and standard medium enriched with 1.0 mg l^{-1} ammonia or urea, including *F*-and *P*-values of one-way ANOVAs. Different symbols per vertical column (^{a,b,c}) indicate statistically significant differences at a 95% level (Tukey's-test).

D. pulex	r (d^{-1})	SM (mm)	AM (d)	S (%)	JL (mm)	W ($\mu g \cdot ind^{-1}$)
Control	0.374 (0.014)^a	1.86 (0.09)^a	7.0 (1.0)	100	0.68 (0.02)^a	3.04 (0.35)^a
"Pulex"	0.296 (0.005)^b	1.86 (0.07)^a	7.6 (0.4)	100	0.78 (0.02)^b	3.98 (0.68)^b
"Ammonia"	0.311 (0.009)^{ab}	1.77 (0.09)^b	7.2 (0.8)	90	0.67 (0.03)^a	2.80 (0.39)^a
"Urea"	0.355 (0.013)^{ab}	1.88 (0.07)^a	7.3 (0.9)	100	0.68 (0.02)^a	2.79 (0.34)^a
	$F_{3,4} = 12.1$	$F_{3,35} = 3.49$	$F_{3,35}=1.07$		$F_{3,35}=52.2$	$F_{3,12}=5.58$
	$P = 0.018$	$P = 0.026$	$P = 0.376$		$p < 0.001$	$P = 0.012$

No ephippia were produced (in contrast to 10% in "Pulex" water) and although clutch sizes were reduced similar to crowded water (Fig. 9.10A) at similar body lengths clutches in crowded water were significantly lower (Table 9.7) than clutch sizes in ammonia water or the control group (Fig. 9.10B). Newborns (of third broods) were significantly larger in crowded water compared to animals born in control medium, or ammonia or urea enriched medium (Table 9.6). The dry-weight of neonates born in crowded water was significantly higher than for neonates in the other water types (Table 9.6). Hence, the observed changes in life-history

parameters of *Daphnia* can not solely be attributed to a simple excretion product, but may be the result of some unknown soluble chemical released from feeding *Daphnia*.

Table 9.7: Regressions of clutch size (CS) on body length (BL) of *Daphnia pulex* grown on *Scenedesmus acutus* in non crowded (Control), crowded ("Pulex") water, and water enriched with 1.0 mg·l^{-1} ammonia, including *t*- and *P*-values of *t*-tests for distinguishing significant differences between regression lines.

	Regression	r^2	*P*-values *t*-tests			
Daphnia pulex				*t*	*P*	df
Control	CS = -41.697 + 24.569*BL	0.839	Control vs."Pulex"	3.56	< 0.001	55
"Pulex"	CS = -27.494 + 16.458*BL	0.906	Control vs. Ammonia	0.93	0.178	53
"Ammonia"	CS = -35.929 + 21.811*BL	0.805	"Pulex" vs. Ammonia	2.25	0.014	52

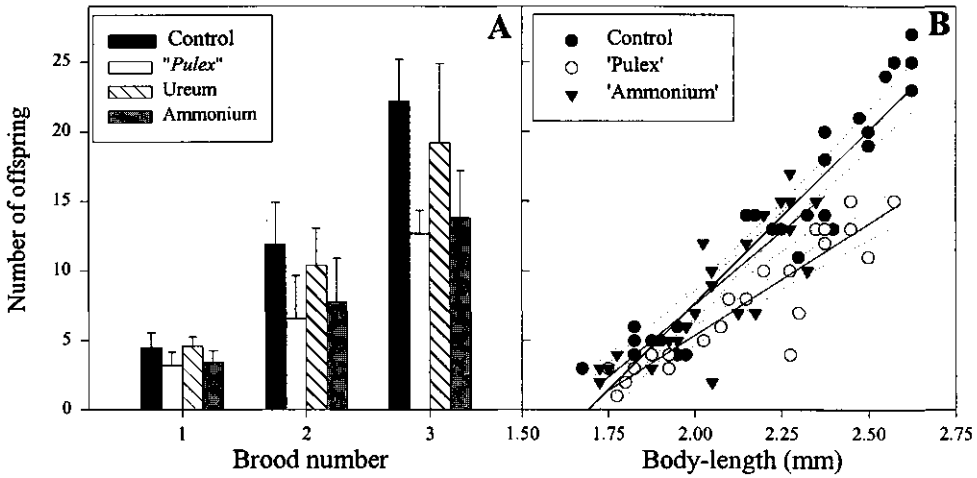


Figure 9.10: Clutch sizes of *Daphnia pulex* grown in standard medium (Control), medium crowded by conspecifics ('Pulex'), and in standard medium enriched with either ammonium or ureum (1 mg·l^{-1}) (Panel A). Clutch sizes in relation to body-length (mm) of the mothers are give in Panel B. Solid lines represent linear regressions, dotted lined the 95% confidence limits. Error bars indicate 1 SD.

9.6 DISCUSSION

Y-tube olfactometer

In their innovative study Van Gool & Ringelberg (1996) showed that *Daphnia* could distinguish between water with odors from different algae when clean odorless water was the alternative. This is in contrast with the results presented here, where *Daphnia* did not alter its

horizontal swimming-behavior to the presence of infochemicals from *Scenedesmus acutus*. The differences may be due to the different *Daphnia* species used in the experiments. In this study among *D. pulex* and *D. magna* a significant difference in response to the presence of chemicals from the green-alga *S. acutus* was detected. However, the experimental animals used in these experiments belonged to clonal cultures, hence, their behavior may not even be representative for the species. Another difference is that in these experiments an artificial medium was used instead of filtered lake-water. The latter still may contain several potential cues, whereas the former, although unlikely, could imply an unknown stress to the animals. To unravel this an additional experiment was performed with *Daphnia galeata* × *hyalina* (clone O2) obtained from the culture collection of the University of Amsterdam. This animal was cultured in aged and filtered lake Maarsseveen (MV) water and fed with *S. acutus*. When *D. galeata* × *hyalina* was offered the choice between filtered MV water and MV water with algal odor again the null hypothesis could not be rejected (*see* Fig. 9.6). Thus even with the same species the results of Van Gool & Ringelberg (1996) could not be repeated. Despite filtration of the MV water and collection in a dark flask, bacterial or photolitical degradation of the algal odors could have occurred, especially regarding the almost 20 hours needed to conduct this experiment with 59 animals. Another possibility may be that the odors are volatile and hence were lost from the vessel during filtration or that they were absorbed to the filter.

The Y-tube olfactometer has been proven very useful in entomological research. However, an important difference between chemical information transfer in air and water is that in the water the diffusivity of a molecule is significantly lower than in the air. According to Strickler (1975) this diffusivity is smaller than the speed of a planktonic animal implying that it would not recognize conspecifics, competitors or predators head on, but rather by swimming in the wake. For algae the question arises if *Daphnia* is capable of smelling the algae from a distance, as most of the algae wander through the water column by the mercy of water movements. Therefore, algae and their exudates may spatially not be separated to a great extent. Moreover, as *Daphnia* respond to algal odor they should also respond to the algal cell itself, but in our experiments they did not. On these aspects conflicting reports appear from literature. *Daphnia* may be attracted by odors associated with edible algal food (Van Gool & Ringelberg, 1996; Laurén-Määttä *et al.*, 1997), by high algal concentrations (Jakobsen & Johnson, 1987), by intermediate food levels (Neary *et al.*, 1994), by the concentration of algal cells rather than the presence of algal odors (Cuddington & McCauley, 1994; Neary *et al.*, 1994), or they may not be attracted at all (Porter *et al.*, 1982). In the latter study, *Daphnia* appeared unable to locate and detect algal patches.

When two different food concentrations were available *D. magna* did not adjust its horizontal position towards one of the concentrations, but the swimming-speed was significantly reduced when swimming in the medium with the higher concentration of algae.

Also both the rate of turning and the vertical component of swimming in *D. magna* have been reported to decrease at increased food levels (Young & Getty, 1987). Cuddington & McCauley (1994) made a similar observation when investigating the swimming-speed of *D. pulex*, suggesting that *Daphnia* is able to alter its behavior to the change in its filtering rate. Also in the presence of chlorophyll-a *D. magna* altered its swimming speed. This indicates that *Daphnia* can detect the green color or associated odor, however, they did not choose for the green colored medium. In these experiments it did seem that *D. magna* was capable of perceiving signals from the environment concerning algal density and green color, but only when they were inside such an environment. They did not adjust their horizontal distribution towards it.

It does seem that the mechanism used by *Daphnia* to locate the algae is linked to their feeding activity. The lower swimming speed in the high algal concentrations suggests that as a result aggregation in high food regions could occur.

Starved animals hardly swam upstream that could indicate a diminished rheotaxis. In their study, Cuddington & McCauley (1994) noted that the ability of *Daphnia* to locate local regions of high food depended on the food concentration. At low food conditions the animals were not able to find these high food regions.

Daphnia responded to medium that contained odors from congeners, but not conspecifics. Since both *Daphnia magna* and *D. pulex* responded, this suggests the existence of species-specific compounds. However, the experiments can not rule out a simple concentration effect. Suppose animal A responds only above a threshold level α, but produces the active compound at a level β with $\beta < \alpha$, while animal B responds below α, but produces the chemical at a level γ with $\gamma > \alpha$, then A will only respond in medium from B and B only in medium from A. Nevertheless, *Daphnia* responded by preference of the clean medium when medium with odors from congeners was the alternative. The experimental animals were well fed, but the runs were performed in medium without any food. Since the dispersal of aggregated animals has been shown to depend strongly on the food availability (Jakobson & Johnson, 1987; Cuddington & McCauley, 1994; Neary *et al.*, 1994; Kleiven *et al.*, 1996), a possible influence of the absence of food can not be ruled out. However, then one would also expect a swim-away response when exposed to medium from conspecifics.

With an Y-tube olfactometer a significant choice between two differently treated media may be found, but when *Daphnia* does not make a choice, this still could imply that the animals perceive the infochemicals, but simply do not respond to them. Only when *Daphnia* shows a clear response, than something can be said about the behaviour of the test-organism. To tackle this problem, it might be important to use a different method than the Y-tube olfactometer, such as a multi-channel circular flow-through chamber (Cuddington & McCauley, 1994), including interactions among organisms.

The responses observed could result in aggregation of animals by reduced swimming speed at high algal densities, by avoidance of areas with competitors. When aggregated *Daphnia* may experience costs due to an increase in exploitative competition (Folt, 1987; Jakobson & Johnson, 1988b; Tessier, 1983; DeMott, 1989 *for review*). Inside the aggregates the animals may thus experience reduced food availability, but will also be exposed to chemicals released from competitors. The results from section 9.5 evidently show that infochemicals released from feeding *Daphnia* influence growth of congeners and conspecifics. The use of an artificial medium (COMBO) is necessary for this type of research to ensure that no infochemicals are already present in the medium and to ensure a constant quality of the medium. In filtered lake-water due to changes in the lake or due to storage differences may occur, obscuring any effects. The pH and conductivity of control medium and crowded medium types were similar hence differences in *Daphnia* response may be attributed to soluble inhibitory substances released from grazing *Daphnia*. As each animal was placed individually in either clean medium or crowded medium, the effects observed must be due to a difference among the media. It is evident that the animals swap from a quantitative to a qualitative reproduction (Cleuvers *et al.*, 1997) even when food is abundant. Since the media differ because of crowding, something in the medium triggers a response in the animals and this can hardly be something else as chemicals dissolved in the crowded medium. Goser (1997) ruled out a possible effect of the algal food and concluded that the *Daphnia* chemicals are "unspecific metabolic substances'. Most probably these chemicals are related to the grazing activity of the animals, and since grazing is essential to *Daphnia*, I thus consider all excretory products essential as well. One could claim that the high densities used to obtain crowded medium are unrealistic, however, they may not be uncommon in shallow lakes, ditches and ponds in the Netherlands. Moreover, Goser & Ratte (1994), Goser (1997) and Cleuvers *et al.* (1997) demonstrated that crowding chemicals might be acting at much lower densities.

Effects on life history parameters of *Daphnia* were remarkable. Mortality was higher in animals reared on crowded medium. Also for the competing rotifers *Brachionus calyciflorus* and *B. rubens* corresponding results have been reported (Halbach, 1969). Similar to findings of Hobæk & Larsson (1990), Matveev (1993), Goser & Ratte (1994) fecundity was considerably reduced by *Daphnia* infochemicals. Interestingly, *D. pulex* produced ephippia in "Cucullata" medium, some in "Pulex" medium and none in control medium, while *D. cucullata* produced no ephippia at all. This latter species has been reported to produce (empty) ephippia in 26% of the broods when exposed to 1:1 crowded medium from *D. pulex* and fish in combination with low food and short daylight (Spaak, 1995). Hobæk & Larsson (1990) found no resting egg formation in *D. magna* when cultured in crowded "Pulex" medium. Ephippia production has been reported to be negatively correlated with food availability and positively with culture density of conspecifics due to an increased encounter rate (Carvalho &

Hughes, 1983). In our experiments the experimental animals did not experience a higher encounter rate. Hence, the observed ephippia production in *D. pulex* may be explained by somekind of infochemically triggering of ephippia production in combination with low light (Kleiven *et al.*, 1992). Parthenogenetic ephippia production as observed in our experiments has reported to occur in several *D. pulex* clones (Hebert *et al.*, 1989). Apparently, just crowded medium is not sufficient for *D. cucullata* to switch to ephippial eggs despite 'stressful' circumstances. *D. cucullata* produced no ephippia, but showed an increase in egg mortality in "Pulex" water. Boersma & Vijverberg (1995) reported 9% - 70% non-developed eggs in *D. cucullata* and explained it by an unknown deficiency of the natural seston. These values are similar to the 22-83% observed in our experiment. Factors such as high pH (Vijverberg *et al.*, 1996), temperature, parasites, food quantity and quality have been suggested to influence egg mortality (refs. in Boersma & Vijverberg, 1995). However, our data indicate that also chemicals released from competitors may cause egg mortality in *Daphnia*, but this phenomenon seems highly species specific and clearly needs further investigation. Apparently, the response in both *Daphnia* species to chemicals released by the other is different (*D. cucullata* produced no ephippia, but had severe egg degeneration), nevertheless the effect on reproduction is similar, namely reproduction is strongly reduced.

The maximum rate of population increase in *D. cucullata* of 0.12 d^{-1} corresponds well with values reported in literature of 0.12 d^{-1} (Ebert & Jacobs, 1991) and 0.11 d^{-1} (Boersma & Vijverberg, 1994). Contrary to the results presented here, Seitz (1984) found that water from mass cultures of *D. cucullata* was good for culturing both *D. cucullata* and *D. hyalina,* while water from *D. hyalina* was good for *D. hyalina,* but not for *D. cucullata.* These deviating results may originate from differences in biomass of animals in his mass cultures and from differences with the mass cultures. Assuming that the excretion of infochemicals is directly related to biomass, equal amounts of 200 *Daphnia*·l^{-1} in the mass cultures as used by Seitz (1984) would mean approximately a 5 fold higher biomass in the *D. hyalina* cultures. For example, *D. pulex* mass cultures contained *ca.* 600-700 animals·l^{-1} and a *ca.* 10 fold higher biomass than Seitz's *D. hyalina* cultures. These observations agree with the proposed reaction norms for *Daphnia* growth and reproduction in response to crowding as reported by Burns (1995).

The maximal value of r for *D. pulex* in the experiments varied between 0.30 d^{-1} (1^{st} life table exp.) and 0.37 d^{-1} (2^{nd} exp.) in the control groups. The deviation between the two experiments may be due to some maternal effects (Brett, 1993). However, this seems not to affect the conclusions since reductions in r in crowded "Pulex" water in both experiments were of similar magnitude. The r-value is somewhat lower than values of 0.40 d^{-1} as reported by Lynch (1989). The lower temperature of 18°C used in our experiments may account partially for this deviation. In the used test tubes *Daphnia* infochemicals may accumulate and may affect growth, or the used experimental tubes may be to small to support maximal

growth. However, measurements on algal concentrations in the test tubes revealed that algal concentrations were 63 ± 12% of initial concentrations (in control group), with maximal clearance rates (mean ± 1 SD) of animals measured in the control group of 1.40 ± 0.42 ml·ind^{-1}·h^{-1}. Although algae could sedimentate to the bottom of the test tubes during the 24 h incubation period, *D. pulex* was frequently observed at the bottom of these tubes. Hence food limitation was not likely to have occurred in the test tubes.

The reduction in population increase *r* was strongest in "Pulex" medium for both species, but relatively strongest in *D. cucullata*. The higher impact of "Pulex" medium may for some part originate from a higher *Daphnia* biomass in the culture vessels. The higher *r*-value for *D. pulex* reared in "Cucullata" water than in "Pulex" medium is caused by the significantly reduced age at maturity and consequently generation time in "Cucullata" medium. This could also be interpreted as a strategy governed by *D. pulex* to establish a better competitive position.

Elevated ammonium levels were measured in the crowded medium types (up to nearly 1 mg·l^{-1}). Nevertheless, neither ammonium nor ureum seemed to be responsible for the differences between the used water-types. Although ammonium had a considerable impact on life-history parameters, the effects of crowding are probably not the result of ammonium, but more likely the effect of an unknown chemical released from feeding daphnids. Illustrative is the significantly different quality (in terms of biomass) of the newborns.

Another explanation for the observed differences in life-history parameters may be an altered feeding behavior. Chemical inhibition of grazing in crowding conditions has been reported for *D. pulex* (Helgen, 1987) and other daphnids (Matveev, 1993). Those effects resemble the feeding rate inhibition of the copepod *Diaptomus tyrelli* by chemicals released from its competitor and predator *Epischura nevadensis* (Folt & Goldman, 1981).

Daphnia responded different to crowding-chemicals from congeners and conspecifics. In both species clutch sizes (of second and the third brood) were more reduced in water from congeners than from conspecifics. Similar results have been found for *D. cucullata* (Seitz, 1984). Hobœk & Larsson (1990) reported a different response for reproduction of *D. magna* to crowded water from conspecifics (slightly stimulating) and the congener *D. pulex* (strongly inhibiting). Also in rotifers intraspecific effects on fecundity were weaker than interspecific (Halbach, 1969). In contrast, Burns (1995) found no differences in response of *D. galeata* or *D. hyalina* to inter- or intraspecific crowding chemicals. Whether different chemicals, different concentrations or whether the animal's ability to distinguish between inter- and intraspecific infochemicals is involved, remains unclear. However, indications for different chemicals exist (Goser, 1997). The crowding chemicals from *D. magna* are unstable (Goser & Ratte, 1994; Goser, 1997), but crowding chemicals from *D. pulex* remain active for prolonged periods (Goser, pers. comm.).

This phenomenon may indicate a chemical feedback mechanism that enables *Daphnia* to respond to a future food limitation. Chemicals associated with high competitor densities, which will occur just before food limitation, will enable the daphnids to switch from a more quantitative reproduction to a more qualitative one (Cleuvers *et al.*, 1997). In that respect, the larger and heavier neonates produced in crowded water can be interpreted as better quality offspring (Tessier & Consolatti, 1991) since they do have higher resistance to starvation than smaller offspring (Gliwicz & Guisande, 1992), which has been confirmed recently by Cleuvers *et al.* (1997).

Overall, it does seem that infochemicals from algae will not affect the energy flow from phytoplankton to consumers such as *Daphnia*. However, examples of rapid reductions in *Daphnia* feeding activity as a result of cyanobacterial excretory products (Haney *et al.*, 1995) have to be kept in mind. By contrast, crowding chemicals may have a clear effect on the phytoplankton-grazer interaction.

CHAPTER 10

Summarizing discussion

"So long as individual scientist believe,
and behave to the belief,
that the essence of success in science
is the freedom to discover the right experiment
and then to do it according to one's own lights,
all the social structures that connect scientists to one another
will be based solely on each scientist's latest piece of individual work:
a hobbesian world of each against all"

- R. Pollack 1997

"Scenedesmus is a freshwater colonial green alga which has a world wide distribution" (Trainor, 1998). However, besides numerous coenobia variants, many *Scenedesmus* produce unicells. For example, *S. abundans* from the field formed unicells in the laboratory (Fott, 1968) and also *S. armatus* did occur mainly as unicells (Tukaj *et al.*, 1996). In culture, unicells may be very common (f.e. Hegewald, 1982; Holtmann & Hegewald, 1986; Trainor, 1998), even at cell density far above *ca.* 1000 cells·ml^{-1}. Hence, low cell density (Egan & Trainor, 1989b) does not seem a prerequisite for unicell development in several *Scenedesmus* strains. In this study, all strains produced unicells and an investigation of literature data shows that the phenomenon of unicellular *Scenedesmus* is widespread in the genus (Table 10.1).

Table 10.1: *Scenedesmus* species for which the formation of unicells has been reported.

S. abundans	Fott (1968)
S. acuminatus	Krienitz (1987); Mladenov & Furnadzieva (1995)
S. acutiformis	Hegewald (1982)
S. acutus	Krienitz (1987); Nagy-Tóth *et al.* (1992); Lampert *et al.* (1994); CHAPTER 8
S. armatus	Swale (1967); Trainor & Egan (1990); Tukaj & Bohdanovicz (1995); Tukaj *et al.* (1996); CHAPTER 8
S. basiliensis	Trainor & Hilton (1963)
S. falcatus	Krienitz (1987); Mladenov & Furnadzieva (1995); CHAPTER 8
S. kissii	Trainor (1995)
S. microspina	Tukaj & Bohdanovicz (1995)
S. obliquus	Hegewald (1982); Holtmann & Hegewald (1986); Wasmund (1992); CHAPTER 8
S. obtusiusculus	Kylin & Das (1967); Krienitz (1987)
S. pectinatus	Holtmann & Hegewald (1986)
S. pseudobernardii	Krienitz (1987)
S. quadricauda	Overbeck & Stange-Bursche (1966); Steenbergen (1978); CHAPTER 8
S. subspicatus	Hessen & Van Donk (1993); Trainor (1993); CHAPTER 8

The species list in table 10.1 is undoubtedly far from complete, but includes already 64 strains. Despite that unicells may be common in *Scenedesmus* they are but occasionally reported from nature (e.g. Krienitz, 1987). This led to a hypothetical seasonal life history of *Scenedemus* with unicells occurring in early spring (Egan & Trainor, 1989a). But why would unicells occur only in spring, especially since sufficient literature data exist on unicellular *Scenedesmus* under a wide range of nutrients and cell densities? And why are there that few reports of unicellular *Scenedesmus* from the field? Trainor (1979) observed that unicells disappeared when incubated in dialysis sacks in the field or when cultured in pond water in

the laboratory. Interestingly, in another study ten years later the same strain produced unicells in water from the same pond (Egan & Trainor, 1989a). Since grazers may be involved in *Scenedemus* plasticity, by both selective grazing on small, unprotected morphs and chemical induction of large protected morphs, they might account for the different observations by Trainor (1979) and Egan & Trainor (1989a). One explanation could be that due to the activity of grazers unicells are produced only in very low numbers, which experience an enormous mortality. Another reason may be that unicells are simply not recognized as *Scenedesmus*. Unicells may resemble species described in at least eight other green algal genera (Trainor, 1998). In a recent study, Kessler and co-workers using sequence analyses of 18S rDNA showed that three taxa of the unicellular *Chlorella* and one of *Kermatia* are in fact unicellular *Scenedesmus* (Kessler *et al.*, 1997)!

An additional consideration is the phenomenon that cultures may respond differently at a later date after years in the laboratory as "*if they turned off a certain process*" (Trainor, 1998). The suggested inactivation of genes by accumulation of storage products in not actively growing cultures (Trainor, 1998) does not apply to cultures used in this study since cultures were maintained actively growing in liquid medium for years by regular transfer into fresh medium. Another alternative explanation may be mutation of colonies into unicells that are competitively superior. Although Boraas *et al.* (1998) proposed mutation as the driving force in changing a unicellular culture into one dominated by colonies under the pressure of grazing, the occurrence of the opposite in our cultures is very unlikely for several reasons:

1) Unicells were formed rapidly, but colonies were always present albeit often in very low numbers.
2) In several cultures a rapid unicell-colony transformation could be observed, either induced by *Daphnia* chemicals or not.
3) In culture, unicells seemed not competitively superior as growth rates between unicells and (induced) colonies were similar.

The capability of unicell production is widespread among the genus *Scenedesmus* (Table 10.1) and perhaps all *Scenedesmus* may have this capacity (Trainor, 1998). The unicells may provide an excellent dispersal mechanism to *Scenedesmus* (Trainor, 1998), and they also experience the lowest sinking losses (Conway & Trainor, 1972; CHAPTER 6).

Scenedesmus, as by definition all photoautotrophic planktonic organisms, are faced with the problem that they have to remain in the euphotic zone of a water column. Thus in an aquatic environment, selection pressure exists for small organisms that have the most efficient uptake of dissolved nutrients and lowest sinking losses (Reynolds, 1984; Lehman, 1988). Assuming that coloniality has evolved as a defense against predation, one could imagine an adaptive trade-off between defensive coloniality and competitively advantageous unicellularity (Boraas *et al.*, 1998). The advantage of unicells may be a prolonged position in

the upper water layers, whereas colonies may sink to deeper water with lower light intensities and presumably lower temperatures.

The apparent stability of colonial *Scenedesmus* in the field could also reflect a constitutive defense as might be expressed under conditions when grazers are always present or when the environment is highly predictable (Dodson, 1989; Brönmark & Petterson, 1994). In surface waters, grazers are always present, but the abundance, activity and taxonomic composition may vary greatly both on spatial and temporal scales. *Daphnia* can easily ingest small *Scenedesmus* coenobia (Lampert *et al.* 1994), but not large eight-celled coenobia (Hessen & Van Donk, 1993; CHAPTER 7). Most coenobia will undoubtedly be too large to be grazed by protist grazers, such as *Paraphysomonas* (Grover, 1989) and *Loxodus* (Goulder, 1972), and also several other zooplankters, such as *Ceriodaphnia* and rotifers may experience reduced ingestion. The grazer-induced colony formation can be interpreted as a defense, since the algae clearly benefit from a reduced feeding activity of grazers (CHAPTER 7). A fixed defense, or a phenotypic stability with four- and eight-celled coenobia as the most dominant morphs, could also be a solution to deal with grazing, but would still confront *Scenedesmus* with the problem of sinking. Especially bristles but also spines reduce the sinking in *Scenedesmus*, however, colonies still experience higher sinking losses than unicells (Conway & Trainor, 1972). One would expect allocation or metabolic costs associated with the induced colony formation, with a demand for additional cementing wall material (Trainor, 1998). Since no metabolic costs were detected and because of the plastic nature of the defense, costs were assigned to sinking out of the euphotic zone (CHAPTER 6). These external or environmental costs (cf. Tollrian & Harvell, 1999) may be the only detectable costs. Enhanced sinking of *Scenedesmus* out of the euphotic zone could, however, also be interpreted as an escape in time since *Scenedesmus* is capable of surviving prolonged periods of darkness (Dehning & Tilzer, 1989). In the dark the coenobia disintegrate and unicells may serve as inocula for subsequent blooms (Dehning & Tilzer, 1989; Egan & Trainor, 1989a,b).

In their study, Boraas and co-workers (1998) reported a 'stable' colonial morph that is competitively inferior to unicells. The colonial form breeds true, but "*active photosynthesis and continued interaction with the predator are essential to maintain the colonial algae*" (Boraas *et al.*, 1998). This statement suggests that also in *Chlorella* the colonies may disintegrate in the dark. Moreover, a continued interaction necessary to maintain the colonies could imply that the induced phenotypic adaptations gradually faded away as has been observed in the ciliate *Paramecium* (Jollos, 1921). Perhaps epigenetic inheritance may be involved, whereby the altered phenotype may persist through many cell divisions even when the inducing stimulus has disappeared (Maynard-Smith, 1990). It has been argued that the epigenetic inheritance systems, responsible for cellular memory, may have played a vital role

in the transition from unicellularity to multicellularity (Jablonka, 1994). Therefore, it may be interesting to examine if *Scenedesmus* pseudounicells form more coenobia than true unicells.

In a system such as described by Boraas *et al.* (1998) with a 'stable' colonial morph and a unicellular morph, clonal replacement will occur when grazing pressure changes. When two *Scenedesmus* species are present in a water body, for example unicellular non-spiny *S. acutus* and four- and eight-celled spined *S. communis*, without grazing *S. acutus* will most likely dominate because of lower sinking losses. However, since colonial *S. communis* is protected against grazing, but unicellular *S. acutus* is not, the former may become the most dominant under grazing conditions, despite higher sedimentation losses (Fig 10.1).

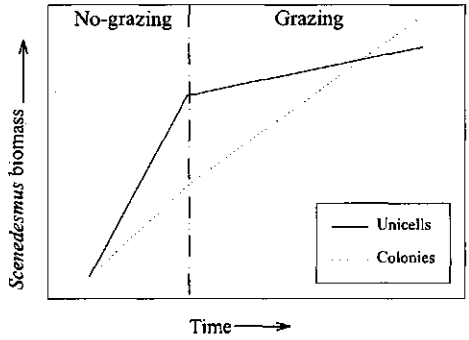


Figure 10.1: Increase in unicellular and colonial *Scenedesmus* subjected to size-dependent sedimentation in the absence and presence of grazing.

In an experiment not reported previously, 5 *Ceriodaphnia* were added to 50 ml suspensions of *S. acutus*, *S. communis* and their '1:1' mixture, all at identical food concentrations of $5 \cdot 10^6$ $\mu m^3 \cdot ml^{-1}$, whereas incubations withouit *Ceriodaphnia* served as controls. After 48 h, growth rates of *Scenedesmus* in *Ceriodaphnia* treatments of the '1:1' suspensions ($t = 5.03$; $P = 0.007$) and the *S. acutus* food suspensions ($t = 3.22$; $P = 0.032$) were significantly reduced because of grazing. However, the *S. communis* suspensions in the presence of *Ceriodaphnia* had identical growth rates to populations grown in its absence ($t = 0.33$; $P = 0.758$). Moreover, after 48 h, in the absence of *Ceriodaphnia* the '1:1' suspensions contained 6.5% *S. communis* on a volume basis, whereas in the presence of *Ceriodaphnia* this proportion was 10.8%. The data of this simple experiment suggest that despite the initial high growth of unicellular *S. acutus* they may eventually be out competed by *S. communis*. However, at higher *Ceriodaphnia* densities, *S. acutus* will be induced (CHAPTER 3) to form protective colonies (CHAPTER 7).

Grazer-induced colonies may be protective against numerous small grazers, but in *Scenedesmus* several strains do not seem to gain an advantage by colony formation, since the coenobia would still be ingestible by *Daphnia*. The trait may reflect a response to grazing in

general rather than to *Daphnia* in particular. In numerous waters, *Scenedesmus* is not exposed to large *Daphnia*, but is confronted with several much smaller grazers such as rotifers, ciliates, and phagotrophic flagellates. Unicells will probably not survive an encounter with one of these grazers, and may therefore use dissolved chemicals to detect these grazers prior to encounter. The phenomenon of grazer-induced coenobia formation could be interpreted as a defense mechanism against grazing, as grazing rates for relatively small *Daphnia* were suppressed when the proportion of eight-celled coenobia in the food was high (Hessen & Van Donk, 1993; Lürling & Van Donk, 1996; Van Donk *et al.*, 1999; CHAPTER 7).

The morphological response of *Scenedesmus acutus* to zooplankton mediated chemicals is related to the amount of algae grazed upon (CHAPTER 3). The type of food seems unimportant as long as it is digestible. Animals fed with ingestible, but undigestible particles, produced no colony-inducing chemicals, neither did starved animals. *Scenedesmus acutus* did not respond to algal homogenates, thus the infochemical originates in the grazer, but most probably as a residual of the digestive process. Moreover, water that had contained carnivorous zooplankton evoked no colony formation (CHAPTER 3). The differences seem directly related to the grazers diet, which, in fact, is not uncommon in the aquatic world. Crucian carp increased its body depth in response to predators (Pike, Perch) with a piscivorous diet. By contrast, perch fed chironomids had no effect (Brönmark & Petterson, 1994). Fathead minnows showed only a fright reaction to pike that had been fed with minnows, but not to pike fed with swordtails (Mathis & Smith, 1993). Also snails (Crowl & Covich, 1990) and sea anemones (Howe & Harris, 1978) showed different responses related to the predators diet. Identification of predators per se could be more advantageous than having to identify all predator species (Brönmark & Petterson, 1994). Thus a response to a general herbivore cue should be adaptive in habitats with variable grazing pressure from a zooplankton assemblage with various herbivores.

Daphnia induced colony formation did occur in both nutrient replete and deplete cultures (CHAPTER 4). In the absence of *Daphnia* infochemicals, cultures consisted mostly of unicells, the proportion of unicells always appeared higher under nutrient-limiting conditions than under non-limited conditions.

At times the pool of dissolved nutrients may become limited, the growth rate may drop and the quality of the algae may change. Especially non-selective grazers, such as members of the genus *Daphnia*, will be confronted with the variability in food quantity and quality. Numerous studies have been devoted to the nutritional inadequacy of nutrient-limited algae as food for *Daphnia*. In most studies that examined the effect of P-limited algae on *Daphnia* growth the food species was a chlorophyte (e.g. Groeger *et al.*, 1991; Mitchell *et al.*, 1992; Urabe & Watanabe, 1992; Sterner, 1993; Sterner *et al.*, 1993; Van Donk *et al.*, 1997; Kilham *et al.*, 1997; Sundbom & Vrede, 1997; Lürling & Van Donk, 1997; DeMott, 1998). Three

mechanisms have been proposed to explain the poor food quality of P-limited algae (mostly chlorophytes):

1) The mineral limitation hypothesis suggesting a direct P limitation of *Daphnia* (e.g. Hessen, 1992; Urabe & Watanabe, 1992; Sterner, 1993).
2) The HUFA-hypothesis suggesting that a biochemical component, such as unsaturated fatty acids, may be limiting to *Daphnia* (e.g. Brett, 1993; Müller-Navarra, 1995; Ahlgren *et al.*, 1997; Brett & Müller-Navarra, 1997).
3) The digestion-resistance hypothesis suggesting that a thickened cell wall reduces the digestibility of P-limited green algae (Van Donk & Hessen, 1993; Van Donk *et al.*, 1997).

Daphnia growth is based on food consumption according the equation: growth = quantity × quality (Brett & Müller-Navarra, 1997). In grazing and growth experiments food suspensions are supplied in equal amounts of carbon or biovolume to *Daphnia* to evaluate the effect of altered food quality. However, in a hypothetical pelagic with just ingestible chlorophytes qualitative and quantitative changes in the algal food population may occur concomitantly. Algal growth rates will drop under nutrient-limitation and P-limited chlorophytes may experience higher sinking losses as a consequence of increased density and size, both negatively affecting the resistance against sedimentation.

Several studies have reported increased sinking rates for nutrient-limited algae (e.g. Smayda, 1974; Titman & Kilham, 1976; Waite *et al.*, 1991). The increase in cell size and concomitant change in biochemical composition seems a general phenomenon in chlorophyte algae. For example, the mean cell volume of an average non-limited green alga appeared around 84 μm^3, while the same alga under P-limitation was 152 μm^3 (based on Van Donk & Hessen, 1993; 1995; Kilham *et al.*, 1997; Sundbom & Vrede, 1997; Lürling & Van Donk, 1997). Assume a spherical cell shape, densities for lipids, proteins and carbohydrates of 0.85, 1.3 and 1.5 $g \cdot ml^{-1}$, respectively, a biochemical composition (percentage of dry-weight) of 60% proteins, 20% carbohydrates and 20% lipids for non-limited and 25% proteins, 50% carbohydrates, and 25% lipids under P-limitation, and 85% water per cell. Based on these assumptions sinking velocities may be estimated according to modified Stoke's equation:

$$v_{SED} = 2 \cdot g \cdot r^2 \cdot (\rho_a - \rho_m) \times (9 \cdot \eta \cdot \varphi_a)^{-1}$$

in which v_{SED} = the sedimentation velocity ($m \cdot d^{-1}$), g = the earth's acceleration (9.8 $m \cdot s^{-2}$), radius of particle (m), ρ_a = density of algal particle ($kg \cdot m^{-3}$), ρ_m = density of medium ($kg \cdot m^{-3}$), η = dynamic viscosity ($kg \cdot m^{-1} \cdot s^{-1}$) and φ_a = form resistance of algal particle (-).

Solely based on size, at 20°C sinking velocities for the hypothetical P-limited chlorophyte are about 1.2 times larger, 0.194 *vs.* 0.236 $m \cdot d^{-1}$. However, taking the shift in biochemical composition into account sinking velocities more than double (2.2 times), 0.194 *vs.* 0.434 $m \cdot d^{-1}$. This theoretical value is close to the 100% higher sinking rate in P-limited *S. quadricauda* (0.4 *vs.* 0.2 $m \cdot d^{-1}$) measured by Titman & Kilham (1976). Thus, in the hypothetical pelagic, cultures could experience lower grazing losses because not only fewer cells fit in the animals'

gut, because of larger cell size, but also more cells may sink thereby reducing the grazable proportion of a population.

Another aspect that has not been taken into account is the phenomenon that P-limited *Scenedesmus* may still built coenobia (CHAPTER 4). The coenobia could not only have a size refuge from small *Daphnia*, but may also have higher sinking rates than P-limited unicells. So far, in many studies changes in quality of P–limited *Scenedesmus* for *Daphnia* have been accredited to mineral P-limitation. The mechanism seems valid and has gained considerable support (Hessen, 1992; Sterner, 1993; DeMott, 1998). The question that arises is whether the mineral limitation hypothesis would be accepted if not unicellular *S. acutus* from high-density cultures had been used as food, but for example large coenobial *Scenedesmus*. In spined *Scenedesmus*, such as *S. armatus* (Shubert & Trainor, 1974) and *S. quadricauda* (Overbeck & Stange-Bursche, 1966), in dilute media with low P-levels cultures were dominated by coenobia, whereas higher P-levels resulted in dominance by unicells. The algal food would probably not support good *Daphnia* growth. However, for examining the mechanism it may be justified to use *Scenedesmus acutus*, but what if a normal procedure would have been to grow *Daphnia* on cryptophytes or diatoms instead of chlorophytes? It is doubtful whether the mineral limitation hypothesis would have been accepted (Brett, *in prep.*). P-limited diatoms and cryptophytes may support good growth in *Daphnia* that is equal to or even higher than the growth of *Daphnia* fed with non P-limited chlorophytes (Müller-Navarra, 1995a; Lürling & Van Donk, 1997). One of the alternative hypotheses, the digestion resistance hypothesis, has been rejected in recent papers by DeMott and co-workers (DeMott, 1998; DeMott *et al.*, 1998). However, the criticism on the digestion resistance hypothesis is derived from indirect measurements, rather than carefully designed experimentation. One could claim that the results of DeMott are at least biased by the use of non-axenic cultures. With high dilution rates and P in excess, the bacteria in the culture will by no means compete severely with the *S. acutus* cells. However, dropping the dilution rate and lowering the P-input significantly will not only result in accumulated photosynthates in the medium, but will also favor the bacteria that are superior competitors for P. Thus, relatively more P may end up in the bacteria that may still be harvested by the *Daphnia*. The cell wall thickening proposed by Van Donk & Hessen (1993) had already been demonstrated in P–limited *Scenedesmus* about 10 years earlier (Tilberg *et al.*, 1984). The cell wall thickness was reduced rapidly after normal photosynthesis had been resumed by the addition of P. That *Daphnia* may have difficulties in digestion of the cell wall has been observed before in *Scenedesmus*. In several studies *Scenedesmus* have been isolated from *Daphnia* faeces (Horn, 1981; Levitan, 1987, Van Donk & Hessen, 1993). Thus, *Scenedesmus* could have evolved several traits to reduce mortality through grazing. A worthwhile attempt could be electron-microscopic analysis of the digestive process or flowcytometric analysis of *Daphnia* faeces sorting various particles for examination of viability.

In non-spiny strains of the subgenus *Acutodesmus* (*Scenedesmus* cf. Kessler *et al.*, 1997), grazer-induced colony formation may be a common strategy. By contrast, in spined members of *Desmodesmus* colony formation seems not easily induced by grazer-chemicals, although reported first in a spined *Scenedesmus* (Hessen & Van Donk, 1993). Nevertheless, in the presence of live *Daphnia* more coenobia were detected and when grazers are present also in spined *Scenedesmus* eight-celled coenobia may be induced (CHAPTER 8). In conclusion, spined *Scenedesmus* respond in a different way to grazers than non-spiny strains. This was also observed in CHAPTER 3, where two detergents were found that appeared effective in inducing colonies in *S. acutus*. However, evaluating the effect on spined *S. subspicatus* revealed that only Na-dodecyl-sulphate was effective in inducing coenobia during the course of a standard biotest. Inasmuch *S.* acutus and *S. subspicatus* strains are often used as test organisms in toxicity-tests (e.g. Twiss *et al.*, 1989; Kühn & Pattard, 1990; Conrad *et al.*, 1993; Corradi & Gorbi, 1993; Saenz *et al.*, 1993; Fournadzhieva *et al.*, 1995; Carrasco & Sabatier, 1997), the variability among species and strains may impose serious implications to these tests. Tukaj & Bohdanowicz (1995) demonstrated diesel-fuel-oil induced morphological changes in three *Scenedesmus* species, but noted that changes in cell shape, unicell production, coenobia organization and abnormalities were species-dependent. So far in toxicity testing merely effects on growth rates have been examined. However, in the plastic *Scenedesmus* morphological changes may occur independent of growth rates (Siver & Trainor, 1981; 1983; Trainor, 1998; CHAPTER 3) and different morphologies may have an effect on species interactions (Lürling & Van Donk, 1996; Van Donk *et al.*, 1998; CHAPTER 7). Therefore, such tests may benefit from the inclusion of examination on morphological appearance of *Scenedesmus*. Especially when testing certain effluents or surface waters using *Scenedesmus* as test organism filter extractables or grazing-associated chemicals could result in induced colony formation and may influence the test. It should be noted that the effect could be greatly reduced by rinsing the filters thoroughly prior to use.

The enormous variability within and among members of the green algal genus *Scenedesmus* together with the taxonomic chaos (Kessler, 1991) puts clear constraints on the use of *Scenedesmus* sp. as a 'standard' test-organism in toxicity tests. Conrad *et al.* (1993) showed that *S. subspicatus* was not suitable for algal herbicide monitoring based on fluorescence and suggested *Chlorella fusca* as an alternative test-organism. However, in a recent study based on 18S rDNA analysis three strains of *Chlorella,* including strain C-1.1.10 used by Conrad *et al.* (1993), had to be placed within the genus *Scenedesmus*! (Kessler *et al.*, 1997).

As a result of environmental heterogeneity several morphotypes may be present simultaneously. Mortality by grazing is only one of the pressures faced by algae. "*The realistic challenge to algae in nature is to resist mortality from a complex array of grazers*

and to exploit nutrients on many different spatial and temporal scales....The conflicting allometries of selection pressure, where large sizes are favored to avoid grazers but small cells are favored for energy and nutrient acquisition, are the types of conflicts that probably generated the morphological and physiological diversities of phytoplankton" (Lehman, 1988). Indeed, *Scenedesmus* strains may respond dramatically to changes in both their biological and chemical environment, and may express considerable phenotypic plasticity within a taxon in the absence of grazers (Trainor, 1998). In this study, the non-spiny *S. acutus* appeared mainly unicellular under a wide range of conditions, unless exposed to grazing-associated chemicals. The formation of eight-celled coenobia, the typical protective morph, occurred within 48 h in non-spiny *Scenedesmus* and could take a few days in spined species. Harvell & Tollrian (1999) have listed four prerequisites for the evolution of an inducible defense:

1). The selective pressure of the inducer has to be variable and unpredictable
2). The necessity of a reliable and detectable cue
3). The defensive response must be effective
4). There should be a trade-off between the tax paid and the benefits of the response

The four specific ecological conditions necessary for the evolution of an inducible anti-grazer response in *Scenedesmus* are indeed met:

Ad 1). Grazers are always present, but grazing pressure may vary considerably on temporal and spatial scales.
Ad 2). The cue appeared to be related to the activity of the inducer, and does seem restricted to herbivorous zooplankton.
Ad 3). The formation of eight-celled coenobia is an effective strategy in reducing mortality through grazing by numerous grazers.
Ad 4). Sinking out to deeper water layers may confront the algae with a serious cost that may offset benefits.

So far, it has become clear that phenotypic plasticity is not only restricted to multicellular organisms, but that it may be widespread among phytoplankton, where both abiotic and biotic factors may affect the morphology, physiology and behavior of the cells. Further research could focus on factors such as light quality and quantity or on other organisms. Diatoms may be worthwhile examining, other chlorophytes that build colonies, flagellates that actively avoid areas with grazers (Hansson, 1996) and definitely cyanobacteria that showed not only some increase in cell size after exposure to grazer-infochemicals, but also a tendency to enhanced production of toxins (CHAPTER 8). It may be worth to examine the toxicity in cyanobacteria, but also in freshwater Haptophyta especially since grazing-activated chemical defense in the marine haptophyte *Emiliania huxleyi* has been demonstrated (Wolfe *et al.*, 1997). Besides colony formation, toxicity and migration, other potential

defensive traits in algae are worthwhile examining, such as cell-wall thickening, mucous formation and the formation of spicules, bristles and spines. Spine formation and spine length in *Scenedesmus* did not change in the presence of *Daphnia* infochemicals (CHAPTER 8), but might occur in combination with coenobia formation (cf. Hessen & Van Donk, 1993). Spines may be considered a constitutive defense, reducing mortality through grazing by numerous protist grazers. Since constructing material is required for the formation of spines, one could expect metabolic costs associated with it. These costs could be reflected in lower growth rates. Comparison of volume-based growth rates of the non-spiny *Scenedesmus* with the spined strains (used in CHAPTER 8), revealed significantly ($t = 2.49$; $P = 0.023$) higher growth rates ($\pm$ 1 SD) for non-spiny strains (1.275 ± 0.233 d^{-1}) than for spined strains (1.039 ± 0.179 d^{-1}). Further research could focus on the grazing success of heterotrophic flagellates and ciliates on spined and non-spined *Scenedesmus* and the effect grazing may have on algal species competition. This could be examined in mixed-species predator-prey continuous cultures (Boraas, 1993).

Scenedesmus was able to detect the presence of grazers, but no evidence was found to support the hypothesis that *Daphnia* may be able to locate regions of high quality algal food by means of chemical cues (CHAPTER 9). However, *Daphnia* did respond in a behavioral way by avoidance of medium that had contained congeners and in life-history shifts that affected growth and reproduction. This chemical warfare could be beneficial to the algae, although *Daphnia* may aggregate because recycling of food increases the digestibility (Kersting, 1991).

The phenomenon of grazer-induced colony formation in *Scenedesmus* can be interpreted as an inducible defense at the expense of higher sinking losses. The phenomenon is not restricted to *Scenedesmus* and because of the enormous plasticity in phytoplankton, numerous species may eventually turn out not only to respond to abiotic, but to biotic agents too. It is, however, of utmost importance that the active compounds are chemically characterized. After chemical characterization the occurrence of the active compounds may be determined in the field, a research area that until now has received little attention. Nevertheless, the future seems bright since organic chemists are currently making progress with the chemical characterization of the *Daphnia* infochemical(s).

REFERENCES

Aaronson, S. (1973). Effect of incubation temperature on the macromolecular and lipid content of the phytoflagellate *Ochromonas danica*. *J. Phycol.* **9**: 111-113.

Ahlgren G., Lundstedt L., Brett M. & Forsberg C. (1990). Lipid composition and food quality of some freshwater phytoplankton for cladoceran zooplankters. *J. Plankton Res.***12**: 809-818.

Ahlgren G., Gustafsson I-B. & Boberg M. (1992). Fatty acid content and chemical composition of freshwater microalgae. *J. Phycol.* **28**: 37-50.

Allaby, M. (1994). The concise oxford dictionary of ecology. Oxford University Press, 415 pp.

Banse, K. (1976). Rates of growth, respiration and photosynthesis of unicellular algae as related to cell size - a review. *J. Phycol.* **12**: 135-140.

Benndorf, J. & Henning, M. (1994). *Daphnia* and toxic blooms of *Microcystis aeruginosa* in Bautzen Reservoir (GDR). *Int. Revue ges. Hydrobiol.* **74**: 233-248.

Bern L. (1990). Size-related discrimination of nutritive and inert particles by freshwater zooplankton. *J. Plankton Res.* **12**: 1059-1067.

Bienfang, P.K. (1981). SETCOL-a technically simple and reliable method for measuring phytoplankton sinking. *Can. J. Fish. Aquat. Sci.* **38**: 1289-1294.

Black, R. & Slobodkin, L. (1987). What is cyclomorphosis? *Freshwater Biol.* **18**: 373-378.

Boersma, M. (1994). On the seasonal dynamics of *Daphnia* species in a shallow eutrophic lake. PhD Thesis, University of Amsterdam, The Netherlands.

Boersma, M. and Vijverberg, J.J. (1994). Resource depression in *Daphnia galeata, Daphnia cucullata* and their interspecific hybrid: life history consequences. *J. Plankton Res.* **16:** 1741-1758.

Boersma, M. and Vijverberg, J.J. (1995a). The significance of nonviable eggs for *Daphnia* population dynamics. *Limnol. Oceanogr.* **40:** 1215-1224.

Boersma, M. & Vijverberg, J.J, (1995b). Possible toxic effects on *Daphnia* resulting from the green alga *Scenedesmus obliquus*. *Hydrobiologia* **294**: 99-103.

Bonner, J.T. (1988). *The evolution of complexity*. Princeton University Press, New Jersey.

Boraas, M.E. (1993). Single-stage predator-prey algal-rotifer chemostat culture. *In*: Walz, N. (ed.) *Plankton regulation dynamics*. Springer-Verlag, Berlin, Heidelberg, New York, pp 51-61.

Boraas, M.E., Seale, D.B. & Boxhorn, J.E. (1998). Phagotrophy by a flagellate selects for colonial prey: a possible origin of multicellularity. *Evol. Ecol.* **12**: 153-164.

Bradley, P.M. (1991). Plant hormones do have a role in controlling growth and development of algae. *J. Phycol.* **27**: 317-321.

Brammer, E.S. (1979). Exclusion of phytoplankton in the proximity of dominant water soldier (*Stratiotes aloides*). *Freswater Biol.* **9**: 233-249.

Brett, M.T. (1993). Resource quality effects on *Daphnia longispina* offspring fitness. *J. Plankton Res.* **15:** 403-412.

Brett, M.T. & Müller-Navarra, D.C (1997). The role of highly unsaturated fatty acids in aquatic food web processes. *Freshwater Biol.* **38**: 483-499.

Brönmark, C. & Miner, J.G. (1992). Predator-induced phenotypical change in body morphology in crucian carp. *Science* **258**: 1348-1350.

Brönmark, C. & Petterson, L.B. (1994). Chemical cues from piscivores induce a change in morphology in crucian carp. *Oikos* **70**: 396-402.

Brown, G.E. & Godin, J.G.J. (1997). Anti-predator responses to conspecific and heterospecific skin extracts by threespine sticklebacks: alarm pheromones revisited. *Behaviour* **134**: 1123-1134.

Brown, G.E. & Smith, R.J.F. (1997). Conspecific skin extracts elicit antipredator responses in juvenile rainbow trout (*Oncorhyynchus mykiss*). *Can J. Zool.* **75**: 1916-1922.

Brown, J.H. & Maurer, B.A. (1986). Body size, ecological dominance, and Cope's Rule. *Nature* **324**: 248-250.

Browne, K.A., Tamburri, M.N. & Zimmer-Faust, R.K. (1998). Modelling quantitative structure-activity relationships between animal behaviour and environmental signal molecules. *J. Exp. Biol.* **201**: 245-258.

Burg, C.A. & Trainor, F.R. (1965). *Scenedesmus* wall ornamentation. II, insertion of bristles. *J. Phycol.* **3**: 53-54.

Burke, S. (1998). Statistics refresher 2: analysis of variance. *Scientific data Management* **2**: 36-41.

Burns, C.W. (1968). The relationship between body size of filter-feeding Cladocera and the maximum size of particles ingested. *Limnol. Oceaonogr.* **13**: 675-678.

Burns, C.W. (1995). Effects of crowding and different food levels on growth and reproductive investment of *Daphnia*. *Oecologia* **101**: 234-244.

Burns, C.W. & Rigler, F.H. (1967). Comparison of filtering rates of *Daphnia* in lake water and in suspensions of yeast. *Limnol. Oceanogr.* **12**: 492-502.

Burns, N.M. & Rosa, F. (1980). *In situ* measurements of the settling velocity of organic carbon particles and 10 species of phytoplankton. *Limnol. Oceanogr.* **25**: 855-864.

Butler, N.M., Suttle, C.A. & Neill, W.E. (1989). Discrimination by freshwater zooplankton between single algal cells differing in nutritional status. *Oecologia* **78**: 368-372.

Butterfield, N.J. (1994). Burgess Shale-type fossils from a Lower Cambrian shallow shelf sequence in northwestern Canada. *Nature* **369**: 477-479.

Butterfield, N.J. (1997). Plankton ecology and the Proterozoic-Phanerozoic transition. *Paleobiology* **23**: 247-262.

Butterfield, N.J. (1999). Microbes, environment, and evolution- a Proterozoic palaeontological perspective. *http://www.astrobiology.sr.unh.edu/abstracts/butterfield.html.*

Cairns, J., Overbaugh, J. & Miller, S. (1988). The origin of mutants. *Nature* **335**: 142-145.

Canter-Lund, H. & Lund, J.W.G. (1995). *Freshwater Algae. Their microscopic world explored.* Biopress Ltd., Bristol, 360 pp.

Carrasco, J.M. & Sabatier, C. (1997). Toxicity of atrazine and chlorsulfuron to algae. *Toxicol. Environm. Chem.* **59**: 89-99.

Carvalho, G.R. & Hughes, R.N. (1983). The effect of food availability, female culture-density and photoperiod on ephippia production in *Daphnia magna* Straus (Crustaceae: Cladocera). *Freshwater Biol.* **13**: 37-46.

Chivers, D.P. & Smith, R.J.F. (1994). Intraspecific and interspecific avoidance of areas markes with skin extract from brook sticklebacks (*Culaea inconstansi*) in a natural habitat. *J. Chem. Ecol.* **20**: 1517-1524.

Chodat, R. (1913). Monographies d'algues en culture pure. Matériaux pour la flore cryptogamique Suisse, pp. 266.

Chodat, R. (1926). *Scenedesmus* étude de génétique, de systématique expérimentele et d'hydrobiologie. *Rev. Hydrobiol.* **3**: 71-258.

Clark, C.W. & Harvell, C.D. (1992). Inducible defenses and the allocation of resources: A minimal model. *Am Nat.* **139**: 521-539.

Cleuvers, M., Goser, B. & Ratte, H.-T. (1997). Life-history shift by intraspecific interaction in *Daphnia magna*: change in reproduction from quantity to quality. *Oecologia* **110**:337-345.

Cloern, J.E. (1977). Effects of light intensity and temperature on *Cryptomonas ovata* (Cryptophyceae) growth and nutrient uptake rates. *J. Phycol.* **13**: 389-395.

Codd, G.A., Bell, S.G. & Brooks, W.P. (1989). Cyanobacterial toxins in water. *Wat. Sci. Tech.* **21**: 1-13.

Cody, M.L. (1974). Optimization in ecology. *Science* **183**: 1156-1164.

Coesel, P.F.M. & Wardenaar, K. (1990). Growth responses of planktonic desmid species in a temperature-light gradient. *Freshwater Biol.* **23**: 551-560.

Colbrook, J.M. (1960). Some observations of zooplankton swarms in Windemere. *J. Anim. Ecol.* **29**: 241-242.

Conde-Porcuna, J.M. (1997). Chemical interference by *Daphnia* on *Keratella*: a life table experiment. *J. Plankton Res.* **20**: 1637-1644.

Conrad, R., Büchel, C., Wilhelm, C., Arsalane, W., Berkaloff, C. & Duval, J-C. (1993). Changes in yield of *in-vivo* fluorescence of chlorophyll a as a tool for selective herbicide monitoring. *J. Appl. Phycol.* **5**: 505-516.

Conway, K. & Trainor, F.R. (1972). *Scenedesmus* morphology and floatation. *J. Phycol.* **8**: 138-143.

Covich, A.P., Crowl, T.A., Alexander, J.E. & Vaughn, C.C. (1994). Predator-avoidance responses in freshwater decapod-gastropod interactions mediated by chemical stimuli. *J. N. Amer. Benthol. Soc.* **13**: 283-290.

Cowles, T.J., Olson, R.J. & Chisholm, S.W. (1988). Food selection by copepods: discrimination on the basis of food quality. *Mar. Biol.* **100**: 41-49.

Crowl, T.A. & Covich, A.P. (1990). Predator-induced life-history shifts in a freshwater snail. *Science* **247**: 949-951.

Cuddington, K.M. & McCauley, E. (1994). Food-dependent aggregation and mobility of the water fleas *Ceriodaphnia dubia* and *Daphnia pulex*. *Can. J. Zool.***72**: 1217-1226.

Davidson, I.R. (1991). Environmental effects on algal photosynthesis: temperature. *J Phycol.* **27**: 2-8.

Davies, J. (1985). Evidence for a diurnal horizontal migration in *Daphnia hyalina lacustris* Sars. *Hydrobiologia* **120**: 103-105.

Dawkins, R. & Krebs, J.R. (1979). Arms races between and within species. *Proc. R. Soc. Lond. B* **205**: 489-511.

Decho, A.W., Browne, K.A. & Zimmer-Faust, R.K. (1998). Chemical cues: Why basic peptides are signal molecules in marine environments. *Limnol. Oceanogr.* **43**: 1410-1417.

Dehning, I. & Tilzer, M.M. (1989). Survival of *Scenedesmus acuminatus* (Chlorophyceae) in darkness. *J. Phycol.* **25**: 509-515.

DeLange, H.J. & Van Donk, E. (1997). Effects of UVB-irradiated algae on life history traits of *Daphnia pulex*. *Freshwater Biol.* **38**: 711-720.

DeMeester, L. (1993). Genotype, fish-mediated chemicals, and phototactic behavior in *Daphnia magna*. *Ecology* **74**: 1467-1474.

DeMott, W.R. (1982). Feeding selectivities and relative ingestion rates of *Daphnia* and *Bosmina*. *Limnol. Oceanogr.* **27**: 318-327.

DeMott, W.R. (1986). The role of taste in food selection by freshwater zooplankton. *Oecologia* **69**: 334-340.

DeMott, W.R. (1989). The role of competition in zooplankton succession. In *Plankton Ecology: succession in plankton communities* (ed. U. Sommer), pp 195-252. Springer-Verlag Berlin, Heidelberg, New York.

DeMott, W.R. (1995). The influence of prey hardness on *Daphnia*'s selectivity for large prey. *Hydrobiologia* **307**: 127-138.

DeMott, W.R. (1998). Utilization of a cyanobacterium and a phosphorus-deficient green alga as complementary resources by daphnids. *Ecology* **79**: 2463-2481.

DeMott, W.R. & Moxter, F. (1991). Foraging on cyanobacteria by copepods: responses to chemical defenses and resource abundance. *Ecology* **72**: 1820-1834.

DeMott, W.R., Zhang, Q.-X. & Carmichael, W. (1991). Effects of toxic cyanobacteria and purified toxins on the survival and feeding of a copepod and three species of *Daphnia*. *Limnol. Oceanogr.* **36**: 1346-1357.

DeMott, W.R., Gulati, R.D. & Siewertsen, K. (1998). Effecys of phosphorus-deficient diets on the carbon and phosphorus balance of *Daphnia magna*. *Limnol. Oceanogr.* **43**: 1147-1161.

Desmarais, K.H. (1997). Keeping *Daphnia* out of the surface film with cetyl alcohol. *J. Plankton Res.* **19**: 149-154

Dicke, M. (1988). Infochemicals in a tritrophic context. *PhD-Thesis*, Agricultural University Wageningen.

Dicke, M. & Sabelis, M.W. (1988). Infochemical terminology: Based on cost-benefit analysis rather

than origin of compounds? *Funct. Ecol.* **2:** 131-139.

Dodson, S.I. (1984). Predation of *Heterocope septentrionalis* on two species of *Daphnia*: morphological defenses and their costs. *Ecoology* **65**: 1249-1257.

Dodson, S.I. (1989). Predator-induced reaction norms. *Bioscience* **39**: 447-452.

Dodson, S.I. & Wagner, A.E. (1996). Temperature affects selectivity of *Chaoborus* larvae-eating *Daphnia.Hydrobiologia* **325**: 157-161.

Doolittle, R.F., Feng, D.-F., Tsang, S., Cho, G. & Little, E. (1996). Determining divergence times of the major kingdoms of living organisms with a protein clock. *Science* **271**: 470-477.

Ebert, D. & Jacobs, J. (1991). Differences in life-history and aging in 2 clonal groups of *Daphnia cucullata* Sars (Crustaceae: Cladocera). *Hydrobiologia* **225**: 245-253.

Egan, P.F. & Trainor, F.R. (1988). Phenotypic plasticity in *Scenedesmus communis* Hegew. (Chlorophyceae). II. Examples of algal cyclo- and noncyclomorphosis. *Am. J. Bot.***78**: 37-52.

Egan, P.F. & Trainor, F.R. (1989). Low cell density: the unifying principle for unicell development in *Scenedesmus* (Chlorophyceae). *Br. Phycol. J.* **24**: 271-283.

Egan, P.F. & Trainor, F.R. (1989). The effect of media and inoculum size on the growth and morphological development of *Scenedesmus communis* Hegew. (Chlorophyceae) in culture. *Arch. Hydrobiol.* **117**: 77-95.

Egan, P.F. & Trainor, F.R. (1989). The role of unicells in the polymorphic *Scenedesmus armatus*. *J. Phycol.* **25**: 65-70.

Egan, P.F. & Trainor, F.R. (1990). Phenotypic plasticity in *Scenedesmus communis* (Chlorophyceae). I. Relationship of *S. communis* to *S. komarekii*. *J. Phycol.* **26**: 367-376.

Egan, P.F. & Trainor, F.R. (1991). Phenotypic plasticity in *Scenedesmus communis* (Chlorophyceae). II. Examples of algal cyclo- and noncyclomorphosis. *Am.J. Bot.* **78**: 37-52.

Elser, J.J., Elser, M.M., MacKay, N.A. & Carpenter, S.R. (1988). Zooplankton-mediated transitions between N- and P-limited algal growth. *Limnol. Oceanogr.* **33**: 1-14.

Enserink E.L. (1995). Food mediated strategies in *Daphnia*: relevance to ecotoxicology. *PhD-thesis*, Agricultural University Wageningen, The Netherlands, 128 p.

Evans, L.V. & Trewavas, A.J. (1991). Is algal development controlled by plant growth substances? *J. Phycol.* **27**: 322-326.

Fialkowska, E. & Pajdak-stós, A. (1997). Inducible defence against a ciliate grazer, *Pseudomicrothorax dubius*, in two strains of *Phormidium* (cyanobacteria). *Proc R. Soc. Lond. B.* **264**: 937-941.

Folch J., Lees M. & Sloane Stanley G.H. (1957). A simple method for the isolation and purification of total lipids from animal tissues. *J. Biol. Chem.* **226**: 497-509.

Folt, C.L. & Goldman, C.R. (1981). Allelopathy between zooplankton: A mechanism for interference competition. *Science* **213**: 1133-1135.

Folt, C.L. (1987). An experimental analysis of costs and benefits of zooplankton aggregation. *In*:

Kerfoot, W.C. & Sih, A. (eds.) *Predation: direct and indirect impacts on aquatic communities.* University Press New England, Hanover, London, pp. 300-314

Forsberg, C., Kleiven, S. & Willén, T. (1990). Absence of allelopathic effects of *Chara* on phytoplankton *in situ. Aquat. Bot.* **38** : 289-294.

Forsyth D. J., Haney, J. F. & James, M. R. (1992). Direct observation of toxic effects of cyanobacterial extracellular products on *Daphnia. Hydrobiologia* **228**: 151-155.

Fott, B. (1968). *Chodatella* stages in *Scenedesmus. Acta Univ. Carol. Biol.* 1967: 189-196.

Fournadzhieva, S., Kassabov, P., Andreeva, R., Petkov, G. & Dittrt, F. (1995). Influence of the herbicide simazine on *Chlorella, Scenedesmus* and *Arthrospira. Algol. Stud.***76**: 97-109.

Francko, D.A. & Wetzel, R.G. (1982). Production and release of cyclic AMP. *J. Freshw. Ecol.* **1**: 365-371.

Fulton III R.S. & Paerl H.W. (1987a). Toxic and inhibitory effects of the blue-green alga *Microcystis aeruginosa* on herbivorous zooplankton. *J. Plankton Res.* **9**: 837-855.

Fulton III R.S. & Paerl H.W. (1987b). Effects of colonial morphology on zooplankton utilization of algal resources during blue-green algal (*Microcystis aeruginosa*) blooms. *Limnol. Oceanogr.* **32**: 634-644.

Gardner, W.S. & Miller III, W.H. (1981). Intracellular composition and net release of free amino acids in *Daphnia magna. Can. J. Fish. Aquat. Sci.* **38**: 157-162.

Gardner & Scavia, D. (1981). Kinetic examination of nitrogen release by zooplankters. *Limnol. Oceanogr.* **26**: 801-810.

Gavis, J., Chamberlin, C. & Lystad, L.D. (1979). Coenobial cell number in *Scenedesmus quadricauda* (Chlorophyceae) as a function of growth rate in nitrate-limited chemostats. *J. Phycol.* **15**: 273-275.

Geel, C. (1997). Photosystem II electron flow as a measure for phytoplankton gross primary production. *PhD-thesis* Agricultural University Wageningen 110 pp.

Geel, C., Versluis, W. & Snel, J.F.H. (1997). Estimation of oxygen evolution by marine phytoplankton from measurement of the efficiency of photosystem II electron flow. *Phot.Res.* **51**: 61-70.

Genstat 5 Committee (1993). Genstat 5 release 3 reference manual. Oxford University Press, pp 796.

George, D.G. & Edward, R.W. (1973). *Daphnia* distributions within Langmuir circulations. *Limnol. Oceanogr.* **18**: 798-800.

George, D.G. & Edward, R.W. (1976). The effect of wind on the distribution of chlorophyll a and crustacean plankton in a shallow eutrophic reservoir. *J. Appl.Phycol.* **13:** 667-692.

Gibbs, G.W. (1973). Cycles of macrophytes and phytoplankton in Pukepuke Lagoon following a severe drought. *Proc. N. Z. Ecol. Soc.* **20**: 13-20.

Gilbert, J.J. (1966). Rotifer ecology and embryological induction. *Science* **151**: 1234-1237.

Gilbert, J.J. & Stemberger, R.S. (1984). *Asplanchna*-induced polymorphism in the rotifer *Keratella slacki. Limnol. Oceanogr.* **29**: 1309-1316.

Gliwicz, Z.M. & Guisande, C. (1992). Family planning in *Daphnia*: resistance to starvation in

offspring born to mothers grown at different food levels. *Oecologia* **91**: 463-467.

Goldman, J.C., Porcella, D.B., Middlebrooks, E.J. & Toerien, D.F. (1972). The effects of carbon on algal growth. *Wat. Res* .**6**: 637-679.

Goldman, J.C. & Carpenter, E.J. (1974). A kinetic approach to the effect of temperature on algal growth. *Limnol. Oceanogr.* **19**: 756-766.

Goldman, J.C., Oswald, W.J. & Jenkins, D. (1974). The kinetics of inorganic carbon limited algal growth. *J. WPCF* **46**: 554-574.

Goldman, J.C., McCarthy J.J. & Peavey, D.G. (1979). Growth rate influence on the chemical composition of phytoplankton in oceanic waters. *Nature* **279**: 310-315.

Gons, H.J (1977). On the light-limited growth of *Scenedesmus protuberans* Fritsch. *PhD-thesis*, University of Amsterdam, pp 120.

Gons, H.J & Mur, L.R (1975). An energy balance for algal populations in light-limiting conditions. *Verh. Int. Verein. Limnol.* **19**: 2729-2733.

Gool, E. van & Ringelberg, J. (1996). *Daphnids* respond to algae-associated odours *J. Plankton Res.* **18**: 197-202..

Goser, B & Ratte, H.T. (1994). Experimental evidence of negative interference in *Daphnia magna. Oecologia* **98**: 354-361.

Goser, B. (1997). Dichteabhängige Änderungen der Entwicklung und Reproduktion bei Cladoceren. *PhD-Thesis*, University of Aachen, ISBN 3-8265-2938-3, pp 210 (*In German*).

Goulden C.E., Henry L.L. & Tessier A.J. (1982). Body size, energy reserves, and competitive ability in three species of cladocera. *Ecology* **63**: 1780-1789.

Gould, S.J. (1997). Full house, the spread of excellence from Plato to Darwin. Three Rivers Press, pp 256.

Gould, S.J. (1999). The evolution of life on earth. http://www.geocities.com/capecanaveral/lab/2948/gould.html.

Goulder, R. (1972). Grazing by the ciliated protozoon *Loxodus magnus* on the alga *Scenedesmus* in a eutrophic pond. *Oikos* **23**:109-115.

Grant, J.W.E. & Bayly, I.A.G. (1981). Predator induction of crests in morphs of *Daphnia carinata* King complex. *Limnol. Oceanogr.* **26**: 201-218.

Groeger A.W., Schram M.D. & Marzolf G.R. (1991). Influence of food quality on growth and reproduction in *Daphnia. Freshwater Biol.* **26**: 11-19.

Gross, E.M., Meyer, H. & Schilling, G. (1996). Release and ecological impact of algicidal hydrolysable polyphenols in *Myriophyllum spicatum. Phytochemistry* **41**: 133-138.

Grover, J.P. (1989). Effects of Si:P supply ratio, supply variability, and selective grazing in the plankton: An experiment with natural algal and protistan assemblage. *Limnol. Ocenanogr.***34**: 349-367.

Guillard, R.R.L. & Lorenzen, C.J. (1972). Yellow-green algae with chlorophyllide c. *J. Phycol.* **8**:10-14.

Guisande, C. (1993). Reproductive strategy as population density varies in *Daphnia magna* (Cladocera). *Freshwater Biol.* **29**: 463-467.

Haardt, H. & Maske, H. (1987). Specific in vivo absorption coefficient of chlorophyll *a* at 675 nm. *Limnol. Oceanogr.* **32**: 608-619.

Halbach, U. (1969). Das zusammenwirken von Konkurrenz und Räuber-Beute-Beziehungen bei Rädertieren. *Verh. Zool. Ges: Zool. Anz. (Suppl.)* **33**: 72-79.

Hall, B.G. (1988). Adaptive evolution that requires multiple spontaneous mutations. *Genetics* **120**: 887-897.

Hallam, A. (1990). Great geological controversies. Oxford University press, Oxford, pp 244.

Han, T.-M. & Runnegar, B. (1992). Megascopic eukaryotic algae from the 2.1 billion-year-old Negaunee iron-formation. *Science* **257**: 232-235.

Hanazato, T. (1990). Induction of helmet development by a *Chaoborus* factor in *Daphnia ambigua* during juvenile stages. *J. Plankton Res.* **12**: 1287-1294.

Hanazato, T. (1991). Effects of a *Chaoborus*-released chemical on *Daphnia ambigua*-reduction in the tolerance of the *Daphnia* to summer water temperature. *Limnol. Oceanogr.* **36**: 165-171.

Haney J.F., Forsyth D.J. & James M.R. (1994). Inhibition of zooplankton filtering rates by dissolved inhibitors produced by naturally occurring cyanobacteria. *Arch. Hydrobiol.* **132**: 1-13.

Haney, J.F., Sasner, J. J. & Ikawa, M. (1995). Effects of products released by *Aphanizomenon flos-aquae* and purified saxitoxin on the movements of *Daphnia carinata* feeding appendages. *Limnol. Oceanogr.* **40**: 263-272.

Hansson, L-A. (1996). Behavioural response in plants: adjustment in algal recruitment induced by herbivores. *Proc. R. Soc. Lond.* B **263**: 1241-1244.

Harris, G.P. (1986). Phytoplankyon Ecology. Structure function and fluctuation. Chapman and Hall Ltd., 384 pp.

Harvell, C.D. (1984). Predator-induced defense in a marine bryozoan. *Science* **224**: 1357-1359.

Harvell, C.D. (1986). The ecology and evolution of inducible defenses in a marine bryozoan: cues, costs, and consequences. *Am. Nat.* **128**: 810-823.

Harvell, C.D. (1991). Coloniality and inducible polymorphism. *Am Nat.***138**: 1-14.

Harvell, C.D. (1992). Inducible defenses and allocation shifts in a marine bryozoan. *Ecology* **73**: 1567-1576.

Harvell, C.D. & Fenical, W. (1989). Chemical and structural defenses of Caribbean gorgonians (*Pseudopterogorgia* spp.): Intracolony localization of defense. *Limnol. Oceanogr.* **34**: 382-389.

Harvell, C.D. & Tollrian, R. (1999). Why inducible defenses? *In:* Tollrian, R. & Harvell, C.D. (eds.) *The ecology and evolution of inducible defenses.* Princeton University Press, New Jersey, pp 3-9.

Hassid W.Z. & Abraham S. (1957). Chemical procedures for analysis of polysaccharides. I.

Determination of glycogen and starch - Determination of glycogen with anthrone reagent. *In*: Golowick S.P. & Kaplan N.O. (eds.) "*Methods in Enzymology*", **3**, Aademic Press NY, 35-36.

Havel, J.E. (1985). Cyclomorphosis of *Daphnia pulex* spined morphs. *Limnol. Oceanogr.* **30**: 853-861.

Havel, J.E. (1987). Predator-induced defenses: a review. *In*: Kerfoot, W.C. & Sih, A. (eds.) *Predation: direct and indirect impacts on aquatic communities*. University Press New England, Hanover, NH, pp: 263-278.

Havel, J.E. & Dodson, S.I. (1987) Reproductive costs of *Chaoborus*-induced polymorphism in *Daphnia pulex*. *Hydrobiologia* **150**: 273-281.

Hebert, P.D.N. & Grewe, P.M. (1985). *Chaoborus*-induced shifts in the morphology of *Daphnia ambigua*. *Limnol. Oceanogr.* **30**: 1291-1297.

Hebert, P.D.N., Beaton, M.J., Schwartz, S.S. & Stanton, D.J. (1989). Polyphyletic origins of asexuality in *Daphnia pulex*. I. Breeding-system variation and levels of clonal diversity. *Evolution*, **43**, 1004-1015.

Hegewald, E. (1977). *Scenedesmus communis* Hegewald, a new species and its relation to *Scenedesmus quadricauda* (Turp.) Bréb. *Arch. Hydrobiol. Suppl.* **51**: 142-155.

Hegewald, E. (1982). Taxonomic-morphological studies of *Scenedesmus* isolates from culture collections. *Arch. Hydrobiol. (Suppl. 60) Algolog. Stud.* **29**: 375-406.

Hegewald, E. & Silva, P. (1988). An annotated catalogue of *Scenedesmus* and nomenclaturally related genera including original descriptions and figures. *Bibl. Phycol.* **80**: 1–587.

Helgen, J.C. (1987). Feeding rate inhibition in crowded *Daphnia pulex*. *Hydrobiologia* **154**: 113-119.

Hessen, D.O. (1992). Nutrient element limitation of zooplankton production. *Am. Nat.***140**: 799-814.

Hessen, D.O. & Van Donk, E. (1993). Morphological changes in *Scenedesmus* induced by substances released from *Daphnia*. *Arch. Hydrobiol.* **127**: 129-140.

Hickey, D.A. (1993). Molecular symbionts and the evolution of the sex. *J. Heredity* **84**: 410-414.

Hobæk, A. & Larsson, P. (1990). Sex determination in *Daphnia magna*. *Ecology* **71**: 2255-2268.

Hofstraat, J.W., Peeters, J.H.C., Snel, J.H.F. & Geel, C. (1994). Simple determination of photosynthetic efficiency and photoinhibition of *Dunaliella tertiolecta* by saturating pulse fluorescence measurements. *Mar. Ecol. Prog. Ser.* **103**: 187-196.

Holm, N.P., Ganf, G.G., Shapiro, J. (1983). Feeding and assimilation rates of *Daphnia pulex* fed *Aphanizomenon flos-aqua*. *Limnol. Oceanogr.* **28**: 677-687.

Holtmann, T. & Hegewald, E. (1986). The influence of nutrient solutions on the variability of the genus *Scenedesmus* subgenus *Acutodesmus*. *Arch. Hydrobiol. (suppl. 73) Algolog. Stud.* **44**: 365-380.

Horn, W. (1981). Phytoplankton losses due to zooplankton grazing in a drinking water reservoir. *Int. Revue ges. Hydrobiol.* **66**: 787-810.

Howe, N.R. & Harris, L.G. (1978). Transfer of the sea anemone pheromone, anthopleurine, by the nudibranch *Aelidia papilosa*. *J. Chem. Ecol.* **4**: 551-561.

Hutchinson, G.E. (1961). The paradox of the plankton. *Am. Nat.* **95**: 137-145.

Irvine, K. (1989). The spatial distribution of zooplankton populations in the presence and absence of fish. *Hydrobiol. Bull.* **23**: 169-178.

Jablonka, E. (1994). Inheritance systems and the evolution of new levels of individuality. *J. theor. Biol.* **170**: 301-309.

Jachner, A. (1995). Chemically induced habitat shift in bleak (*Alburnus alburnus* (L.)). *Arch. Hydrobiol.* **133**:71-79.

Jachner, A. (1996). Alarm reaction in bleak (*Alburnus alburnus* (L.), *Cyprinidae*) in response to chemical stimuli from injured conspecifics. *Hydrobiologia* **325**: 151-155.

Jachner, A. (1997). The response of bleak to predator odour of unfed and recently fed pike. *J. Fish Biol.* **50**: 878-886.

Jakobsen, P.J. & Johnson, G.H. (1987). Behavioural response of the waterflea *Daphnia pulex* to a gradient in food concentration. *Anim. Behav.* **35:** 1891-1895.

Jakobsen, P.J. & Johnson, G.H. (1988a). Size-specific protection against predation by fish in swarming waterfleas, *Bosmina longispina*. *Anim. Behav.* **36:** 986-990.

Jakobsen, P.J. & Johnson, G.H. (1988b). The influence of food limitation on swarming behaviour in the waterflea *Bosmina longispina*. *Anim. Behav.* **36:** 991-995.

Jeffries, M, (1990). Evidence of induced defences in a pondweed. *Freshwater Biol.* **23**: 265-269.

Jollos, V. (1921). Experimentelle Protistenstudien. *Arch. Protistenkd.* **43**: 1-22.

Kats, L.B., Petranka, J.W. & Sih, A. (1988). Antipredator defenses and the persistence of amphibian larvae with fishes. *Ecology* **69**: 1865-1870.

Keefe, M.L. (1993). Chemically mediated avoidance behavior in wild brook trout, *Salvelinus fontinalis*: the response to familiar and unfamiliar predaceous fishes and the influence of fish diet. *Can J. Zool.* **70**: 288-292.

Kerfoot, W.C. (1987). Cascading effects and indirect pathways. *In* Kerfoot, W.C. & Sih, A. (eds.) *Predation. Direct and indirect impacts on aquatic communities*. Univ. Press of New England, pp 57-70.

Kersting, K. (1991). Recycling of food by *Daphnia magna* Straus (Cladocera): a possible role in swarming. *Hydrobiol. Bull.* **25**: 73-75.

Kessler, E. (1991). *Scenedesmus*: Problems of a highly variable genus of green algae. *Bot. Acta* **104**: 169-171.

Kessler, E., Schäfer, M., Hümmer, C., Kloboucek, A. & Huss, V.A.R. (1997). Physiological, biochemical, and molecular characters for the taxonomy of the subgenera of *Scenedesmus* (Chlorococcales, Chlorophyta). *Bot. Acta* **110**: 244-250.

Kilham, S.S., Kreeger, D.A., Goulden, C.E. & Lynn, S.G. (1997). Effects of nutrient limitation on biochemical constituents of *Ankistrodesmus falcatus*. *Freshwater Biol.* **38**: 591-596.

Kilham, S.S., Goulden, C.E., Lynn, S.G., Kreeger, D.A. & Herrera, L. (1999) COMBO: A defined freshwater culture medium for algae and zooplankton. *Hydrobiologia* **377**: 147-159.

Kirk, J.T.O. (1994). *Light and photosynthesis in aquatic systems*. Cambridge University Press,

Cambridge.

Kleiven, O.T., Larsson, P. & Hobæk, A. (1992). Sexual reproduction in *Daphnia magna* requires three stimuli. *Oikos* **65**: 197-206.

Kleiven, O.T., Larsson, P. & Hobæk, A. (1996). Direct distributional response in *Daphnia pulex* to a predator kairomone. *J. Plankton Res.* **18:** 1341-1348.

Kogan, S.I., Chinnova, G.A. & Kravchenko, M.E. (1972). The effect of macrophytes on certain algae in joint cultivation. *Izv. Akad. Nauk.Turkm. SSR Ser. Biol. Nauk.***3**: 3-8.

Kolber, Z. & Falkowski, P.G. (1993). Use of active fluorescence to estimate phytoplankton photosynthesis *in situ. Limnol. Oceanogr.* **38**: 1646-1665.

Komárek, J. & Ružička, J. (1969). Effect of temperature on growth and variability of *Scenedesmus quadricauda* (Turp.) Breb. *In* Fott, B. [ed.] *Studies in Phycology*. Stuttgart. pp 262-292.

Kondrasov, A.S. (1993). Classification of hypotheses on the advantages of amphimixis. *J. Heredity* **84**: 372-387.

Krienitz, K. (1987). Studies to the morphology and taxonomy of the subgenus *Acutodesmus* (Chlorellales). *Arch. Hydrobiol. suppl. (Suppl. 78) Algolog. Stud.* **46**: 1-37.

Kuhlmann, H.-W. & Heckmann, K. (1985). Interspecific morphogens regulating prey-predator relationships in protozoa. *Science* **227**: 1347-1349.

Kuhlmann, H.-W. & Heckmann, K. (1994). Predation risk of typical ovoid and "winged" morphs of *Euplotes* (Protozoa, Ciliophora). *Hydrobiologia* **284**: 219-227.

Kühn, R. & Pattard, M. (1990). Results of the harmful effects of water pollutants to green algae (*Scenedesmus subspicatus*) in the cell multiplication inhibition test. *Wat. Res.***24**: 31-38.

Kusch, J. (1993). Induction of defensive morphological changes in ciliates. *Oecologia* **94**: 571-575.

Kusch, J. & Kuhlmann, H.-W. (1994). Cost of *Stenostomum*-induced morphological defence in the ciliate *Euplotes octocarinatus. Arch. Hydrobiol.* **130**: 257-267.

Kvam, O.V. & Kleiven, O.T. (1995). Diel horizontal migration and swarm formation in *Daphnia* in response to *Chaoborus. Hydrobiologia* **307**: 177-184.

Kylin, A. & Das, G. (1967). Calcium and strontium as micronutrients and morphogenetic factors for *Scenedesmus*. *Phycologia* **6**: 201-210.

Lake, J.A. (1988). Origin of the eukaryotic nucleus determined by rate-invariant analysis of rRNA sequences. *Nature* **331**: 184-186.

Lampert, W. (1977). Studies on the carbon balance of *Daphnia pulex* De Geer as related to environmental conditions. II. The dependence of carbon assimilation on animal size, temperature, food concentration and diet species. *Archiv. Hydrobiol. Suppl.* **48**: 310-355.

Lampert, W. (1978). Release of dissolved organic carbon by grazing zooplankton. *Limnol. Oceanogr.* **23**: 831-834.

Lampert, W. (1981). Toxicity of the blue-green *Microcystis aeruginosa*: Effective defence mechanism against grazing pressure by *Daphnia. Verh. int. Ver. Limnol.* **21**: 1436-1440.

Lampert W. (1987). Feeding and nutrition in *Daphnia*. In: Peters R.H. & De Bernardi R. (eds.),

"*Daphnia*" *memorie dell'Istituto Italiano di Idrobiologia* **45**: 143-192.

Lampert, W., Rothhaupt, K.O. & von Elert, E. (1994). Chemical induction of colony formation in a green alga (*Scenedesmus acutus*) by grazers (*Daphnia*). *Limnol. Oceanogr.* **39**: 1543-1550.

Larsson, P. & Dodson, S.I. (1993). Invited review - chemical communication in planktonic animals. *Arch. Hydrobiol.* **129**: 129-155.

Laurén-Määttä, C., Hietala, J. & Walls, M. (1997). Responses of *Daphnia pulex* populations to toxic cyanobacteria. *Freshwater Biol.* **37**: 635-647.

Lauridsen T.L. & Lodge D.M. (1996). Avoidance by *Daphnia magna* of fish and macrophytes: Chemical cues and predator-mediated use of macrophyte habitat. *Limnol. Oceanogr.* **41**: 794-798.

Lawton, L. A., Edwards, C. & Codd, G.A. (1994). Extraction and high-performance liquid chromatographic method for the determination of microcystins in raw and treated waters. *Analyst* **119**: 1525-1530.

Lehman, J.T. (1980). Release and cycling of nutrients between planktonic algae and herbivores. *Limnol. Oceanogr.* **25**: 620-632.

Lehman, J.T. (1988). Selective herbivory and its role in the evolution of phytoplankton growth strategies. *In* Sandgren, C.D. (ed.) *Growth and reproductive strategies of freshwater phytoplankton*. Cambridge Univ. Press, pp 369-387.

Levitan, C. (1987). Formal stability analysis of a planktonic freshwater community. *In* Kerfoot, W.C. & Sih, A. (eds.) *Predation. Direct and indirect impacts on aquatic communities*. Univ. Press of New England, pp 71-100.

Liley, N.R. (1982). Chemical communication in fish. *Can. J. Fish. Aquat. Sci.* **39**: 22-35.

Lively, C.M. (1986). Competition, comparitive life histories, and maintenance of shell dimorphism in a barnacle. *Ecology* **67**: 858-864.

Logan, G.A., Hayes, J.M., Hieshima, G.B. & Summons, R.E. (1995). Terminal Proterozoic reorganization of biochemical cycles. *Nature* **376**: 53-56.

Loose, C. J., von Elert, E. & Dawidowicz, P. (1993). Chemically-induced diel vertical migration in *Daphnia* - a new bioassay for kairomones exuded by fish. *Arch. Hydrobiol.* **126**: 329-337.

Lowry O.H., Rosebrough W.J., Farr A.L. & Randall R.J. (1951). Protein measurement with Folin phenol reagent. *J. Biol. Chem.* **193**: 265-275.

Lukavský, J. (1991). Motile cells in *Scenedesmus obliquus* in outdoor mass culture. *Arch. Protistenkd.* **140**: 345-348.

Lüning J. (1992). Phenotypic plasticity of *Daphnia pulex* in the presence of invertebrate predators: morphological and life history responses. *Oecologia* **92**: 383-390.

Lürling, M. (1998). Effect of grazing-associated infochemicals on growth and morphological development in *Scenedesmus acutus* Meyen (Chlorophyceae). *J. Phycol.* **34**: 578-586.

Lürling, M. (1999). Grazer-induced coenobia formation in clonal cultures of *Scenedesmus obliquus* (Chlorococcales; Chlorophyceae). *J. Phycol.* **35**: 19-23.

Lürling, M. & Van Donk, E. (1996). Zooplankton-induced unicell-colony transformation in *Scenedesmus acutus* and its effect on growth of herbivore *Daphnia*. *Oecologia* **108**: 432-437.

Lürling, M. & Van Donk, E. (1997a). Life history consequences for *Daphnia pulex* feeding on nutrient limited phytoplankton. *Freshwater Biol.***38**: 693-709.

Lürling, M. & Van Donk, E. (1997b). Morphological changes in *Scenedesmus* induced by infochemicals released in situ from zooplankton grazers. *Limnol. Oceanogr.* **42**: 783-788.

Lürling, M. De Lange, H.J.& Van Donk, E. (1997). Changes in food quality of the green alga *Scenedesmus* induced by *Daphnia* infochemicals: biochemical composition and morphology. *Freshwater Biol.* **38**: 619-628.

Lürling, M. & Beekman, W. 1999. Grazer-induced defenses in *Scenedesmus* (Chlorococcales; Chlorophyceae): coenobium and spine formation. *Phycologia* **xx**: xxx-xxx.

Lürling, M. & Beekman, W. *Subm.* Filter-extractables (detergents) induce morphological changes in the green alga *Scenedesmus* (Chlorophyceae). *Limnol.Oceanogr.* **xx**: xxx-xxx.

Lynch, M. (1980). *Aphanizomenon* blooms: Alternate control and cultivation by *Daphnia pulex*. *In*: Kerfoot, W.C. (ed.) *Evolution and ecology of zooplankton communities*. University Press of New England, Hanover, pp 299-304.

Lynch, M. (1989). The life history consequences of resource depression in *Daphnia pulex*. *Ecology* **70:** 246-256.

MacArthur, R.H. (1960). On the relative abundance of species. *Am. Nat.* **94**: 25-36.

Macháček, J. (1993). Comparison of the response of *Daphnia galeata* and *Daphnia obtusa* to fish-produced chemical substance. *Limnol. Oceanogr.* **38**: 1544-1550.

Magurran, A.E. (1989). Acquired recognition of predator odor in the european minnow (*Phoxinus* phoxinus). Ethology **82**: 216-223.

Malej, A. & Harris, G.P. (1993). Inhibition of copepod grazing by diatom exudates- a factor in the development of mucus aggregates. *Mar. Ecol. Progr. Ser.* **96**: 33-42.

Malone, B.J. & McQueen, D.J. (1983). Horizontal patchiness in zooplankton populations in two Ontario kettle lakes. *Hydrobiologia* **99:** 101-124.

Marčenko, E. (1973). On the nature of bristles in *Scenedesmus Arch. Mikrobiol.* **88**: 153-161.

Margulis, L. (1993). Symbiosis in cell evolution. Freeman, W.H. New York, 452 pp.

Massalski, A, Trainor, F.R. &. Shubert., E. (1971). Wall ultrastructure of *Scenedesmus* culture N46. *rch. Microbiol.* **96**: 145-153.

Mathis, A. & Smith, R.J.F. (1993). Intraspecific and cross-superorder response to chemical alarm signal by brook stickleback. *Ecology* **74**: 2395-2404.

Mathis, A., Chivers, D.P. & Smith, R.J.F. (1993). Population differences in responses of fathead minnows (*Pimephales promelas*) to visual and chemical stimuli from predators. *Ethology* **93**: 31-40.

Mattoni, R.H.T., Keller, E.C. Jr. & Myrick, H.N. (1965). Industrial photosynthesis. A means to a beginning. *Bioscience* **15**: 403-407

Matveev, V. (1993). An investigation of allelopathic effects of *Daphnia*. *Freshwater Biol.* **29:** 99-105.

Maynard Smith, J. (1990). Models of a dual inheritance system. *J. theor. Biol.* **143**: 41-53.

Maynard Smith, J. (1997). The theory of evolution, 3rd edition, Cambridge University Press, 354 pp.

McCauley E. & Downing J.A. (1985). The prediction of cladoceran grazing spectra. *Limnol. Oceanogr.* **30**: 202-212.

McCollum, S.A. & Vanbuskirk, J. (1996). Costs and benefits of a predator-induced polyphenism in the gray treefrog *Hyla chrysoscelis*. *Evolution* **50**: 583-593.

Meyer E. & Walther A. (1988). Metods for the estimation of protein, lipid, carbohydrate and chitin levels in fresh water invertebrates. *Arch. Hydrobiol.* **113**: 161-177.

Meyer, J.S., Ingersoll, C.G., McDonald, L.L. & Boyce, M.S. (1986). Estimating uncertainty in population growth rates: jackknife vs. bootstrap techniques. *Ecology* **67**: 1156-1166.

Mikheeva, T.M. & Kruchkova, H.M. (1980). Morphological changes in *Chlamydomonas sp* and *Scenedesmus acuminatus* in the presence of zooplankton. *Botanica* **5**: 60-63 (*In Russian*).

Milinski, M. (1979). Can an experienced predator overcome the confusion of swarming prey more easily? *Anim. Behav.* **27:** 1122-1126.

Mitchell S.F., Trainor F.R., Rich P.H. & Goulden C.E. (1992). Growth of *Daphnia magna* in the laboratory in realtion to the nutritional state of its food species, *Chlamydomonas reinhardtii*. *J. Plankton Res.***14**: 379-391.

Mladenov, R. & Furnadzieva, S. (1995). Morphological variability of the clonal cultures of *Scenedesmus falcatus* Chod. and *Scenedesmus acuminatus* (Lagerh.) Chod. *Arch. Hydrobiol. (Suppl. 111) Algolog. Stud.* **79**: 93-107.

Mladenov, R. & Furnadzieva, S. (1997). Morphological variability of the clonal cultures of *Scenedesmus pectinatus* Meyen. *Arch Hydrobiol. (Suppl. 118) Algolog. Stud.* **84**: 113-121.

Morgan, K.C. & Kalff, J. (1975). The winter dark survival of an algal flagellate *Cryptomonas erosa* (Skuja). *Verh. Internat. Verein. Limnol.* **19**: 2734-2740.

Morgan, K.C. & Kalff, J. (1979). Effect of light and temperature interactions on growth of *Cryptomonas erosa* (Cryptophyceae). *J. Phycol.* **15**: 127-134.

Muck, P & Lampert, W. (1984). An experimental study on the importance of food conditions for the relative abundance of calanoid copepods and cladocerans. *Arch. Hydrobiol./Suppl.* **66**: 157-179.

Müller, W.F.G. (1997). Evolution of protozoa to metazoa. *Theory Bioscienc.***116**: 145-168.

Müller-Navarra, D.C. (1995a). Evidence that a highly unsaturated fatty acid limits *Daphnia* growth in nature. *Arch. Hydrobiol.***132**: 297-307.

Müller-Navarra, D.C. (1995b). Biochemical versus mineral limitation in *Daphnia*. *Limnol. Oceanogr.* **40**: 1209-1214.

Mur, L.R. (1971). *Scenedesmus* in brak water (in *Dutch*) PhD-thesis, University of Amsterdam 167 pp.

Myklestad, S. (1977). Production of carbohydrates by marine diatoms. II. Influence of the N/P ratio in

the growth medium on the assimilation ratio, growth rate and production of cellular and extracellular carbohydrates by *Chaetocerus affinis* var. *willei* (Gran) Hustedt and *Skeletonema costatum* (Grev.) Cleve. *J. Exp. Mar.Biol.Ecol.* **29**: 161-179.

Nagy-tóth, F. (1964). Contributii la cunosterea variatiei talurilor de *Scenedesmus quadrispina* Chod. sub influenta substantelor stimulatoare de crestere. *Contrib. Bot.* Cluj pp. 321-328.

Nagy-tóth, F., Péterfi, L.I. & Fodorpataki, L. (1992). Effects of carbon sources on the morphology and structure of *Scenedesmus acutus* Meyen. *Acta Bot. Hung.* **37**: 295-316.

Neary, J., Cash, K. & McCauley, E. (1994). Behavioural aggregation of *Daphnia pulex* in response to food gradients. *Funct. Ecol.* **8**: 377-383.

Nilshammar, M. & Walles, B. (1974). Electron microscope studies on cell differentiation in synchronized cultures of the green alga *Scenedesmus*. *Protoplasma* **79**: 317-332.

Nygaard, K. & Hessen, D.O. (1994). Diatom kills by flagellates. *Nature*: **367**: 520.

Ojala, A. (1993). Effects of temperature and irradiance on the growth of two freshwater photosynthetic cryptophytes. *J. Phycol.* **29**: 278-284.

Ostrofsky M.L., Jacobs F.G. & Rowan J. (1983). Evidence for the production of extracellular herbivore deterrents by *Anabaena flos-aqua*. *Freshwater Biol.***13**: 501-506.

Otten, J.H. & Willemse, M.T.M. (1988). First steps to periphyton. *Arch. Hydrobiol.* **112**: 177-195.

Overbeck, J. & Stange-Bursche, M.-E. (1966). Experimentelle Untersuchungen zum Coenobienform wechsel von *Scenedesmus quadricauda* (Turp.) Bréb. *Ber. Deutsch. Bot. Ges.* **78**: 357-372.

Palmer, A.R. (1985). Quantum changes in gastropod shell morphology need not reflect speciation. *Evolution* **39**: 699-705.

Parejko, K & Dodson, S.I. (1990). Progress towards characterization of a predator-prey kairomone: *Daphnia pulex* and *Chaoborus americanus*. *Hydrobiologia* **198**: 51-59

Pennak, R.W. (1973). Some evidence for aquatic macrophytes as repellents for a limnetic species of *Daphnia*. *Int. Rev. ges. Hydrobiol.* **58**: 569-576.

Pérasso, R., Baroin, A., Qu, L.H., bachellerie, J.P. & Adoutte, A. (1989). Origin of the algae. *Nature* **339**: 142-144.

Perrin, N. (1989). Population density and offspring size in the cladoceran *Simocephalus vetulus* (Müller). *Funct. Ecol.* **3**: 29-36.

Peters, R.H. (1984). Methods for the study of feeding, grazing and assimilation by zooplankton. *In:* Downing, J.A. & Rigler, F.H. (eds.). *A manual on methods for the assessment of secondary production in fresh waters.* IBP Handbook **17**, 2nd edition, Blackwell, Oxford, pp 336-412.

Peters, R.H. (1987). Metabolism in *Daphnia*. *In:* Peters, R.H. & de Benardi, R. (eds.) *Daphnia. Mem. Ist. Ital. Idrobiol.* **45**: 193-243.

Peters, R.H. & Rigler, F.H. (1973). Phosphorus release by *Daphnia*. *Limnol. Oceanogr.* **18**: 821-839.

Peters-Regehr, T., Kusch, J. & Heckmann, K. (1997). Primary structure and origin of a predator released protein that induces defensive morphological changes in *Euplotes*. *Eur. J. Protistol.* **33**: 389-395.

Petterson, L.B. & Brönmark, C. (1997). Density-dependent costs of an inducible morphological defense in crucian carp. *Ecology* **78**: 1805-1815.

Pfeiffer, W. (1974). Pheromones in fish and amphibia. *In:* Birch, M.C. (ed.) *Pheromones*, North Holland Publishing Co., Amsterdam, pp 269-296.

Pianka, E.R. (1983). *Evolutionary ecology*. Harper & Row, New York.

Pijanowska, J. (1994). Fish enhancement patchiness in *Daphnia* distribution. *Verh.int. Ver.Limnol.* **25:** 2366-2368.

Pijanowska, J. (1997). Alarm signals in *Daphnia*? *Oecologia* **112**: 12-16.

Piorreck M., Baasch K-H. & Pohl P. (1984). Biomass production, total protein, chlorophylls, lipids and fatty acids of freswater green and blue-green algae under different nitrogen regimes. *Phytochemistry* **23**: 207-216.

Porter, K.G. (1973). Selective grazing and differential digestion of algae by zooplankton. *Nature* **244**: 179-180.

Porter, K.G. (1975). Viable gut passage of gelatinous green algae ingested by *Daphnia.Verh. Int.Ver.Limnol.* **19**: 2840-2850.

Porter, K.G. (1976). Enhancement of algal growth and productivity by grazing zooplankton. *Science* **192**: 1332-1334.

Porter, K.G. (1977). The plant-animal interface in freshwater ecosystems. *Amer. Scient.* **65**: 159-170.

Porter, K.G., Gerritsen, J. & Orcutt, J.D. (1982). The effect of food concentration on swimming patterns, feeding behavior, ingestion, assimilation, and respiration by *Daphnia. Limnol. Oceanogr.* **27**: 935-949.

Porter K.G., Gerritsen J. & Orcutt Jr J.D. (1983). Functional response and fitness in a generalist filter feeder, *Daphnia magna* (Cladocera, Crustaceae). *Ecology* **64**: 735-742.

Portielje, R. & Lijklema, L. (1995). Carbon dioxide fluxes across the air-water interface and its impact on carbon availability in aquatic systems. *Limnol. Oceanogr.* **40**: 690-699.

Ramos-Cárdenas, A. & de Lara-Isassi, G. (1985). The effect of nutrients in the development of algal populations (*Scenedesmus spp.*). *Arch. Hydrobiol.Beih. Ergebn. Limnol.* **20**: 63-69.

Ratzlaff, W. (1974). Swarming in *Moina affinis*. *Limnol. Oceanogr.* **19:** 993-994.

Rausch, H., Larsen, N. & Schmitt, R. (1989). Phylogenetic relationships of the green alga *Volvox carteri* deduced from small-subunit ribosomal RNA comparisons. *J. Molec. Evol.* **29**: 255-265.

Reed, J.R. (1969). Alarm substances and fright reaction in fishes. *Trans. Amer. Fish. Soc.* **4**: 664-668.

Reede T. (1995). Life history shifts in response to different levels of fish kairomones in *Daphnia*. *J. Plankton Res.***17**: 1661-1667.

Reinikainen, M., Ketola, M. & Walls, M. (1994). Effects of the concentration of toxic *Microcystis aeruginosa* and an alternative food on the survival of *Daphnia pulex*. *Limnol. Oceanogr.* **39**: 424-432.

Reinikainen, M., Ketola, M., Jantunen, M. & Walls, M. (1995) Effects of *Microcystis aeruginosa*

exposure and nutritional status on the reproduction of *Daphnia pulex*. *J. Plankton Res.* **17**:431-436.

Reynolds, C.S. & Wiseman, S.W. (1982). Sinking losses of phytoplankton in closed limnetic systems. *J. Plankton Res.***4**: 489-522.

Reynolds, C.S., Morison, H.R. & Butterwick, C. (1982). The sedimentary flux of phytoplankton in the south basin of Windemere. *Limnol. Oceanogr.* **27**: 1162-1175.

Reynolds, C.S. (1984). The ecology of freshwater phytoplankton. Cambridge, 384 pp.

Rhee, G-Y. & Gotham, I.J. (1981). The effect of environmental factors on phytoplankton growth: Temperature and the interaction of temperature with nutrient limitation. *Limnol. Oceanogr.* **26**: 635-648.

Rhoades, D.F. (1979). Evolution of plant chemical defenses. *In*: Rosenthal, G.A. & Janzen, D.H. (eds.) *Herbivores: Their interaction with secundary metabolites*. Academic press, New York, pp 3-54.

Riessen, H.P. (1984). The other side of cyclomorphosis: Why *Daphnia* lose their helmets? *Limnol. Oceanogr.* **29**: 1123-1127.

Riessen, H.P. (1992). Cost-benefit model for the induction of an anti-predator defense. *Am. Nat.* **140**: 349-362.

Ringelberg, J. (1987). Light induced behaviour in *Daphnia*. In:Peters R.H. and R. De Bernardi (Eds) "*Daphnia*". *Mem. Ist. Ital. Idrobiol.* **45**: 285-323.

Rothhaupt, K.O. (1990). Population growth rates of two closely related rotifer species: effects of food quantity, particle size and nutritional quality. *Freshwater Biol.* **23**: 561-570.

Rothhaupt, K.O. (1995). Algal nutrient limitation affects rotifer growth rate but not ingestion rate. *Limnol. Oceanogr.* **40**: 1201-1208.

Ruvinsky, A.O. (1990). Sex, evolution and origin of multicellularity. *In* Hoshi, M & Yamashita, O (eds.) *Advances in invertebrate reproduction* 5, Amsterdam, Elsevier Science Publishers, pp 55-60.

Ruvinsky, A.O. (1997). Sex, meiosis and multicellularity. *Acta Biotheoretica* **45**: 127-141.

Ryther, J.H. (1954). Inhibitory effects of phytoplankton upon the feeding of *Daphnia magna* with reference to growth, reproduction and survival. *Ecology* **35**: 522-533.

Saenz, M.E., Accorinti, J. & Tortorelli, M.C. (1993). Toxicity of paraquat to a green alga *Scenedesmus acutus*. *J. Environ. Sci. Health B* **28**: 193-204.

Sakwińska, O. (1998). Plasticity of *Daphnia magna* life history traits in response to temperature and information about a predator. *Freshwater Biol.* **39**: 681-687.

Sandgren, C.D. (1988). The ecology of chrysophyte flagellates: their growth and perennation strategies as freshwater phytoplankton. *In:* Sandgren, C.D. (ed.). *Growth and reproductive strategies of freshwater phytoplankton*. Cambridge University Press, Cambridge, pp 442.

Sathyendranath, S., Lazzara, L. & Prieur, L. (1987). Variations in the spectral values of specific

absorption of phytoplankton. *Limnol. Oceanogr.* **32**:

Schaffer, W.N. & Rosenzweig, M.L. (1978). Homage to the Red Queen. I. Coevolution of predators and their victims. *Theor. Pop. Biol.* **14**: 135-157.

Scheffer, M., Hosper, H.S., Meijer, M.-L., Moss, B. & Jeppesen, E. (1993). Alternative equilibria in shallow lakes. *Trends Ecol. Evol.* **8**: 275-279.

Schindler, D.W. (1971). Carbon, nitrogen and phosphorus and the eutrophication of freshwater lakes. *J. Phycol.* **7**: 321-329.

Schindler, D.W., Brunskill, G.J. & Emerson, S. (1972). Aymospheric carbon dioxide: its role in maintaining phytoplankton standing crops. *Science* **177**: 1192-1194.

Schlegel, M. (1994). Molecular phylogeny of eukaryotes. *Trends ecol. Evol.* **9**: 330-335.

Schopf, W.J., Hayes, J.M. & Walter, M.R. (1983). Evolution of earth's earliest ecosystems: recent progress and unsolved problems. *In*: Schopf, W.J. (ed.). *Earth's earliest biosphere: its origin and evolution.* Princeton University Press, Princeton, pp 361-384.

Schopf, W.J. (1994). The early evolution of life: solution to Darwin's dilemma. *Trends ecol. Evol.* **9**: 375-377.

Schlichting, C.D. (1989). Phenotypic integration and environmental change. *Bioscience*, **39**: 460-464.

Schreiber, U., Neubauer, C. & Schliwa, U. (1993). PAM fluorometer based on medium frequency pulsed Xe-flash measuring light: a highly sensitive new tool in basic and applied photosynthesis research. *Photosynth. Res.* **36:** 65-72.

Seitz, A. (1984). Are there allelopathic interactions in zooplankton? Laboratory experiments with *Daphnia*. *Oecologia* **62**: 94-96.

Senger, H. & Fleischhacker, P. (1978). Adaptation of the photosynthetic apparatus of *Scenedesmus obliquus* to strong and weak light conditions. I. Differences in pigments, photosynthetic capacity, quantum yield and dark reactions. *Physiol. Plant.* **43**: 35-42.

Šetlík, I., Berková, E., Doucha, J., Kubín, Š., Vendlová, J. & Zachleder, V. (1972). The coupling of synthetic and repproduction processes in *Scenedesmus quadricauda*. *Algol. Stud.* **7**: 172-213.

Sheldrake, R. (1991). *The rebirth of nature. The greening of science and God.* Century, London, 330p.

Shubert, E. L. & Trainor, F.R. (1974). *Scenedesmus* morphogenesis. Control of the unicell stage with phosphorus. *Br. Phycol. J.* **9**: 1-7.

Simpson, G.G. (1953). *The major features of evolution*. Columbia University Press, New York.

Sih, A. & Kats, L.B. (1994). Age, experience, and the response of streamside salamander hatchlings to chemical cues from predatory sunfish. *Ethology* **96**: 253-259.

Siver, P & Trainor F.R. (1981). Morphological control and physiology of *Scenedesmus* strain 170. *Phycologia* **20**: 1-11.

Siver, P & Trainor F.R. (1983). Effect of growth rate on unicell production in two strains of *Scenedesmus* (Chlorophyta). *Phycologia* **22**: 127-131.

Siver, P.A. & Freeda, S.J. (1982). The interaction of growth rate and cell cycle on the number of cells in a *Scenedesmus* coenobium. *Proc. Penn. Ac. Sci.* **56**: 133-137.

Skelly, D.K. (1992). Field evidence for a cost of behavioral antipredator response in a larval amphibian. *Ecology* **73**: 704-708.

Skelly, D.K. & Werner, E.E. (1990). Behavioral and life-historical responses of larval American toads to an odonate predator. *Ecology* **71**: 2313-2322.

Skulberg, O.M. & Skulberg, R. (1990). Research with algal cultures. NIVA's culture collection of algae. *NIVA-report* ISBN 82-551743-6.

Smayda, T.J. (1970). The suspension and sinking of phytoplankton in the sea. *Oceanogr. Mar. Biol. Ann. Rev.* **8**: 353-414.

Smayda, T.J. (1974). Some experiments on the sinking characteristics of two freshwater diatoms. *Limnol. Oceanogr.***19**: 628-635.

Smith, R.E.H. & Kalff, J. (1982). Size-dependent phosphorus uptake kinetics and cell quota in phytoplankton. *J. Phycol.* **18**: 275-284.

Snell, T.W. & Morris, P.D. (1993). Sexual communication in copepods and rotifers. *Hydrobiologia* **255/256**: 109-116.

Sokal, R.R. & Rohlf, F.J. (1995). Biometry: the principles and practice of statistics in biological research, 3rd Edition. W.C. Freeman and Co., New York, 887 pp.

Sommer, U. (1988). Phytoplankton succession in microcosm experiments under simultaneous grazing pressure and resource limitation. *Limnol. Oceanogr.* **33**: 1037-1054

Sommer, U. (1989). Nutrient status and nutrient competition of phytoplankton in a shallow, hypertrophic lake. *Limnol. Oceanogr.* **34**: 1162-1173.

Sommer, U., Gliwicz, Z.M., Lampert, W. & Duncan, A. (1986). The PEG-model of seasonal succession of planktonic events in fresh waters. *Arch. Hydrobiol.* **106**: 433-471.

Spaak, P. (1995). Sexual reproduction in *Daphnia*: interspecific differences in a hybrid species complex. *Oecologia* **104**: 501-507.

Spitze, K. (1992). Predator-mediated plasticity of prey life history and morphology: *Chaoborus americanus* predation on *Daphnia pulex*. *Am. Nat.* **139**: 229-247.

Stanley, S.M. (1973). An ecological theory for the sudden origin of multicellular life in the Late Precambrian. *PNAS* **70**: 1486-1489.

Stearns, S.C. (1992). The evolution of life histories. Oxford University Press, Oxford, pp 249.

Steenbergen, C.L.M. (1975). Light-dependent morphogenesis of unicellular stages in synchronized cultures of *Scenedesmus quadricauda* (Turp.) Bréb. (Chlorophyceae). *Acta Bot. Neerl.* **24**: 391-396.

Steenbergen, C.L.M. (1978). Pleomorphism of *Scenedesmus quadricauda* (Turp.) Bréb. (Chlorophyceae) in synchronized cultures. *Mitt. Int. Verein. Limnol.* **21**: 216-223.

Steenbergen, C.L.M. & Bär-Gilissen, M.J.B. (1977). Nuclear divisions and cytokinesis during unicell and coenobia formation in synchronized cultures of *Scenedesmus quadricauda* (Turp.) Breb. (Chlorophyceae). *Acta Bot. Neerl.* **26**:177-182.

Steinberg, S., Dicke, M. & Vet, L.E.M. (1993). Relative importance of infochemicals from first and second trophic level in long-range host location by the larval parasitoid *Cotesia glomerata*. *J. Chem. Ecol.* **19**: 47-59.

Stemberger, R.S. (1988). Reproductive costs and hydrodynamic benefits of chemically induced defenses in *Keratella testudo*. *Limnol. Oceanogr.* **33**: 593-606.

Stemberger, R.S. (1990). Foood limitation, spination, and reproduction in *Brachionus calyciflorus*. *Limnol. Oceanogr.***35**: 33-44.

Stemberger, R.S. & Gilbert, J.J. (1984). Spine development in the rotifer *Keratella cochlearis*. Induction by cyclopoid copepods and *Asplanchna*. *Freshwater Biol.* **14**: 639-647.

Stemberger, R.S. & Gilbert, J.J. (1987). Defenses of planktonic rotifers against predators. *In*: Kerfoot, W.C. & Sih, A. (eds.) *Predation: direct and indirect impacts on aquatic communities*. University Press New England, Hanover, NH, pp: 227-239.

Sterner, R.W. (1989). The role of grazers in phytoplankton succession. *In*: Sommer, U. (ed.) *Plankton Ecology:succession in plankton communities*.. Springer-Verlag Berlin, Heidelberg, New York, pp 107-170

Sterner R.W. (1993). *Daphnia* growth on varying quality of *Scenedesmus*: mineral limitation of zooplankton. *Ecology* **74**: 2351-2360.

Sterner, R.W. & Smith, R.F. (1993). Clearance, ingestion and release of N and P by *Daphnia obtusa* feeding on *Scenedesmus acutus* of varying quality. *Bull. Mar. Sci.* **53**: 228-239.

Sterner, R.W., Hagemeier, D.D., Smith, R.F. & Smith, W.L. (1993). Phytoplankton nutrient limitation and food quality for *Daphnia*. *Limnol. Oceanogr.* **38**: 857-871.

Stibor H. (1992). Predator induced life-history shifts in a freshwater cladoceran. *Oecologia* **92**: 162-165.

Stibor, H. & Macháček, J. (1998). The influence of fish-exuded chemicals on the carbon budget of *Daphnia*. *Limnol. Oceanogr.***43**: 997-1000.

Stirling, G. (1995). *Daphnia* behaviour as a bioassay of fish presence or predation. *Funct. Ecol.* **9**: 778-784.

Strickler, J.R. (1975). Intra- and interspecific information flow among planktonic copepods: Receptors. *Verh. Internat. Verein. Limnol.* **19**: 2951-2958.

Stryer, L. (1988). *Biochemistry*. 3rd edition, Freeman, W.H. & company, New York, pp 1089.

Stutzman P. (1995). Food quality of gelatinous colonial chlorophytes to the freshwater zooplankters *Daphnia pulicaria* and *Diaptomus oregonensis*. *Freshwater Biol.* **34**: 149-153.

Sundbom, M. & Vrede, T. (1997). Effects of fatty acids and phosphorus content of food on the growth, survival and reproduction of *Daphnia*. *Freshwater Biol.* **38**:665-674.

Swale, E.M.F. (1967). A clone of *Scenedesmus* with *Chodatella*-stages. *Br. phycol. Bull.***3**: 281-293.

Szecpanska, W. (1987). Allelopathy in helophytes. *Arch. Hydrobiol. Beih. Ergeb. Limnol.* **27** 173-179.

Takabayashi, J. & Dicke, M. (1992). Response of predatory mites with different rearing histories to volatiles of uninfested plants. *Entomol. exp. appl.* **64**: 187-193.

Tessier, A.J. (1983). Cohorence and horizontal movements of patches of *Holopedium gibberum* (Cladocera). *Oecologia* **60**: 71-75.

Tessier, A.J. & Consolatti, N.L. (1991). Resource quantity and offspring quality in *Daphnia. Ecology* **72:** 468-478.

Thompson, P.A., Harrison, P.J. & Parslow, J.S. 1991. Influence of irradiance on cell volume and carbon quota for ten species of marine phytoplankton. *J. Phycol.* **27**: 351-360.

Timms, R.M. & Moss, B. (1984). Prevention of growth of potentilay dense phytoplankton populations by zooplankton grazing, in the presence of zooplanktivorous fish, in a shallow wetland ecosystem. *Limnol. Oceanogr.* **29**: 472-486.

Tilberg, J.-E., Barnard, T. & Rowley, J.R. (1984). Phosphorus status and cytoplasmic structures in *Scenedesmus* (Chlorophyceae) under different metabolic regimes *J.Phycol.* **20**: 124-136

Titman, D & Kilham, P. (1976). Sinking in freshwater phytoplankton: Some ecological implications of cell nutrient status and physical mixing processes. *Limnol. Oceanogr.***21**: 409-417.

Tollrian, R. (1993). Neckteeth formation in *Daphnia pulex* as an example of continuous phenotipic plasticity: morphological effects of *Chaoborus* kairomone concentration and their quantification. *J. Plankton Res.* **15**: 1309-1318.

Tollrian, R. (1995). Predator-induced morphological defenses: Costs, life-history shifts, and maternal effects in *Daphnia pulex. Ecology* **76**: 1691-1705.

Tollrian, R. & von Elert, E. (1994). Enrichment and purification of *Chaoborus* kairomone from water: Further steps towards its chemical characterization. *Limnol. Oceanogr.* **39**: 788-796.

Tollrian, R. & Harvell, C.D. (1999).The evolution of inducible defenses: current ideas. *In*: Tollrian, R & Harvell, C.D. (eds.) *The ecology and evolution of inducible defenses.* Princeton University Press, New Jersey, pp 306-321.

Trainor, F.R. (1963). Zoospores in *Scenedesmus obliquus. Science* **142**: 1673-1674.

Trainor, F.R. (1964). The effect of composition of the medium on morphology in *Scenedesmus obliquus. Can. J. Bot* .**42**: 515-518.

Trainor, F.R. (1964). Spine distribution in several *Scenedesmus* cultures. *Am. J. Bot.* **51**: 995-1001.

Trainor, F.R. (1969). *Scenedesmus* morphogenesis. Trace elements and spine formation.*J. Phycol.***5**: 185-190.

Trainor, F.R. (1971). Unicells in soil extract in *Scenedesmus* strain 170. *Br. Phycol. J.* **6**: 171-174.

Trainor, F.R. (1979). *Scenedesmus* AP 1 (Chlorophyceae): polymorphic in the laboratory but not in the field. *Phycologia* **18**: 273-277.

Trainor, F.R. (1991). Discovering the various ecomorphs of *Scenedesmus*: the end of a taxonomic era. *Arch. Protistenkd.* **139**: 125-132.

Trainor, F.R. (1992a). Cyclomorphosis in *Scenedesmus communis* Hegew. Ecomorph expression at low temperature. *Br. Phycol. J.* **27**: 75-81.

Trainor, F.R. (1992b). Cyclomorphosis in *Scenedesmus armatus* (Chlorophyta): An ordered sequence of ecomorph development. *J. Phycol.* **28**: 553-558.

Trainor, F.R. (1993). Cyclomorphosis in *Scenedesmus subspicatus* R. Chod. Cell behaviour during the unicell-colony transformation of a phenotypicly plastic organism. *Arch. Protistenkd.* **143**: 55-61.

Trainor, F.R. (1995). The sequence of ecomorph formation in a phenotypicly plastic, multispined *Scenedesmus* species (Chlorophyceae). *Arch. Hydrobiol.* **133**: 161-171.

Trainor, F.R. (1998). Biological aspects of *Scenedesmus* (Chlorophyceae)-phenotypic plasticity. *Nova Hedwigia, Beiheft* **117**, 367 pp.

Trainor, F.R. & Burg C. (1965). Detection of bristles in *Scenedesmus* species. *J. Phycol.* **1**: 139-144.

Trainor, F.R. & Roskosky, F.G. (1967). Control of unicell formation in a soil *Scenedesmus*. *Can. J. Botany* **45**: 1657-1664.

Trainor, F.R. & Rowland, H.L. (1968). Control of colony and unicell formation in a *Scenedesmus*. *J. Phycol.* **4**: 310-317.

Trainor, F.R., Rowland, H.L., Lylis, J.C., Winter, P.A. & Bonanomi, P.L. (1971). Some examples of polymorphism in algae. *Phycologia* **10**: 113-119.

Trainor, F.R. & Shubert, L.E. (1974). *Scenedesmus* morphogenesis. Colony control in dilute media. *J. Phycol.* **10**: 28-30.

Trainor, F.R. & Egan, P.F. (1988). The role of bristles in the distribution of a *Scenedesmus* (Chlorophyceae). *Br. phycol. J.* **23**: 135-141.

Trainor, F.R. & Egan, P.F. (1990). The implications of polymorphism for the systematics of *Scenedesmus*. *Br. phycol. J.* **25**: 275-279.

Trainor, F.R. & Egan, P.F. (1991). Discovering the various ecomorphs of *Scenedesmus*: The end of a taxonomic era. *Arch. Protistenkd.* **139**: 125-132.

Tukaj, Z. & Bohdanowicz, J. (1995). Diesel-fuel oil indiced morphological changes in *Scenedesmus* species (Chlorococcales). *Algolog. Stud.* 77: 83-94.

Tukaj, Z., Kubinova, A. & Zachleder, V. (1996). Effect of irradiance on growth and reproductive processes during the cell cycle in *Scenedesmus armatus* (Chlorophyta) *J. Phycol.* **32**: 624-631

Turner, E.J., Zimmer-Faust, R.K., Palmer, M.A., Luckenbach, M. & Pentcheff, N.D. (1994). Settlement of oyster (*Crassostrea virginica*) larvae: Effects of water flow and a water-soluble chemical cue. *Limnol. Oceanogr.* **39**: 1579-1593.

Turpin, D.H. & Harrison, P.J. (1980). Cell size manipulation in natural marine planktonic, diatom communities. *Can. J. Fish. Aquat. Sci.* **37**: 1193-1195.

Twiss, M.R., Nalewajko, C. & Stokes, P.M. (1989). Assay technique for determining the effect of copper on photosynthesis in *Scenedesmus*. *J. Phycol.* **25** Suppl. p11.

Uherkovich, G. (1966). Die *Scenedesmus*-Arten Ungarns. Akadémiai Kiadó, Budapest, 173 pp.

Urabe, J. & Watanabe, Y. (1992). Possibility of N and P limitation for planktonic cladocerans: An experimental test. *Limnol. Oceanogr.* **37**: 244-251.

Valentine, J.W. (1978). The evolution of multicellular plants and animals. *Sci. Am.* **239**: 105-118.

Van Donk, E. (1997). Defenses in phytoplankton against grazing induced by nutrient limitation, UV-B stress and infochemicals. *Aquatic Ecology* **31**: 53-58.

Van Donk, E., Gulati, R.D. & Grimm, M.P. (1990). Restoration by biomanipulation in a small hypertrophic lake: first-year results. *Hydrobiologia* **191**: 285-295.

Van Donk, E., Grimm, M.P., Gulati, R.D. & Klein Breteler, J.P.G. (1990). Whole-lake food-web manipulation as a means to study community interactions in a small ecosystem. *Hydrobiologia* **200/201**: 275-289.

Van Donk, E. & Hessen, D.O. (1993). Grazing resistance in nutrient-stressed phytoplankton. *Oecologia* **93**: 508-511.

Van Donk, E. & Hessen, D.O. (1995). Reduced digestibility of UV-B stressed and nutrient-limited algae by *Daphnia magna*. *Hydrobiologia* **307**: 147-151.

Van Donk, E., Lürling, M., Hessen, D.O. & Lokhorst, B. (1997). Changed cell wall morphology in nutrient deficient phytoplankton and the significance for grazers. *Limnol. Oceanogr.* **42**: 357-364.

Van Donk, E., Lürling, M. & Lampert, W. (1999). Consumer-induced changes in phytoplankton: inducibility, costs, benefits and impacts on grazers. *In*: Tollrian, R & Harvell, C.D. (eds.) *The ecology and evolution of inducible defenses*. Princeton University Press, New Jersey, pp 89-103.

Van Kooten, O & Snel, J.F.H. (1990).The use of chlorophyll fluorescence nomenclature in plant stress physiology. *Photosynth. Res.***25**: 147-150.

Vanni M.J. & Lampert W. (1992). Food quality effects on life history traits and fitness in the generalist herbivore *Daphnia*. *Oecologia* **92**: 48-57.

Van den Hoek, C., Mann, D.G. & Jahns, H.M. 1995. *Algae: an introduction to phycology*. Cambridge University Press, Cambridge, 623 pp.

Van Valen,L. (1973). A new evolutionary law. *Evol. Theor.* **1**: 1-30.

Vellai, T., Takács, K. & Vida G. (1998). A new aspect to the origin and evolution of eukaryotes. *J. Mol. Evol.* **46**: 499-507.

Vet, L.E.M. & Dicke, M. (1992). Ecology of infochemical use by natural enemies in a tritrophic context. *Annu. Rev. Entomol.* **37**: 141-172.

Vidal, G. (1984). The oldest eukaryotic cells. *Sci Am.***250**: 32-41.

Vijverberg, J, Kalf, D.F. & Boersma, M. (1996). Decrease in *Daphnia* egg viability at elevated pH. *Limnol. Oceanogr.* **41**: 789-794.

Vincent, W. (1980). The physiological ecology of a *Scenedesmus* population in the hypolimnion of a hypertrophic pond.II. Heterotrophy. *Br. Phycol. J.* **15**: 35-41.

Visser, P.M., Massault, L., Huisman, J. & Mur, L.R. (1996). Sedimentation losses of Scenedesmus in relation to mixing depth. *Arch. Hydrobiol.* **136**: 289-308.

Von Elert, E. (*in press*). Fatty acids released by *Daphnia*: Do they induce colony formation in *Scenedesmus acutus*?. *Verh. Internat. Verein. Limnol.*

Von Elert, E. & Franck, A. (*in press*). Colony formation in *Scenedesmus*: grazer-mediated release and chemical feature of the infochemical. *J. Plankton Res.*

Von Frisch, K. (1941). Über einen Schreckstoff der Fischhaut und seine biologische Bedeutung. *Z. Vgl. Physiol.* **29**: 321-333.

Vossbrinck, C.R., Maddox, J.V., Friedman, S., DeBRunner-Vossbrinck, B.A. & Woese, C.R. (1987). Ribosomal RNA sequence suggests microsporidia are extremely ancient eukaryotes. *Nature* **326**: 411-414.

Wahle, R.A. (1992). Body-size dependent anti-predator mechanisms of the American lobster. *Oikos* **65**: 52-60.

Waite A.M., Thompson, P.A. & Harrison, P.J. (1992). Does energy control the sinking rates of marine diatoms? *Limnol. Oceanogr.* **37**: 468-477.

Walls, M. & Ketola, M. (1989). Effects of predator-induced spines on individual fitness in *Daphnia pulex*. *Limnol. Oceanogr.* **34**: 390-396.

Wasmund, N. (1992). Temperature and salinity demand of *Scenedesmus abundans* (Kirchn.) Chod. and *Scenedesmus obliquus* (Turp.) Kütz. (Chlorophyceae). *Limnologica* **22**: 249-263.

Watanabe, M.F. & Oishi, S. (1985). Effects of environmental factors on toxicity of a cyanobacterium (*Microcystis aeruginosa*) under culture conditions. *Appl. Env. Microbiol.* **49**: 1342-1344.

Weers P.M.M. & Gulati R.D. (1997). Effect of the addition of polyunsaturated fatty acids to the diet on the growth and fecundity of *Daphnia galeata*. *Freshwater Biology* **38**: 721-729.

West-Eberhard, M. (1989). Phenotypic plasticity and the origins of diversity. *Ann. Rev. Ecol. Syst.* **20**: 249-278.

Wiackowski, K. & Szkarlat, M. (1996). Effects of food availability on predator-induced morphological defence in the ciliate *Euplotes octocarinatus* (Protista). *Hydrobiologia* **321**: 47-52.

Willmer, P. (1990). *Invertebrate relationships: patterns in animal evolution*. Cambridge University Press.

Wium-Andersen, S. Anthoni, U. Christophersen, C. & Houen, G. (1982). Allelopatic effects on phytoplankton by substances isolated from aquatic macrophytes (Charales). *Oikos* **39**: 187-190.

Wium-Andersen, S. (1987). Allelopathy among aquatic plants. *Arch. Hydrobiol. Beih. Ergeb. Limnol.* **27**: 167-172.

Wolfe, G., Steinke, M. & Kirst, G.O. (1997). Grazer-activated chemical defense in a unicellular marine alga. *Nature* **387**: 894-897.

Wood, A.M. & Leatham, T. (1992). The species concept in phytoplankton ecology. *J. Phycol.* **28**: 723-729.

Wudkevich, K., Wisenden, B.D., Chivers, D.P. & Smith, R.J.F. (1997). Reactions of *Gammarus lacustris* to chemical stimuli from natural predators and injured conspecifics. *J. Chem. Ecol.* **23**: 1163-1173.

Yoshioka, P.M. (1982). Predator-induced polymorphism in the bryozoan *Membranipora*

membranacea (L.). *J. Exp. Mar. Biol. Ecol.* **62**: 233-242.

Young, S. & Getty, C. (1987). Visually guided feeding behaviour in filter feeding cladoceran, *Daphnia magna*. *Anim. Behav.* **35**: 541-548.

Zimmer-Faust, R.K. & Tamburri, M.N. (1994). Chemical identity and ecological implications of a waterborne, larval settlement cue. *Limnol. Oceanogr.* **39**: 1075-1087.

SUMMARY

In aquatic systems, the phytoplankton – zooplankton relation is of major importance because it is the first step in the pelagic food chain. It is well known that zooplankton feed with a highly variable success on phytoplankton, primarily owing to algal characteristics such as size, shape, cell wall texture, nutritional quality and toxicity. Algae are present in a broad variety of shapes and may express an enormous variability in their morphology, physiology and behavior depending on environmental variables. Because algae depend on solar energy they have to remain in the upper water layers as long as possible. Moreover, they have to compete with other algae for dissolved nutrients. This means that in an aquatic environment selection pressure exists for small organisms since these have the most efficient uptake of nutrients and light and lowest sinking losses. By contrast, mortality through grazing by an entire assemblage of protozoan and metazoan grazers will exert a strong selection for traits that reduce this mortality through grazing. An effective way to resist grazing is by a dramatic increase in size. However, this confronts the algae with conflicting allometries of selection pressures.

Since algae are small relative to their predatory enemies, they may not survive an encounter with a grazer. Therefore, it may be profitable to detect a grazer before they encounter each other in order to elicit a defensive strategy. In a predictable environment temperature and day length could be good predictor of danger. However, in aquatic systems, grazing fluctuates considerably on temporal and spatial scales and chemical cues may be used instead. All organisms exchange constantly chemicals with their environment and those chemicals that are essential in the biology of the grazer and are detectable by the algae may prove potential indicators of danger. They convey information and are referred to as infochemicals. This thesis focuses on the role of infochemicals in the interaction between algae and zooplankton, with emphasis on the *Scenedesmus* (algae) - *Daphnia* (waterflea) relation.

In the presence of filtered medium from a *Daphnia* culture, the non-spiny *Scenedesmus acutus* formed numerous eight-celled colonies (coenobia) (CHAPTER 2). However, in control populations, i.e. in the absence of *Daphnia*-infochemical, *S. acutus* remained unicellular and formed only four-celled colonies when cultures reached stationary phase. The induced colony formation appeared reversible as eight-celled colonies gradually disappeared from the treated populations.

A prerequisite for further exploration of the phenomenon of *Daphnia*-induced colony formation is the development of a reliable biotest (CHAPTER 3). Inoculum algal density, carbon availability and filter-type are some of the factors that affected the *Daphnia*-induced colony formation. Analysis of filter extractables revealed that at least two detergents might cause *S. acutus* to shift rapidly from a completely unicellular population to one dominated with colonies. The production of the *Daphnia*-infochemical is related to the amount of food

processed by the animals. Starved animals or animals fed with ingestible but non-digestible beads were ineffective in inducing colonies. Neither algal homogenates nor auxins and several organic carbon sources have colony inducing activity. The infochemical does seem to originate from the *Daphnia*-food interaction, or better from the grazer-algal food interaction as several herbivorous zooplankters were able to induce colonies in *S. acutus*, whereas carnivorous zooplankton and fish were ineffective (CHAPTER 3). Simple excretion products, such as ammonia and urea alone or in combination with organic carbon sources were ineffective as colony inducing agents (CHAPTERS 3 & 4).

Scenedesmus plasticity has, however, not only been shaped by the activity of grazers, but also by other selective forces. Several factors are known that may influence the growth and morphological development in *Scenedesmus* and among them nutrient availability (CHAPTER 4) and temperature (CHAPTER 5) are important ones. In culture, with relatively high algal densities carbon limitation may occur. The availability of inorganic carbon appeared ineffective in inducing colonies, but had a clear effect on cell size. Neither N- nor P-limitation resulted in the formation of numerous, eight-celled coenobia. In general, under nutrient limitation cultures were dominated by unicells. However, despite the limitation, by adding *Daphnia* water, colonies still could be induced. One of the criticisms on use of artificial growth media is the excessive amount of nutrients in most of them. However, using media of various strengths showed no differences in morphological appearance of *S. acutus*, both in the absence and presence of *Daphnia* water. It appears that as long as cell division is not hampered grazer-induced colony formation may occur.

Temperature not only affected growth, but also the morphological development in *S. acutus* (CHAPTER 5). At low temperatures growth was reduced, but cell- and colony size increased. Under a broad range of temperatures from 9° to 29°C, the addition of *Daphnia* water significantly increased the proportion of eight-celled coenobia. The smaller size at higher temperature supports the hypothesis of a trade-off between sinking and size.

An analysis of potential costs associated with grazer-induced colony formation was initially directed on metabolic costs (CHAPTER 6). However, no reductions in growth and photosystem II efficiency were detected in induced colonies. Higher sinking losses of induced colonial *Scenedesmus* populations were measured. Hence, costs may be assigned to enhanced sinking out of the euphotic zone into darker and colder water layers, thereby significantly reducing growth rates. The strategy may, however, not be completely lethal, as *Scenedesmus* is known to be capable of surviving for prolonged periods on the sediments.

One of the prerequisites for interpreting the grazer-induced colony formation as an induced defense is that the response has to be effective in reducing mortality through grazing. In CHAPTER 7, the grazing success of several zooplankton species, such as the rotifers *Keratella* and *Brachionus* and the cladocerans *Bosmina*, *Ceriodaphnia* and *Daphnia*, was analyzed. Food intake was reduced in all smaller grazers, but not in the largest *Daphnia*

species. Moreover, growth of the small *Daphnia cucullata* was reduced when offered colonial *S. acutus*. These reductions appeared not the result of an altered biochemical composition of induced colonies.

So far, the effect of grazing-associated infochemicals had only been examined for the non-spiny *S. acutus*. In CHAPTER 8, 23 different *Scenedesmus* strains, 9 different other chlorophytes, 2 diatom species and 5 strains of cyanobacteria were investigated. In 35% of the *Scenedesmus* a positive response to the addition of *Daphnia* water was observed. Most responding appeared the non-spiny strains, i.e. 64% in contrast to the 4% for spined *Scenedesmus*. Not only is the trait colony formation only one of the potential defensive traits, it also appeared that the biotest was only suited for examining non-spiny *Scenedesmus*.

The grazer-induced colony formation appeared not to be restricted to the genus *Scenedesmus*, since two *Coelastrum* strains were responsive too. Also in the diatom *Synedra* and the cyanobacterium *Microcystis* cell size was increased in the presence of *Daphnia* water. Moreover, the latter showed a tendency to higher toxin levels when cultured in the presence of medium from a *Daphnia* culture (CHAPTER 8).

Another phenomenon often observed in the presence of live *Daphnia*, is the aggregation of live cells onto fecal pellets (CHAPTER 8). These large aggregates will undoubtedly be inedible to grazers and may be an additional process affecting the energy flow from algae to their consumers.

In CHAPTER 9, experiments were performed to evaluate the ability of *Daphnia* to locate algae by means of chemical cues. No evidence for such a mechanism was detected. However, the animals did seem to avoid water with odors from congeners. Moreover, water from crowded *Daphnia* cultures had clear effects on growth and reproduction in two *Daphnia* species and may have an effect on the phytoplankton-grazer interaction.

Summarizing the various experiments described in this thesis, the phenomenon of grazer-induced colony formation in *Scenedesmus* can be interpreted as an inducible defense at the expense of higher sinking losses. The phenomenon is not restricted to *Scenedesmus* and because of the enormous plasticity in phytoplankton, numerous species may eventually turn out not only to respond to abiotic but to biotic agents as well.

SAMENVATTING

De predator-prooi relatie tussen algen en watervlooien is één van de belangrijkste interacties in een aquatisch systeem, omdat het de eerste schakel vormt in de pelagische voedselketen. Het is bekend dat algen (het fytoplankton) een rijke schakering aan verschijningsvormen ten toon spreiden, maar ook dat niet iedere alg even kwetsbaar is voor graas. Met name de grootte, de vorm, de celwandstructuur en eventuele giftigheid bepalen in een belangrijke mate het graassucces door watervlooien (het zoöplankton) op de verschillende algen.

Omwille van hun fotosynthetiserende activiteit, zijn algen in sterke mate afhankelijk van zonlicht. Dit betekent dat ze zo lang mogelijk in de bovenste waterlagen moeten zien te verblijven. Daarnaast dienen ze er een zo efficiënt mogelijke nutriëntenvoorziening op na te houden om de concurrentie om voedingsstoffen in hun voordeel te beslechten. Kortom, in het open water ligt er een sterke selectiedruk op de ontwikkeling van kleine algen met de thermodynamisch meest voordelige oppervlakte/volume- ratio en kleinste sedimentatie verliezen. Algen hebben echter niet alleen te maken met uitzinken en concurrentie, ook de aanwezigheid van een hele batterij aan grazers (de watervlooien) zal een sterke selectiedruk doen gelden op de ontwikkeling van mechanismen om mortaliteit door graas te minimaliseren. Eén van de meest effectieve afweermechanismen is een aanzienlijke toename in grootte. Dat houdt echter in dat algen te maken hebben met selectiemechanismen, die tegengesteld werken.

Vanwege hun geringe omvang zullen algen in het algemeen een ontmoeting met een grazer niet overleven. Het is voor deze algen daarom noodzaak deze grazers waar te nemen voordat ze elkaar tegen komen. Door afwezigheid van organen is het gebruik van visuele en akoestische informatie onmogelijk voor de alg onmogelijk. In een voorspelbare leefomgeving kunnen temperatuur en daglengte als betrouwbare voorspellers voor de aanwezigheid van grazers dienen ware het niet dat de graasdruk een enorme variatie laat zien in zowel ruimtelijke als temporele zin. De continue uitwisseling van chemische stoffen door organismen met hun omgeving maakt het mogelijk om dergelijke uitscheidingsproducten als voorspellers voor de aanwezigheid van grazers te gebruiken. Deze chemische verbindingen bevatten in zo'n geval informatie en worden ook wel aangeduid met de term: *infochemicaliën*. In dit proefschrift is er met name gekeken naar de rol van infochemicaliën in de interactie tussen algen en watervlooien, waarbij de nadruk lag op de interactie tussen de groenalg *Scenedesmus* en de watervlo *Dafnia*.

In aanwezigheid van gefiltreerd water uit een *Dafnia* cultuur, vormde de alg *Scenedesmus acutus* voornamelijk acht-cellige kolonies (HOOFDSTUK 2). In afwezigheid van dit *Dafnia* water bleef de cultuur gedomineerd door enkelcellige *Scenedesmus* en werd er een toename aan viercellige kolonies waargenomen naarmate de populaties verouderden. De geïnduceerde kolonievorming bleek een omkeerbaar proces.

Om de fenotypische plasticiteit in *S. acutus* en dan met name de *Dafnia*-geïnduceerde kolonievorming nader te onderzoeken, werd een biotest ontwikkeld (HOOFDSTUK 3). Verschillende factoren, zoals beginconcentratie alg, hoeveelheid en dichtheid aan watervlooien, voedingstoestand van de watervlo, hoeveelheid koolstof voor de alg, en het gebruikte filtertype, bleken een aanzienlijke invloed te hebben op de kolonievorming in *S. acutus*. Zo werd gevonden dat kolonievorming geïnduceerd kan worden door detergenten vrijkomend uit bepaalde membraanfilters. Het natuurlijke kolonie-inducerende stofje, uit de *Dafnia*, blijkt sterk gerelateerd aan de hoeveelheid voedsel die door de watervlo is verwerkt. Gehongerde watervlooien en beesten die onverteerbaar 'voedsel' voorgeschoteld kregen produceerden geen kolonie-inducerende infochemicaliën. Ook simpele *Dafnia* uitscheidingsproducten, zoals ammonium en ureum, verschillende organische koolstofverbindingen, gehomogeniseerde algen en plantengroeihormonen waren niet effectief als kolonie-inducerende substanties (HOOFDSTUK 3 & 4). Daarnaast werd alleen een respons in *S. acutus* waargenomen wanneer blootgesteld aan gefiltreerd medium uit een herbivore zoöplankton cultuur, maar niet wanneer medium uit een carnivore zoöplankton- of viscultuur werd toegediend (HOOFDSTUK 3).

Het is bekend dat diverse omgevingsfactoren de kolonievorming in *Scenedesmus* kunnen beïnvloeden. Twee van de belangrijkste factoren zijn de aanwezigheid en concentratie van voedingsstoffen (HOOFDSTUK 4) en de temperatuur (HOOFDSTUK 5). Zowel de hoeveelheid koolstof, als de concentraties aan stikstof en fosfor waren nauwelijks van invloed op de kolonievorming in *S. acutus*. In het algemeen werden voedingsstofarme kweken gekenmerkt door een dominantie aan enkelcelligen. Desalniettemin kon in iedere kweek, gecultiveerd in medium variërend in sterkte van zeer voedselarm tot zeer rijk, de vorming van achtcellige kolonies verkregen worden door water uit een *Dafnia* kweek toe te dienen. Dus zolang de celdeling niet volledig geblokkeerd is, is *Dafnia*-geïnduceerde kolonievorming mogelijk.

De temperatuur had niet alleen een aanzienlijk effect op de groei van *S. acutus*, maar ook op de morfologische ontwikkeling (HOOFDSTUK 5). Bij lage temperatuur werd de groei gereduceerd en de vorming van kolonies gestimuleerd. Desondanks stimuleerde de toediening van *Dafnia* water de vorming van achtcellige kolonies. In warmer water waren cel- en koloniegrootte kleiner dan in kouder water. De geringere grootte in warmer water kan duiden op een aanpassing om uitzinken te voorkomen en ondersteunt daarmee de hypothese dat er een balans kan bestaan tussen defensieve kolonievorming en versnelde sedimentatie uit de eufotische zone.

De grazer-geïnduceerde kolonievorming suggereert dat er kosten verbonden zijn aan deze flexibele afweer, omdat anders de beschermende kolonievorming altijd gehandhaafd zou worden. Vanwege extra celwandmateriaal nodig als cement tussen de cellen in een kolonie en de veranderde oppervlakte/volume-ratio en daarmee samenhangende zelfbeschaduwing en

verminderde voedingsstoffen opname, werd een geringere groei van geïnduceerde kolonies verwacht. Er werd echter geen lagere groeisnelheid van kolonies gemeten en ook de efficiëntie van het fotosysteem II was niet verschillend van die van ééncelligen (HOOFDSTUK 6). Metabole kosten konden niet worden aangetoond. Kosten verbonden aan kolonievorming bleken te kunnen worden toegeschreven aan het versneld uitzinken van kolonies.

Om grazer-geïnduceerde kolonievorming daadwerkelijk als een afweer te kunnen interpreteren, dient er een duidelijk voordeel voor de alg te zijn. Hiertoe werden verschillende zoöplankton soorten, zoals de rotiferen *Keratella* and *Brachionus*, en de cladoceren *Bosmina, Ceriodafnia* en *Dafnia*, gevoerd met ééncellige- en kolonievormige *Scenedesmus* (HOOFDSTUK 7). De voedselopname was beduidend lager voor alle kleinere grazers wanneer kolonies als voedsel werden aangeboden, maar dit gold niet voor de grotere *Dafnia*. Daarnaast groeide de kleine *Dafnia cucullata* beduidend slechter op kolonievormige *S. acutus*. De lagere groei bleek niet het gevolg van een veranderde biochemische samenstelling van de kolonies, maar kon worden toegeschreven aan een geringere voedselopname.

Om een indruk te verkrijgen van de algemeenheid van het fenomeen grazer-geïnduceerde kolonievorming, werden 23 verschillende *Scenedesmus* stammen, negen andere groenalgen, twee diatomeeën en vijf cyanobacteriën onderzocht (HOOFDSTUK 8). In 35% van de *Scenedesmus* werd een positieve respons op de toediening van *Dafnia* water gevonden. Het vaakst bleek het om een niet-stekelige *Scenedesmus* te gaan. Kolonievorming is slechts één van de mogelijke afweermechanismen en het is dan ook mogelijk dat andere stammen anders reageren, maar duidelijk werd in ieder geval dat de biotest ontwikkeld voor *S. acutus* vooral geschikt is voor de niet stekelige *Scenedesmus* stammen. Grazer-geïnduceerde kolonievorming is niet beperkt tot het geslacht *Scenedesmus*, daar dit fenomeen ook in twee *Coelastrum* soorten kon worden aangetoond. Bovendien werd er een geringe toename in de koloniegrootte van de diatomee *Synedra* en de cyanobacterie *Microcystis* gevonden. Deze laatste liet ook een geringe toename in het toxine gehalte zien (HOOFDSTUK 8). Een ander regelmatig optredend fenomeen is de vorming van grote meercellige aggregaten in aanwezigheid van levend zoöplankton. Het samenklonteren van cellen kan naast de geïnduceerde kolonievorming een belangrijk effect hebben op de energiestroom van algen naar grazers.

In HOOFDSTUK 9 zijn experimenten uitgevoerd om te achterhalen of watervlooien bij het lokaliseren van algen gebruik maken van infochemicaliën. Er is echter geen bewijs voor het bestaan van zo'n mechanisme gevonden. Water waarin concurrenten hadden gezeten werd daarentegen gemeden door de *Dafnia*. Bovendien had dit water een negatief effect op de groei en voortplanting van de *Dafnia* en daarmee op de energiestroom tussen algen en watervlooien.

Samenvattend kan gesteld worden dat het fenomeen van grazer-geïnduceerde kolonievorming in *Scenedesmus* een geïnduceerd afweermechanisme is, met als voordeel een lagere mortaliteit door graas, en als nadeel een verhoogde sedimentatie van kolonies. Het

fenomeen is niet beperkt tot *Scenedesmus* en rekeninghoudende met de enorme plasticiteit in algen, kan uiteindelijk blijken dat vele, zoniet alle algen op een of andere wijze reageren op biotische omgevingsfactoren.

CURRICULUM VITAE

Miquel Franciscus Lucas Leonardus Wilhelmus Lürling werd op 16 september 1968 geboren te 's-Hertogenbosch. In 1986 behaalde hij het V.W.O. diploma aan het Rooms Katholieke Gymnasium 'Beekvliet' te Sint-Michielsgestel. Vervolgens werd begonnen met de studie Milieuhygiëne aan de Landbouwuniversiteit te Wageningen. Nadat hij in 1988 het propedeutisch examen had gehaald, werd hij opgeroepen ter vervulling van zijn militaire dienstplicht. In 1990 werd begonnen aan de doctoraalfase Milieuhygiëne waarbinnen hij koos voor de richting Waterkwaliteit. In 1992 werd begonnen met een afstudeervak Waterkwaliteitsbeheer. Aansluitend volgde een stage bij het Centrum voor Limnologie van het Nederlands Instituut voor Oecologisch Onderzoek te Nieuwersluis. In het kader van een tweede afstudeervak Aquatische Oecologie verbleef hij enige maanden bij het Rijks Instituut voor Kust en Zee te Middelburg. In januari 1994 behaalde hij met lof het doctoraal diploma.

Van juni 1994 tot april 1998 is hij als onderzoeker in opleiding (OiO) verbonden geweest aan de leerstoelgroep Aquatische Oecologie en Waterkwaliteitsbeheer van de Landbouwuniversiteit te Wageningen. Hier werd het onderzoek, zoals beschreven in dit proefschrift, uitgevoerd onder supervisie van mw. Dr. E. van Donk en Prof. Dr. W.J. Wolff. In het kader van zijn onderzoek verbleef hij 4 maanden aan het Max-Planck-Instituut voor Limnologie te Plön (Duitsland). Vanaf 1 december 1998 is hij als post-doctoraal onderzoeker werkzaam bij het Centrum voor Limnologie van het Nederlands Instituut voor Oecologisch Onderzoek te Nieuwersluis.

LIST OF PUBLICATIONS

Lürling, M. (1999). Grazer-induced coenobia formation in clonal cultures of *Scenedesmus obliquus* (Chlorococcales; Chlorophyceae). *Journal of Phycology* **35**: 19-23.

Van Donk, E., Lürling, M. & Lampert, W. (1999). Consumer-induced changes in phytoplankton: inducibility, costs, benefits and the impact on grazers. *In:* Tollrian, R. & Harvell, D.C. (eds.) *The ecology and evolution of inducible defenses*. Princeton University Press, New Yersey, pp 89-103.

Lürling, M. (1998). Effect of grazing-associated infochemicals on growth and morphological development in *Scenedesmus acutus* Meyen (Chlorophyceae). *Journal of Phycology* **34**: 578-586.

Lürling, M. & Van Donk, E. (1997). Morphological changes in *Scenedesmus* induced by infochemicals released *in situ* from zooplankton grazers. *Limnology and Oceanography*, **42**: 783-788.

Lürling, M. & Van Donk, E. (1997). Life history consequences for *Daphnia pulex* feeding on nutrient-limited phytoplankton. *Freshwater Biology*, **38**: 693-709.

Lürling, M., De Lange, H.J. & Van Donk, E. (1997). Changes in food quality of the green alga *Scenedesmus* induced by *Daphnia* infochemicals: biochemical composition and morphology. *Freshwater Biology*, **38**: 619-628.

Van Donk, E., Lürling, M., Hessen, D.O. & Lokhorst, G.M. (1997). Altered cell wall morphology in nutrient-deficient phytoplankton and its impact on grazers. *Limnology and Oceanography*, **42**: 357-364.

Lürling, M. & Van Donk, E. (1996). Zooplankton-induced unicell-colony transformation in *Scenedesmus acutus* and its effect on growth of herbivore *Daphnia*. *Oecologia*, **108**: 432-437.

Lürling, M., Blom, G. & Van Donk, E. (1995). Resuspensie, sedimentatie en lichtklimaat in de Breukeleveense Plas. *H_2O*, **28**: 194-198.

PAPERS IN PRESS:

Lürling, M., Van Donk, E. & Beekman, W. Algal concentration affects grazer-induced colony formation in *Scenedesmus*. *Verhandlungen internationale Vereinigung für theoretische und angewandte Limnologie.*

Van der Grinten, E., Lürling, M. & Burger-Wiersma, T.. Is *Microcystis* really toxic to *Daphnia*? *Verhandlungen internationale Vereinigung für theoretische und angewandte Limnologie.*

Lürling, M. & Beekman, W. Grazer-induced defense in *Scenedesmus* (Chlorococcales; Chlorophyceae): coenobium and spine formation. *Phycologia.*

PAPERS SUBMITTED:

Lürling, M. & Van Donk, E. Response of *Daphnia* to infochemicals released from crowded congeners and conspecifics: Costs of living together. Submitted to *Aquatic Ecology.*

Lürling, M. & Beekman, W. Filter-extractables (detergents) induce morphological changes in *Scenedesmus* (Chlorophyceae). Submitted to *Limnology and Oceanography.*

Lürling, M. & Van Donk, E. Grazer-induced colony formation in *Scenedesmus*: Costs of being colonial? Submitted to *Oikos.*

Lürling, M. & Van Donk, E. Grazer-induced colony formation in *Scenedesmus acutus* Meyen (Chlorophyceae): Ecomorph expression at different temperatures. Submitted to *Journal of Phycology.*

Lürling, M. & Van Donk, E. Effects of *Daphnia* infochemicals on growth and morphology in the green alga *Scenedesmus acutus* Meyen (Chlorophyceae; Chlorococcales) cultured under different phosphorus conditions. Submitted to *Freshwater Biology.*

Roozen, F., Lürling, M. & Plath, K. Response of *Daphnia* to algal infochemicals: Is it real? Submitted to *Journal of Plankton Research.*

PAPERS IN PREPARATION:

De Lange, H.J. & Lürling, M. UVB irradiated algae cause different effects on ingestion and assimilation by *Daphnia, Bosmina* and *Brachionus.*