

## Chemical warfare in freshwater

allelopathic effects of  
macrophytes on phytoplankton

## Chemische oorlogsvoering in zoetwater

allelopathische effecten van  
waterplanten op fytoplankton

Gabi Mulderij

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# Chemical warfare in freshwater

een wetenschappelijke proeve op het gebied van  
de Natuurwetenschappen, Wiskunde en Informatica

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*Niemand die het weten kan  
hoeveel ik van je hou  
Niemand die mij troosten kan  
in mijn verdriet om jou  
Niemand die begripen zal  
hoe vreselijk ik je mis  
Niemand die beseffen zal  
hoe erg die pijn wel is*

Lieve Paps,

Zie je?

Het is me gelukt!

Jammer dat we het laatste  
stukje niet meer samen  
konden doen...

Cover: Krabbescheer, *Stratiotes aloides* (Willem Kolvoort)

Lay-out & cover design: Gabi Mulderij & Elaine Sullivan



*Stratiotes aloides* L. (Water soldier) surrounded by filamentous algae in a ditch near Lake Naardermeer, The Netherlands.

Picture: G. Mulderij





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# Introduction



Mixed stand of *Stratiotes aloides*, *Nuphar lutea*, *Potamogeton natans* and filamentous algae in a ditch near Lake Naardermeer, The Netherlands.

Picture: G. Mulderij



# 1

## General introduction

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# General introduction

## 1.1 Eutrophication of shallow lakes: the role of macrophytes

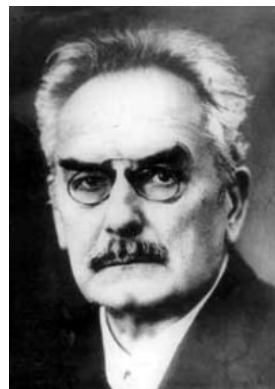
Since the 1950's many shallow lakes in Europe have changed from oligo-/mesotrophic systems, characterized by clear water with a diverse submerged aquatic macrophyte community, to hypertrophic and mainly turbid systems, dominated by extensive (sometimes harmful) phytoplankton blooms (Simons et al. 1994, Simons & Nat 1996). Lakes in Sweden (Blindow 1992ab), Denmark (Sand-Jensen et al. 2000), Great Britain (Moss 1980), Poland (Królikowska 1997) Germany (Körner 2002), and The Netherlands (Best et al. 1984, Gulati & Van Donk 2002) have virtually lost all their submerged macrophytes. Discharges of nutrients (mainly N and P) from human activities (domestic, industrial and agricultural) were the main cause of this transition from a clear to a turbid state (Jeppesen 1998). This process, by which (aquatic) ecosystems become enriched with nutrients, is known as *Eutrophication*.

Eutrophication primarily reflects increases in the biomass of primary producers and changes in the competition among them (Philips et al. 1978). The nutrient levels in previously oligotrophic-mesotrophic (clear water) systems increased, thereby stimulating the growth of autotrophic organisms (Schindler 1977, Hecky & Kilham 1988). Shallow aquatic ecosystems can support several types of autotrophic organisms: vascular plants, epiphyton, macroalgae and phytoplankton. The increased lake water turbidity, due to increases in phytoplankton densities, deteriorated the underwater light climate, and caused changes in ecological interactions between the different autotrophs (e.g., Sand-Jensen & Borum 1991). Consequently, shading by primary producers, like phytoplankton and epiphytes, was shown to be the main reason for the shift from a dominance of vascular plants to that of phytoplankton (Philips et al. 1978, Sand-Jensen & Borum 1991).

Eutrophication not only caused a decline in macrophyte abundance, but also lead to a decline in species richness of the macrophytes (Körner 2001). Additionally, excessive nutrient loadings in freshwater ecosystems as well as in coastal and estuarine areas, can lead harmful blooms of, for example, toxic cyanobacteria (e.g., Carmichael 2001) or toxic dinoflagellates (e.g., Burkholder & Glasgow 1997). These

blooms can cause adverse effects on other phytoplankton groups and on zooplankton, fish, domestic animals and even on humans (e.g., Chorus & Bartram 1999). To overcome these problems, a return to the pristine situation would be ideal. This return, however, is complicated. Even after a reduced nutrient loading, a turbid lake may be difficult to restore (Meijer 2000) because of the initiation of self-perpetuating processes (Chapter 2), which stabilize one of the two alternative stable states (e.g., Scheffer 1990, Scheffer et al. 1993a). Nowadays, with improved knowledge on the mechanisms involved in the process of eutrophication, modern restoration studies focus on the control of episodic events, like sudden increases in the turbidity or the occurrence of harmful phytoplankton blooms (Berg et al. 1987), and also aim at increasing the abundance and dominance of macrophytes, thereby increasing the water transparency of lakes and stabilizing the ecosystem (e.g., Gulati & Van Donk 2002). Macrophytes play a key role in these processes.

The presence of aquatic macrophytes (angiosperms and macroalgae, Wetzel 2001) is an extremely important factor for the biological structure and water quality of shallow lakes (e.g., Carpenter & Lodge 1986, Jeppesen et al. 1998a, Scheffer 1998). They play a crucial role in stabilizing the clear water state in shallow mesotrophic and eutrophic lakes (Perrow et al. 1997, Scheffer 1998). Submerged macrophytes can stimulate changes to a clear water state by a variety of mechanisms which will partly be described in detail in Chapter 2. One of these mechanisms is *Allelopathy* (Anthoni et al. 1980, Wium-Andersen et al. 1982, Jasser 1995, Gross et al. 1996). Molisch (1937, Fig. 1.1) defined allelopathy as both detrimental and beneficial biochemical interactions between all classes of plants (including micro-organisms).



**Figure 1.1** Professor Hans Molisch (1856–1937), German Plant Physiologist and President of the University of Vienna, was the first to coin the term ‘allelopathy’, in 1937. The word allelopathy is derived from two separate Greek words: *allelon*, which means *of each other*, and *pathos*, which means *to suffer* or rather *feeling* or *sensitive* (Gross 1999).



Submerged macrophytes have been shown to reduce phytoplankton and epiphyton growth by the excretion of inhibitory/allelopathically active compounds. The release of such compounds may be an effective mechanism of submerged macrophytes to gain competitive advantage over other photoautotrophs (e.g., Wium-Andersen 1987, Gross 1999). Nakai et al. (1999) proposed to use allelopathy as a mechanism to control undesired phytoplankton growth in aquatic ecosystems. Several studies focussed on this issue, but the knowledge of allelopathy in aquatic habitats is still fragmentary. Many aquatic macrophytes have inhibitory allelopathic effects on phytoplankton and/or on macrophyte (Table 1.1, page 38), but Wium-Andersen (1987), for example, stated that it is not enough to show that macrophytes produce allelopathic substances. More importantly, they should excrete these substances to have an (inhibitory) effect on epiphyton and phytoplankton. Willis (1985) proposed six criteria that need to be fulfilled to demonstrate allelopathy:

1. A consistent pattern of inhibition of one species by another must be shown.
2. The inhibitor species must produce a allelopathic chemical.
3. This chemical should be released into the environment.
4. There should be a mode of transportation or accumulation of the chemical.
5. The target organism should be able to take up the allelopathic chemical.
6. The observed inhibition cannot be explained only by physical factors or other biotic factors, like competition and grazing.

However, only very few studies fulfil all these criteria (Willis 1985, Legrand et al. 2003). It is difficult to prove the presence of allelopathy in natural ecosystems because physical, biotic and chemical processes (often occurring concurrently), interfere with each other. Moreover, some of these criteria may be too selective (Fistarol 2004). They only include inhibitory effects and consider mostly terrestrial habitats or consider allelopathic chemicals that need to be taken up, although there are some that act on the cell membranes.

## 1.2 The macrophytes studied

Before going into detail about allelopathic interactions (Section 1.3 and further), I will first focus on the ecology of two aquatic macrophytes with completely different life forms: *Chara* sp. (Stoneworts) and *Stratiotes aloides* (Water soldier, Water aloë or Crab's claw). Thereafter I will describe the allelopathic effects of substances from these two macrophytes on the growth of several phytoplankton species. Both these macrophytes are typically rapid colonizers (Erixon 1979, Van den Berg et al. 1998a, 1998b) and there are indications that they both produce allelopathic substances, which may affect the growth of phytoplankton (e.g., Blindow & Hootsmans 1991, Jasser 1995).

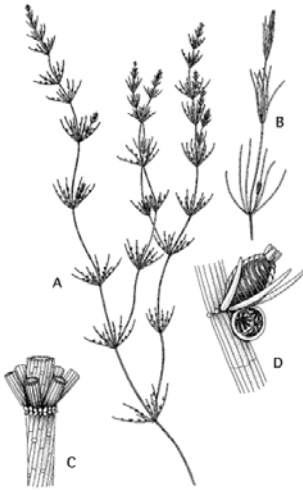
Biomanipulation studies have shown that charophytes are important plants in shallow lakes (Gulati & Van Donk 2002). They plants are often associated with patches of clear water in shallow, relatively turbid lakes. Sedimentation of suspended particles and restriction of resuspension were shown to be important mechanisms in charophyte beds (Van den Berg et al. 1998a). There are some indications that *Chara* sp. possess allelopathic activity (Blindow & Hootsmans 1991, Berger & Schagerl 2003, 2004), although Forsberg et al. 1990 argued that evidence for this phenomenon *in situ* has not been obtained.

*S. aloides* is a species that is nowadays often restricted to nature reserves, where it is an important species. Observations on *S. aloides* stands showed clear water conditions in close proximity, but turbid water further away. The observations of clear water surrounding both macrophytes cannot completely be explained by processes like sedimentation, nutrient limitation or shading. Another process that also seems to be involved is allelopathy between photoautotrophs (e.g., Jasser 1995).

In summary, although we have indications of allelopathy for both macrophytes, previous studies showed contradicting results (Brammer 1979, Forsberg et al. 1990, Blindow & Hootsmans 1991, Jasser 1995). Therefore, new experiments were designed to study the role of allelopathy for both macrophytes, excluding alternative explanations like e.g. nutrient or light competition. Further, the differential response of phytoplankton species was tested and the allelopathic activity of the two macrophytes was compared. Additionally, the role and importance of allelopathy *in situ* were estimated with a model and finally experiments were performed to identify the allelopathic substances in *S. aloides* exudates and extracts.

### 1.2.1 Charophytes (*Chara sp.*)

Charophytes or stoneworts/brittleworts (Figs. 1.2 - 1.4) are members of the green algae, Chlorophyta, the largest, most diverse and structurally complex group of algae. The reproduction in charophytes is primarily sexual. The gametangia (Fig. 1.2d & 1.4b) develop in the axis at the nodes. Mature plants have series of multicellular nodes interspersed with long single cells (internodes). From these nodal cells, branches and branchlets develop. Several cells growing out of a node can form the protection of oogonia (female gametangia, Figs. 1.2d & 1.4b). They form a spiral around the oogonium. Male gametangia (antheridia) are developed from nodal cells (Figs. 1.2d & 1.4b). The positioning of the gametangia differs among different species. Identification of charophytes is often based on these characteristics (Bruinsma et al. 1998).



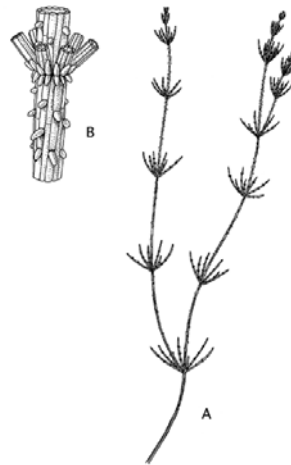
**Figure 1.2** *Chara globularis* var. *globularis*.

**A** normal habit.

**B** habit of deeper waters.

**C** stem with stipules.

**D** gametangia.

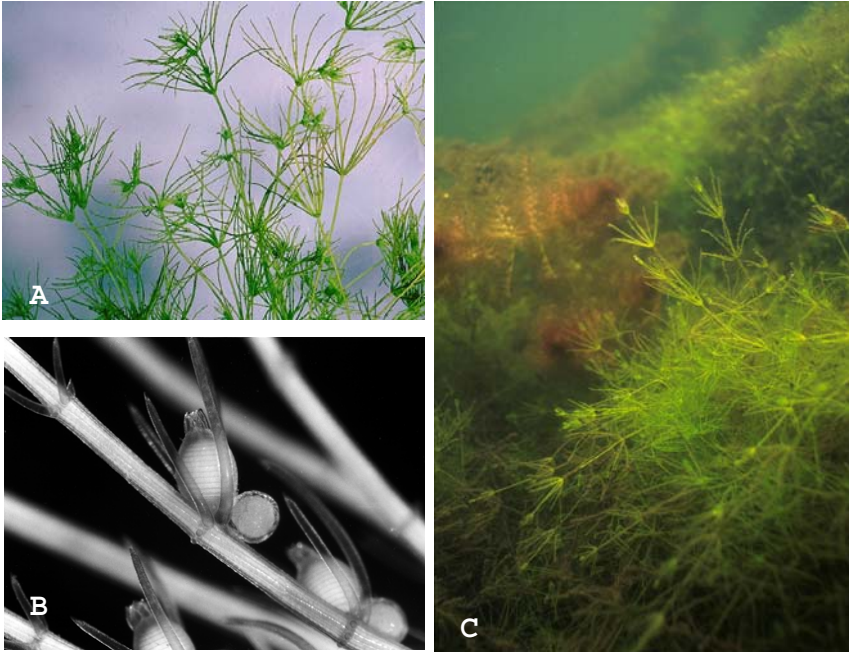


**Figure 1.3** *Chara contraria* var. *contraria*.

**A** normal habit.

**B** stem with stipules.

Figures: Bruinsma et al. 1998.



**Figure 1.4** Freshwater charophytes.

**A** *Chara* sp. close-up (<http://ag.arizona.edu/azaqua/aquaplants/photos/chara.jpg>).

**B** Oogonia (oval structures) and antheridia (round structures) of *Chara globularis* var. *globularis* (<http://www.kranswieren.nl/>).

**C** *Chara* sp.

Picture B: A. van Beem

Picture C: W. Kolvoort

Charophytes occur in a variety of habitats (Bruinsma et al. 1998) ranging from freshwater (lakes, ponds, streams) to brackish ecosystems (lagoons, estuaries), on all continents, except Antarctica. In general, charophytes grow in relatively nutrient-poor, stagnant to slightly running waters. They are bottom-dwelling plants (Pereya-Ramos 1981) that can form dense carpets (Blindow 1992a) concentrated near the sediment, anchored by rhizoids in sandy or muddy substrates.

Charophytes are important study objects in restoration and biomanipulation projects because they grow close to the sediment and because of their positive effect on water transparency. There is a great need for the return and expansion of charophytes in shallow lakes.

Some of the charophytes, especially the *Chara* species, have a very strong smell of sulphur-containing substances (like thiols). Because of the unpleasant smell, charophytes are also called *Skunkweed*. These sulphur-containing compounds are reported to have allelopathic properties against phytoplankton (Anthoni et al. 1980, Wium-Andersen et al. 1982, 1983). Moreover, observations of natural charophyte beds showed that they hardly contain any other macrophyte species and that they are rarely overgrown by epiphytes (Wium-Andersen et al. 1982, Wium-Andersen 1987). These observations support the idea that charophytes could possess allelopathic activity.

Contradictory to the findings of Wium-Andersen et al. 1982, Blindow (1987) recorded higher epiphyton densities on three charophyte species than on *Potamogeton pectinatus* in a lake in southern Sweden. This might suggest that allelopathy in charophytes is less likely or not always present. Also Forsberg et al. (1990) argued that allelopathy is unlikely to be of ecological importance under natural conditions. However, Mulderij et al. (2003, Chapter 3) and Berger & Schagerl (2003, 2004) provided new evidence for allelopathy in *Chara*. Among the different charophytes *Chara globularis* seems to have the most prominent allelopathic properties (Wium-Andersen et al. 1982, Blindow & Hootsmans 1991).

### 1.2.2 *Water soldier (Stratiotes aloides)*

*S. aloides* (Fig. 1.5) is a species native to Europe and Siberia, but it also appears in Central Asia (Cook & Urmi-König 1983). Fossil records of this plant go back to the Middle Eocene, 50 million years ago (Cook & Urmi-König 1983). *S. aloides* L. is a member of the *Hydrocharitaceae* (water weeds), just like frogbit (*Hydrocharis morsus ranae*), waterweeds (*Elodea*), eelgrass (*Vallisneria spiralis*) etc. Nowadays, *S. aloides* is the only surviving member of its genus that used to consist of 16 species (Cook & Urmi-König 1983).

*S. aloides* is a dioecious, perennial aquatic macrophyte that can grow vigorously and will tend to outcompete many plants when growing in small water systems (Bloemendaal & Roelofs 1988). It shows vegetative as well as generative propagation, but the first is the most frequently observed, although opinions of authors vary as to the vegetative reproduction of *S. aloides* (Kornatowski 1983/84). *S. aloides* produces turions and offsets that develop into new plants (Kornatowski 1979).



**Figure 1.5** *Stratiotes aloides* L.

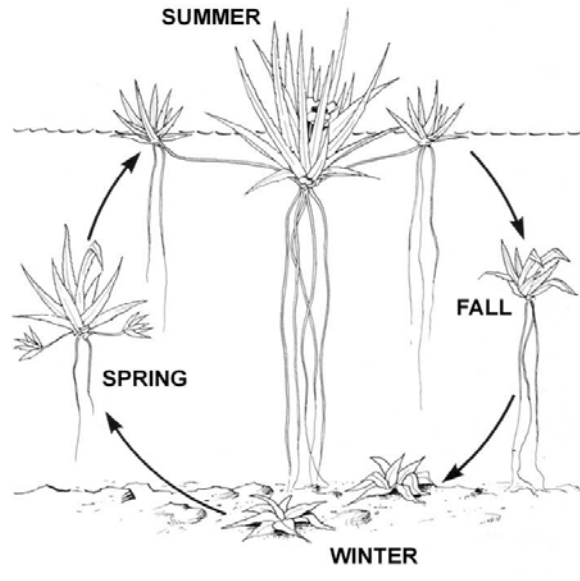
- A** *S. aloides* plants surrounded by filamentous algae.
- B** *S. aloides* stand in a ditch in Giethoorn (De Wieden), The Netherlands.
- C** Close up of *S. aloides* plant.
- D** Extremely dense *S. aloides* stand in the Danube Delta, Romania.

Pictures A – C: G. Mulderij

Picture D: A. Mauchamp

An important part of the life cycle of *S. aloides* is submerged (Bloemendaal & Roelofs 1988, Fig. 1.6). The plants overwinter as turions or entire (but rootless) plants on the lake bottom, where the decay of the macrophytes creates a thick layer of sapropel. The sinking process of the plants is probably mediated by increases in the weight of decaying leaves and decreases of photosynthesis in autumn (Cook & Urmi-König 1983). The macrophyte becomes buoyant in spring because of increased photosynthesis and remains floating on the water surface until autumn (De Geus-Kruyt & Segal 1973).

Removal of peat created numerous shallow lakes with peaty sediments (broads) in The Netherlands. Water soldier was a common species in these peat bog lakes. It is a characteristic species in the transition zone between submerged macrophytes and terrestrial vegetation. It occurs in relatively shallow (< 2m) lakes at sheltered places. This shelter protects the plants against wind and wave exposure and causes accumulation of the sapropel, stimulating the growth of the macrophyte.



**Figure 1.6** Life-cycle of *Stratiotes aloides* L. (Bloemendaal & Roelofs 1988).

*S. aloides* is a common species in Polish lakes (Strzałek 2004) and used to be very abundant in The Netherlands, but in a great number of Dutch canals, broads and ditches, *S. aloides* stands declined dramatically (Higler 1977) due to multiple environmental stress factors, such as eutrophication, iron limitation, sulphide and ammonium toxicity etc. (Roelofs 1991, Smolders et al. 1996, 2003) or due to the regulation of rivers (Ořahel'ová & Banášová 1997). In some areas where the inlet of alkaline sulphate-rich river water has strongly decreased, the species has recovered re-

markably since the end of the 1990's. Other factors that seem to control the growth of *S. aloides* are water temperature, water depth, type of substrate, and severity of winter (Erixon 1979).

Apart from playing an important role in terrestrialization processes, stands of *Stratiotes* also form an essential habitat for some fauna species: e.g. *Aeshna viridis*, an endangered dragonfly species with a larval distribution strongly associated with lakes where *S. aloides* occurs (Rantala et al. 2004). Suutari et al. (2004) demonstrated that the plants act as oviposition substrate for *A. viridis* females. Larvae of this dragonfly were almost exclusively found in patches of *S. aloides* (Rantala et al. 2004), where they were less susceptible to predation by fish (e.g., European Perch, *Perca fluviatilis*) than larvae on other aquatic macrophytes. So, *S. aloides* may facilitate the distribution of *A. viridis*.

Moreover, the decline of the number of Black terns (*Chlidonias niger*) in Europe, is attributed to the previously described strong decline of *S. aloides*, the Black terns most favoured nesting substrate (Kreidel 1984, Beintema 1997). Prevention of a further decline in the number of *S. aloides* stands may prevent extinction of *Aeshna viridis* and thereby stabilizes populations of Black tern, as these dragonflies occur in chick-diets of Black terns (Baarspul et al. 1997).

Compared with other submerged macrophytes, the role of *S. aloides* in the inhibition of phytoplankton growth is still poorly understood. As the life cycle of this macrophyte is mainly submerged (Fig. 1.6), allelopathy may be a useful strategy in the competition for light with, for example, phytoplankton. During the submerged phase, the macrophyte has to compete with phytoplankton for light, while this competitive interaction is less strong when the plant floats on the water surface, later in the season. Brammer (1979) studied the exclusion of phytoplankton in the proximity of *S. aloides*, but was not able to show unequivocally that allelopathic substances caused the low phytoplankton densities close to *S. aloides* plants. According to Brammer (1979), the availability of potassium or sodium rather than the presence of an inhibiting substance released by *S. aloides* might be the most influential growth-limiting factor for phytoplankton. In addition to potassium depletion, also co-precipitation of phosphates with calcium has been considered as an important mechanism that inhibits phytoplankton growth in the proximity of *Stratiotes* (Brammer 1979, Brammer & Wetzel 1984).



Others who studied the allelopathic activity of *S. aloides* are Jasser (1995) and Usenko et al. 2002. Extracts of this macrophyte showed inhibitory effects on phytoplankton growth that became stronger when the extract concentration increased (Jasser 1995). Usenko et al. (2002) showed that phenolic acids, present in the tissue of *Stratiotes*, may cause allelopathic effects on phytoplankton. These studies, however, do not provide evidence that allelopathic substances are excreted by the macrophyte and subsequently inhibit phytoplankton growth. The use of exudates or cell-free filtrates (instead of extracts) appears to be a better alternative for experiments to demonstrate allelopathic effects of macrophytes on phytoplankton. Chapters 4 & 5 (Mulderij et al. 2005a, 2005b) describe laboratory experiments with *S. aloides* exudates that inhibit the growth of several phytoplankton species grown under different conditions.

### 1.3 Allelopathy background

*Allelopathy* is often defined as direct or indirect effect (stimulatory or inhibitory) of one species, including micro-organisms, on adjacent organisms, through the production of chemical compounds that are subsequently released into the environment (Molisch 1937). Allelopathy generally implies a negative interaction (e.g., Lampert & Sommer 1997, Putnam & Tang 1986), but allelochemicals can also exert a beneficial influence (Rice 1984, 1995, Callaway 1995). In this thesis I will mainly consider harmful (Chapter 4 - 8), but also some beneficial (Chapter 9) effects of allelopathic substances from macrophytes on the growth and development of algae and cyanobacteria.

The term allelopathy was coined by Molisch in 1937, but the first observations on chemical interactions between plants date back to ca. 300 B.C., when Theophrastus observed effects of chickpea on other plants (*sensu* Weston & Duke 2003). Thereafter (ca. 1 A.D.), Pliny observed harmful effects of several plants on cropland and recorded allelopathic effects of walnut trees (*sensu* Vyvyan 2002). The International Allelopathy Society (1996) redefined allelopathy as any process involving secondary metabolites, produced by plants, algae, bacteria and/or fungi, that influence the growth and development of biological and agricultural systems. The term *Allelopathy* may, however, be used for too many processes, since it was also used for biochemical interactions between aquatic plants and animals and among animals (Gross 1999). Further, Rizvi & Rizvi (1992) suggested to add predator-prey interactions and Gopal & Goel (1993) used the term to encompass a wide array of inter-

actions, i.e. those between terrestrial plants, aquatic macrophytes, plant litter, phytoplankton, bacteria and macroinvertebrates. Other authors argued against including animal interactions. Inderjit & Dakshini (1995) included only plants and microbes, while Legrand et al. (2003) defined allelopathy strictly as chemical interaction among competing micro-algae and bacteria in which one or more allelochemicals inhibit the growth of competing algae or bacteria. I do not include interactions with animals in the term *Allelopathy*, and focus on the effects of chemical compounds from one primary producer on another.

In aquatic ecosystems, Harder (1917, *sensu* Inderjit & Dakshini 1994) was the first to note algal allelopathy. Later, Akehurst (1931) suggested that algal allelopathy plays a role in the succession of phytoplankton. Research on allelopathic interactions was initially conducted mainly by botanists who studied the effect of one plant on the other, but recent allelopathy researches aim more at elucidating which specific natural products cause allelopathic interactions and what their ecophysiological impacts are (Einhellig 1995). Allelopathy research is becoming more interdisciplinary, and now involves organic chemists, biologists and ecologists.

Plants may interfere with each other through chemical interaction and through resource depletion. As these processes may act simultaneously, it may be difficult to prove the role of allelochemicals in such interactions, even if the responsible allelochemical has been chemically identified (Putnam & Tang 1986, Inderjit & Del Moral 1997). Therefore, many scientists have questioned the existence of allelopathy (Forsberg et al. 1990, Begon et al. 1995). Nevertheless, many scientists still consider the importance of allelopathic interactions to be important and outline arguments in favour of allelopathy in a series of reviews (e.g., Gross 1999, 2003a, Van Donk & Van de Bund 2002).

### **1.3.1 Terrestrial allelopathy**

Allelopathy has been observed in many ecosystems and ecological situations, in all stages of succession, from pioneer (Rice 1972) to climax stages (Rice & Pancholy 1973, 1974). Such interactions have been reported worldwide, from temperate regions to the (sub-)tropics, in a range of ecosystems such as deserts (Bennett & Bonner 1953), savannas (Parvez et al. 2003), prairie ponds (Crawford 1979), biofilms on rocks (Jüttner & Wu 2000), rice fields (Hirai et al. 2000) and other agricultural ecosystems (Kato-Noguchi 2003). Extensive allelopathy research has been carried out with terrestrial plants (e.g., Putnam & Tang 1986, Inderjit & Dakshini 1994). Studies on allelopathic interactions are much more common in terrestrial than in aquatic

ecosystems, because of the economical importance of crop protection and agriculture (Whittaker & Feeny 1971).

### 1.3.2 *Aquatic allelopathy*

Compared with terrestrial allelopathy, we are just starting to understand how allelopathy can play a role in aquatic ecology. An increasing number of studies, however, show the importance of aquatic allelopathy and the identification of allelopathic compounds in these systems.

Studies on ecological interactions in aquatic ecosystems mediated by chemical cues have often been carried out in marine ecosystems. The most intensively studied allelopathic interaction in marine habitats is the interaction between phytoplankton species (Legrand et al. 2003), but marine angiosperms, macroalgae and corals are also found to excrete allelopathic substances (e.g., Harrison & Chan 1980, Thacker et al. 1998, Jin & Dong 2003). Investigations of allelopathy in brackish waters are scarce and describe interactions between phytoplankton and macrophytes (e.g., Van Vierssen & Prins 1985, Della Greca et al. 2000) and among phytoplankton species (Fistarol et al. 2003). Allelopathic interactions in marine and brackish ecosystems will not be discussed in detail in this thesis.

Although freshwater chemical ecology lags behind terrestrial and marine chemical ecology (Burks & Lodge 2002), the number of studies in freshwater ecosystems is increasing. In these ecosystems, macrophytes (emergent, floating and submerged), macroalgae, microalgae and cyanobacteria seem to affect neighbouring aquatic organisms with the excretion of chemical cues.

In freshwater ecosystems four different allelopathic interactions between or among macrophytes (MPH) and phytoplankton (PHY) as primary producers are observed: MPH→MPH (effect of one macrophyte on another), PHY→PHY, PHY→MPH and MPH→PHY. The studies presented in this thesis deal with the allelopathic effect of macrophytes on phytoplankton (MPH→PHY) in freshwater ecosystems, and therefore I will only give a brief overview of current knowledge on this interaction in the following in this sections.

Many species of aquatic macrophytes are known to have allelopathic effects on phytoplankton growth (Table 1.1, page 38). *Myriophyllum* (e.g., Nakai et al. 2000, Leu et al. 2002), *Ceratophyllum* (e.g., Körner & Nicklisch 2002, Gross et al. 2003b),

and *Chara* (e.g., Berger & Schagerl 2003, 2004, Mulderij et al. 2003, Chapter 3) are submerged macrophyte genera that frequently are subject of allelopathy studies.

*S. aloides* has never been a popular macrophyte in allelopathy studies. The few studies carried out on this macrophyte only suggest rather than prove allelopathic interactions between the macrophyte and phytoplankton (Brammer 1979, Jasser 1994, 1995). A later study demonstrated that phenolic acids from *S. aloides* may be responsible for inhibition of phytoplankton growth (Usenko et al. 2002). More detailed research on this macrophyte is presented in Chapters 4 - 9.

Not only submerged aquatic macrophytes seem to have allelopathic activity (Table 1.1, page 38). The emergent macrophytes *Juncus effusus* and *J. acutus*, for example, inhibited the growth of the green alga *Selenastrum capricornutum* (Della Greca et al. 1996, 2003). Ervin & Wetzel (2000), however, showed that rather than affecting other organisms, *Juncus* exudates were autoinhibitory to *Juncus* seedlings. Another emergent macrophyte, *Typha domingensis*, produced allelochemicals against *Salvinia minima* (Gallardo-Williams et al. 2002).

## **1.4 Which substances cause allelopathic effects?**

From studies on terrestrial allelopathy it is known that many different chemicals, such as phenolics, terpenoids, alkaloids, polyacetylenes, fatty acids, peptides can cause allelopathic effects: (e.g., Whittaker & Feeny 1971, Rizvi et al. 1992). Although not studied in as much detail as in terrestrial ecosystems, there is a wide variety of substances with allelopathic activity in aquatic ecosystems (Gopal & Goel 1993, Gross 1999, 2003a). According to Wolfe (2000) chemical interactions in water are an important part of the ecology of aquatic organisms. This section, focusses on allelochemicals with special emphasis on those originating from macrophytes.

The most common substances, responsible for allelopathic effects, are phenolic compounds and they seem to be effective herbivore deterrents in terrestrial (Feeny 1970, Hagermann & Butler 1991), marine (Steinberg & Van Altena 1992), and freshwater ecosystems (Nakai et al. 2000, Usenko et al. 2002). Della Greca et al. (1992, 1996, 2003) observed substances like phenylpropanoids, phenalene metabolites, phenantrenoids, phenylpropane glycerides, dihydroxyphenanthrene, and tetrahy-

dropyrene with algicidal activity in *Acorus gramineus*, *Eichhornia crassipes*, *Juncus acutus*, and *J. effusus*, respectively.

*Myriophyllum*, a common aquatic macrophyte, contains algicidal polyphenolic compounds (Planas et al. 1981, Nakai et al. 2000, 2001) of which gallotannins and ellagitannins were most dominant (Gross et al. 1996, Leu et al. 2002). Several studies (Gross et al. 1996, Leu et al. 2002) have demonstrated the effect of an allelopathic substance (*Tellimagrandin II*) from *Myriophyllum spicatum* – a substance that has also been extracted from *Myriophyllum brasiliense* (named with the synonym *Eugeniin*, Saito et al. 1989). Another macrophyte species, *M. verticillatum*, affected the growth of the cyanobacterium *Synechococcus leopoliensis* with three phenylpropanoid glucosides (Aliotta et al. 1992).

Phenolics, however, are not the only group of secondary metabolites with allelopathic activity. In studies with microalgae, identification of allelopathic substances showed that the inhibitory substances were polyunsaturated fatty acids, amino acids, and organic acids (e.g., Proctor 1957, Gross et al. 1991). Elakovich & Yang (1996) reported that alkaloids of the floating-leaved macrophyte *Nuphar lutea* (nupharolutine and 6,6'-dihydroxythiobinupharidine) were responsible for growth limiting effects on another floating-leaved macrophyte: *Lemna minor*.

The non-rooting aquatic macrophyte *Ceratophyllum demersum* (Rigid Hornwort) inhibited phytoplankton photosynthesis possibly by excreting labile sulphur compounds (Wium-Andersen et al. 1983). *Chara globularis* also contains biologically active sulphur compounds (dithiolane and trithiane) with algicidal properties (Anthoni et al. 1980). Wium-Andersen et al. (1982) isolated similar substances from charophytes in freshwater and brackish water areas infested with *Chara baltica*, *C. hispida*, *Nitella translucens*, and *Tolypella nidifica* and showed that dithiolane and trithiane affected the growth of an epiphytic diatom and a natural phytoplankton population. More recently, Berger & Schagerl (2003) showed that two new allelopathic compounds with lipophilic characteristics in extracts of the charophyte *C. aspera* were responsible for the growth inhibition of cyanobacteria. Allelopathy studies with extracts, however, do not provide evidence for the excretion of allelopathic substances by the donor. Mulderij et al. (2003, Chapter 3) conducted experiments with exudates of *Chara* and phytoplankton to prove that allelopathic compounds are indeed excreted by the charophytes.

For *S. aloides* little is known about the excretion of allelopathic substances that may inhibit phytoplankton growth. Smolders et al. (2000) showed that, compared with other aquatic macrophytes, *S. aloides* tissue has a low content of phenolic compounds. Later, Usenko et al. (2002) reported these compounds cause inhibitory effects on phytoplankton growth. Chapters 4 & 5 describe experiments with *S. aloides* exudates and phytoplankton and in Chapter 9 the first results from the chemical analysis of allelopathic compounds from *S. aloides* are presented (Chapter 9).

## 1.5 Possible modes of action

Rizvi et al. (1992), who reviewed current knowledge on terrestrial allelopathy, demonstrated many modes of action in these habitats. In section 1.4 it was noted that in aquatic ecosystems a great diversity of substances can cause allelopathic effects on target organisms. It will be rather simplistic to assume a common mode of action when the diversity of substances with allelopathic activity is so large. Single substances could be responsible for allelopathic effects, but it may be more realistic to assume additive or synergistic inhibition from combinations of allelochemicals (Inderjit et al. 2002, Berger & Schagerl 2003).

More information on the mode of action of allelopathic substances has now become available. Some substances (e.g., phenolic compounds) act as effective inhibitors of microalgal exoenzymes, such as alkaline phosphatase (Gross et al. 1996, Gross 1999). Many algae have alkaline phosphatase, an enzyme that makes organic phosphorus available for uptake when orthophosphate is limiting.

Flores & Wolk (1986) showed that filamentous nitrogen-fixing cyanobacteria can directly kill related strains. *Chlorella*, *Cosmarium*, *Pediastrum*, *Phormidium* and *Scenedesmus* were reported to be killed in the presence of *Aphanizomenon gracile* (Lefèvre 1950, *sensu* Legrand et al. 2003). Similarly, the freshwater dinoflagellate *Peridinium bipes* caused damage and subsequently cell death of the cyanobacterium *Microcystis aeruginosa* (Wu et al. 1998). *Peridinium aciculiferum*, an other dinoflagellate, inhibited the growth and caused blistering and lysis in the cryptophyte *Rhodomonas lacustris* (Rengefors & Legrand 2001).

Inhibition of photosynthesis is a widespread mode of action caused by different aquatic allelochemicals. Such chemicals released from cyanobacteria or higher plants were often shown to inhibit the photosynthesis of target organisms (e.g., Smith & Doan 1999). Some cyclic sulphur compounds (Wium-Andersen et al. 1982, Wium-Andersen 1987), trithiane and dithiane (Anthoni et al. 1980) originating from *Chara* inhibited the photosynthesis of the diatom *Nitzschia* and natural phytoplankton. Allelopathic compounds often interfere with photosystem II (Gross et al. 1991). Körner & Nicklisch (2002) showed that the submerged aquatic macrophytes *Myriophyllum spicatum* and *Ceratophyllum demersum* inhibited the PS II activity of phytoplankton. *M. spicatum* (Leu et al. 2002) and the cyanobacterium *Trichormus doliolum* (Von Elert & Jüttner 1996) inhibited the photosynthetic electron transport and oxygen production of cyanobacteria and other photoautotrophs. Inhibition of the photosynthetic electron transport in PSII has been described more often (e.g., Gleason & Case 1986; Srivastava et al. 1998). Allelopathic effects on photosynthesis were also observed for *Oscillatoria latevirens* (Bagchi et al. 1993), *Scytonema hofmanni* (Mason et al. 1982), and *Pandorina morum* (Harris & Caldwell 1974). The benthic cyanobacterium *Fischerella muscicola* excretes allelochemicals (*Fischerellin A* and *B*) that inhibit the growth of other cyanobacteria in similar ways as described in the previous studies (Papke et al. 1997, Srivastava et al. 1998).

The motile green alga *Chlamydomonas reinhardtii* was shown to settle to the bottom due to paralysis, when exudates (containing anatoxin-a and microcystin-LR) from the cyanobacterium *Anabaena flos-aquae* were added (Kearns & Hunter 2001). Algal extracellular products from *C. reinhardtii*, in turn, inhibited heterocyst spacing of *A. flos-aquae* and regulate the toxin production of this cyanobacterium (Kearns & Hunter 2000). Another allelopathic substance (cyanobacterin) produced by *Scytonema hofmanni* was shown to disrupt the thylakoid membrane structure in *Euglena gracilis* (Gleason 1990). Finally, allelopathic substances can negatively influence shoot formation and elongation of angiosperms (Kulshretha & Gopal 1983, Frank & Dechoretz 1980).

## 1.6 Differential phytoplankton sensitivity and ecological relevance of allelopathy

Limnologists have been intrigued by the pronounced seasonal fluctuations in the abundance and composition of phytoplankton in aquatic ecosystems. Environmental conditions (e.g., nutrient limitation) influence both donor and target species (Section 1.7), but also without apparent abiotic limitations we find differences in sensitivity to allelopathic substances, because different target species may show varying responses to the same allelochemical (see Chapters 3 & 5).

Cyanobacteria are reported to be more sensitive to allelochemicals than chlorophytes (e.g., Planas et al. 1981, Körner & Nicklisch 2002). In a study with aqueous extracts of the aquatic macrophyte *Ceratophyllum demersum* and natural phytoplankton, Jasser (1995) showed a decline of the biomass of cyanobacteria, but a simultaneous increase in the biomass of chlorophytes. Gross et al. (1991) who showed that the allelochemical, *fischerellin*, produced by three species of the cyanobacterium *Fischerella*, inhibited other cyanobacteria (*Anabaena*, *Synechococcus*, *Phormidium*, *Synechocystis*) and, to a lesser extent, chlorophytes (*Ankistrodesmus*, *Nannochloris*, *Scenedesmus*). Later, Gross et al. (1996) described that extracts of *Myriophyllum spicatum* inhibited various filamentous and coccoid cyanobacteria (*Anabaena*, *Synechococcus* and *Trichormus*) more strongly than chlorophytes (*Scenedesmus*, *Stigeoclonium*). Also, Usenko et al. (2002) observed that species of Cyanophyta were inhibited by exometabolites from macrophytes like *Ceratophyllum*, *Potamogeton*, *Stratiotes*, *Trapa* etc., but species of Chlorophyta and Bacillariophyta were stimulated. Leu et al. (2002), on the contrary, showed that both cyanobacteria (*Anabaena* and *Synechococcus*) and the chlorophyte *Chlamydomonas* were equally sensitive to allelochemicals from *M. spicatum*. Recently, Mulderij et al. (2005b, Chapter 5) studied the sensitivity of cyanobacteria and chlorophytes to exudates from *S. aloides*.

So, differential sensitivity of phytoplankton was observed when species of different functional phytoplankton groups (e.g., cyanobacteria, diatoms, green algae) were compared, but it also exists between species that belong to the same group. Kogan & Chinnova (1972) showed differential sensitivity between the three cyanobacteria *Anabaenopsis intermedia*, *Anabaena karakumica*, and *A. robusta* to exudates from *Ceratophyllum demersum*. Also Gross et al. (2003b) showed that *Anabaena* sp. and *Synechococcus elongatus* were more sensitive to *Ceratophyllum* extracts than *A. variabilis*. Berger & Schagerl (2003) conducted experiments with nine species of cyanobacteria and showed that *Microcystis aeruginosa* and *Cylindrospermum* sp. were



resistant to substances from *C. aspera*, while other cyanobacteria (*Anabaena* spp. and *Microcystis flos-aquae*) were not. Later, they (Berger & Schagerl 2004) demonstrated that *M. aeruginosa* and *Cylindrospermum* sp. were not resistant to *C. aspera*. Nakai et al. (1999) showed that the cyanobacterium *Microcystis* was more sensitive to substances from *Myriophyllum spicatum* and *Ceratophyllum demersum* than the cyanobacterium *Anabaena*. Körner & Nicklisch (2002) showed that *Microcystis*, was more sensitive to *M. spicatum* than the cyanobacterium *Aphanizomenon*. The green algae *Chlorella* and *Selenastrum*, showed another response than *Scenedesmus*, when exposed to exudates from a mixture of *Chara globularis* and *C. contraria* (Mulderij et al. 2003, Chapter 3). Blindow & Hootsmans (1991) showed that *Chara globularis* exudates inhibited the growth of the green alga *Scenedesmus*, but apparently they cannot inhibit *Ankistrodesmus*.

Both Keating (1977, 1978) and Rojo et al. (2000) report allelopathic activity of macrophyte exudates which may influence the dynamics of phytoplankton populations. Differential sensitivity of phytoplankton, within and between functional phytoplankton groups, may therefore favour other phytoplankton species and thereby influence species succession (Körner & Nicklisch 2002, Legrand et al. 2003). An example is the alternating dominance of the dinoflagellate *Peridinium* and the cyanobacterium *Microcystis* in the Sea of Galilee (Vardi et al. 2002.).

Allelopathy was also mentioned in management approaches for natural and agricultural ecosystems (e.g., Rizvi et al. 1992, Einhellig 1995, Anaya 1999). Pesticides could successfully be replaced by biological agents (allelochemicals). According to Legrand et al. (2003), little success has been achieved with such replacements in aquatic ecosystems, what is perhaps related to the instability of allelopathic compounds (Nakai et al. 1999). For controlling blooms of cyanobacteria, however, Howard et al. (1996) suggested several management approaches including killing of cyanobacteria with chemical and biological agents. Although they mentioned chemical and biological agents, they specify only a chemical agent (e.g., copper sulphate), that causes disintegration of cyanobacterial blooms. Other ways to control cyanobacterial blooms may be addition of leaf litter (Ridge et al. 1999) or decomposing (barley) straw (Newman & Barrett 1993, Pillinger et al. 1994). Our present state of understanding of allelopathy, with special emphasis on the use of allelochemicals in biological control of nuisance species and its consequences, is still too fragmentary.

## 1.7 Allelopathy and environmental influences

The importance of the singular roles of macrophytes in shallow aquatic ecosystems, described in Section 1.1 and Chapter 2, changes with plant species, composition, and density, but also lake morphology, nutrient status and climate can play a role (e.g., Moss et al. 1997, Scheffer 1998, Jeppesen et al. 1999a). Dynamics and concentrations of allelochemicals change with season or time and spatially (e.g., Blindow & Hootsmans 1991, Gross 2000). Stress factors may cause increased production of allelochemicals (e.g., Tang et al. 1995, Reigosa et al. 1999, Granéli & Johansson 2003), but can also make target organisms more sensitive to allelochemicals (e.g., Einhellig 1995, Tang et al. 1995). In both cases the result is an enhanced allelopathic effect on the target species.

Light and nutrient limitation of target (Granéli & Johansson 2003) and donor (Rengefors & Legrand 2001) species are often mentioned as important factors influencing the extent of allelopathic effects (Ervin & Wetzel 2003). Fitzgerald (1969) studied the competition or antagonism among bacteria, algae and aquatic weeds and showed that nitrogen limitation and not phosphorus limitation of the donor species stimulates allelopathy. Gross (2003b) described that total bioactive hydrolysable tannin levels in the submerged freshwater angiosperm *Myriophyllum spicatum* were highly influenced by light, while nitrogen availability had an effect on tellimagrandin II levels, but not on total bioactive hydrolysable tannin levels. Ray & Bagchi (2001) showed a negative relationship between the addition of phosphate or magnesium and the production of an algicide by the cyanobacterium *Oscillatoria laetevirens*. Earlier, Wu et al. (1991) studied the production of geosmin by *Anabaena*. Cells without any artificial supply of nitrogen (only gaseous nitrogen) produced more geosmin than cells that received excess nitrogen.

The allelopathic activity of the planktonic cyanobacterium *Trichormus doliolum* was shown to be affected by phosphorus (Von Elert & Jüttner 1997). The release of allelochemicals increased 30-fold under P-limited growth of the cyanobacterium. Wu & Jüttner (1988) demonstrated that, rather than nutrient depletion, the growth rate of *Fischerella muscicola* was important for the synthesis of allelochemicals. However, Rengefors & Legrand (2001) who cultured the dinoflagellate *Peridinium*, observed that it may sometimes be difficult to determine whether nutrient limitation or growth limitation enhances allelochemical production.

Contradictory to the previously described observations, Gross et al. (1994) suggested that neither phosphorus nor nitrate, nor light limitation increased the production of allelochemicals in the cyanobacterium *Fischerella*. An energy shortage caused by nitrate depletion or light limitation resulted in a dramatic decrease of internal concentrations of allelochemicals.

Von Elert & Jüttner (1996) suggested that target organisms, like cyanobacteria and chlorophytes, are more susceptible to allelochemicals from the cyanobacterium *Trichormus doliolum* under light limited conditions. Later, however, Von Elert & Jüttner (1997) demonstrated that phosphorus and not light was the controlling factor. Experiments of Mulderij et al. 2005b (Chapter 5) showed that the light intensity for culturing phytoplankton influenced the sensitivity of phytoplankton to allelopathic substances of the macrophyte *S. aloides*.

As indicated by Gross (2003b) light and also processes like oxidation, polymerisation, or cleavage may influence the stability of allelochemicals. The study by Twist & Codd (1997) is an example of light as a factor influencing the stability of allelopathic compounds. They investigated the stability of the cyanobacterial hepatotoxin, *nodularin*, and observed photodegradation of the allelochemical after exposure to ultraviolet radiation. The pH may also influence the production of allelochemicals. For *Oscillatoria laetevirens*, Ray & Bagchi (2001) showed that pH negatively affects the algicidal production. Monahan & Trainor (1970) found that the green alga *Hormotila* stimulated the growth of another green alga (*Scenedesmus*) at pH 6.3, but inhibited it at pH 7.7. Other examples of pH dependent allelopathic activity are from studies on marine phytoplankton species (Legrand et al. 2003). In some cases, the temperature also influences allelopathic activity, as revealed by studies with terrestrial crops (Reigosa et al. 1999) and investigations of marine environments (Legrand et al. 2003).

## 1.8 Thesis outline

This thesis mainly focusses on the allelopathic effect of two structurally different macrophytes on phytoplankton growth. Chapter 2 gives an overview of current knowledge on the role of aquatic macrophytes (floating, submerged and emergent) on food webs in shallow aquatic ecosystems.

The experimental studies in this thesis describe the role of two typically rapid colonizers, the macrophytes *Chara* sp. and *S. aloides*. The project started with basic experiments to identify the inhibitory effect of *Chara* on phytoplankton growth. Previous studies showed that *Chara* (in particular *C. globularis*) can inhibit phytoplankton growth. Most of those studies were, however, performed with extracts of aquatic macrophytes. The use of extracts indicates the presence/production of allelopathic substances in/by macrophytes, but it does not *per se* mean that they are indeed excreted. To show that allelopathic substances are really active after production and excretion, we conducted experiments with macrophytes exudates or cell-free filtrates of the macrophyte culture water.

Chapter 3, describes the response of three green algae to semi-continuous addition of cell-free filtrates from a mixed culture of *Chara contraria* and *C. globularis*. The exudates from *Chara* can influence phytoplankton growth in two ways: changes in the duration of the lag phase (i) and the growth rate (ii). With our experimental set-up, we were able to demonstrate allelopathic effects of *Chara* spp. on phytoplankton.

Then several experiments with *S. aloides*, were performed. Chapter 4 reports on the allelopathic effect of the aquatic macrophyte, *S. aloides*, on the green alga *Scenedesmus obliquus*. *S. aloides* not only influenced the length of the lag phase, but also changed the exponential growth rate and affected the morphology of *Scenedesmus*.

Natural phytoplankton communities do not only contain green algae. Therefore, we also tested the allelopathic activity of *S. aloides* exudates on different phytoplankton species (green algae, toxic and non-toxic cyanobacteria), as described in Chapter 5. Moreover, we tested the effect of light intensity on the sensitivity of phytoplankton to allelopathic substances from *S. aloides*. With this experimental set-up we could also compare the responses of phytoplankton species and strains.

Laboratory experiments only, will not provide answers about the impact of allelopathy in natural habitats. Therefore, [Chapter 6](#) focussed on the allelopathic activity of role of *S. aloides* on the growth of phytoplankton in a field survey and in incubation experiments conducted *in situ*.

In addition to phytoplankton, filamentous algae also seem to be affected by exudates from *S. aloides*. Field observations showed gaps free of filamentous algae surrounding *S. aloides* plants. Experiments with artificial macrophytes showed the relative importance of chemical and physical processes that may cause the gaps free of filamentous algae. These experiments are described in [Chapter 7](#).

A review of the literature showed that only a few ecological models show the importance of allelopathic interactions. These models are often too theoretical and only consider interactions between phytoplankton species. With the knowledge gained from preceding studies, we developed a model to get insight into the effects of macrophyte allelopathy on phytoplankton populations in natural shallow aquatic ecosystems. [Chapter 8](#) reports the contribution of allelopathy in the field, analysed with a model that simulated the single and combined effects of shading, resuspension, nutrient competition, and allelopathy on the development of phytoplankton.

[Chapter 9](#) deals with a first attempt to chemically characterize allelopathic substances from *S. aloides*. With biotests (agar diffusion assays & liquid culture tests) and solid phase extractions (SPE) we attempted to identify allelopathic substance(s) from *S. aloides*.

In [Chapter 10](#) I discuss the major conclusions of this thesis and the importance of allelopathy in shallow aquatic ecosystems. In this concluding part of the thesis I also propose follow-up research studies.

**Table 1.1** List of allelopathically active aquatic macrophytes (submerged and emergent).

Genus/species	Evidence <sup>a</sup>	Identified compound(s)	Active against	Mode of action	References
<i>Acorus gramineus</i>	L	Phenylpropanoids	Algae and cyanobacteria	Growth	Della Greca et al. 1989
<i>Alternanthera philoxeroides</i>	–	–	<i>Brassica campestris</i> , <i>Oryza sativa</i>	–	Paria & Mukherjee 1981 <sup>b</sup>
<i>Azolla caroliniana</i>	L*	Unknown	<i>Lemna paucicostata</i>	Growth	Sutton & Portier 1989
<i>Berula erecta</i>	L*	Falcarindiol 1 and 2, or unknown	<i>Nitzschia palea</i>	Photosynthesis	Wium-Andersen et al. 1987
<i>Brasenia schreberi</i>	L*	Unknown	9 Cyanobacteria, lettuce Duckweed, lettuce	Growth	Elakovich & Wooten 1987 Elakovich & Wooten 1989
<i>Cabomba caroliniana</i>	L/L*	Unknown	Cyanobacteria, duckweed, lettuce, <i>Hydrilla verticillata</i>	Growth	Nakai et al. 1999 Elakovich & Wooten 1989 Jones 1993 <sup>b</sup>
<i>Carex sp.</i>	L/F	Unknown	<i>Aldrovanda vesiculosa</i>	Growth	Kaminski 1987
<i>Carex hudsonii</i>	L	Unknown	<i>Phragmites communis</i>	Growth in coexistence cultures	Szczepańska 1977
<b><i>Chara</i></b>					
<i>Chara sp.</i>	L*	Trithiane	<i>Nitzschia palea</i>	Photosynthesis	Wium-Andersen 1987
<i>C. aspera</i>	L*	Unknown	Phytoplankton	Growth	Berger & Schagerl 2003, 2004 Van Donk & Van de Bund 2002
<i>C. contraria</i>	L*	Unknown	Cyanobacteria	Growth	Berger & Schagerl 2004
<i>C. delicatula</i>	L*	Unknown	Cyanobacteria	Growth	Berger & Schagerl 2004
<i>C. fragilis</i>	L*	Unknown	Cyanobacteria	Total biomass decline	Jasser 1995

<sup>a</sup> L = laboratory study, F = field study, L/F = both, \* only extract tested.

<sup>b</sup> *sensu* Elakovich & Wooten 1995.

Table 1.1 Continued

Genus/species	Evidence <sup>a</sup>	Identified compound(s)	Active against	Mode of action	References
<i>C. globularis</i>	L, F L*	Dithiane, trithiane unknown	Diatoms (cultures), natural phytoplankton, <i>Scenedesmus communis</i> Cyanobacteria chlorophyta, bacillariophyceae	Photosynthetic carbon fixation Growth	Anthoni et al. 1980 Berger & Schagerl 2004 Blindow & Hootsmans 1991 Wium-Andersen et al. 1982
<i>C. globularis/</i> <i>C. contraria</i>	L	Unknown	Two green algae	Growth	Mulderij et al. 2003, Chapter 3
<i>C. hispida</i>	F	Unknown	Phytoplankton	Growth	Horecka 1991
<i>C. rudis</i>	L*	Unknown	<i>Anabaena cylindrica</i> , <i>Cylindrospermum</i> sp.	Growth	Berger & Schagerl 2004
<i>C. tomentosa</i>	L*	Unknown	<i>Anabaena torulosa</i> , <i>Aphanizomenon flexuosum</i>	Growth	Berger & Schagerl 2004
<i>C. vulgaris</i>	F/L	Unknown	Phytoplankton Cyanobacteria	Growth	Crawford 1979 Berger & Schagerl 2004
<i>Ceratophyllum</i> <i>demersum</i>	L, F, L*	Elemental sulfur, labile sulphur compound and/or unknown	Phytoplankton, <i>Nitzschia palea</i> , cyanobacteria, lettuce, <i>Hydrilla verticillata</i> , <i>Myriophyllum spicatum</i> , <i>Lepidium sativum</i>	Photosynthetic carbon fixation, PS II activity, photosynthesis, growth	Elakovich & Wooten 1989 Gross et al. 2003b Jasser 1994, 1995 Jones 1993 <sup>b</sup> Kleiven & Szczepańska 1988 Kogan & Chinnova 1972 Körner & Nicklisch 2002 Mjelde & Faafeng 1997 Nakai et al. 1999 Wium-Andersen et al. 1983 Wium-Andersen 1987

<sup>a</sup> L = laboratory study, F = field study, L/F = both, \* only extract tested.<sup>b</sup> *sensu* Elakovich & Wooten 1995.

Table 1.1 Continued

Genus/species	Evidencea	Identified compound(s)	Active against	Mode of action	References
<i>Egeria densa</i>	L	Unknown	Cyanobacteria	Growth	Nakai et al. 1999
<i>Eichhornia crassipes</i>	L*/L	Phenylene metabolites Root exudates Culture water of seedlings	<i>Porphyridium aerugineum</i> , (algae) <i>Green algae</i> , <i>Anabaena azollae</i> <i>Chlamydomonas reinhardtii</i>	Growth	Della Greca et al. 1992 Shu-Wen et al. 1991 Sun et al. 1990 Sun et al. 1991 <sup>b</sup>
<b><i>Eleocharis</i></b>					
<i>E. acicularis</i>	L/L*	Unknown	Cyanobacteria, duckweed, lettuce, <i>Potamogeton</i>	Growth	Elakovich & Wooten 1989 Nakai et al. 1999 Oborn et al. 1954 <sup>b</sup>
<i>E. cellulosa</i>	L*	Unknown	<i>Lemna paucicostata</i> , <i>Hydrilla verticillata</i>	Growth	Sutton & Portier 1989, 1991 <sup>b</sup>
<i>E. coloradoensis</i>	L, L*	Root leachate/extract	<i>Potamogeton nodosus</i> , <i>P. foliosus</i> , <i>P. pectinatus</i> , <i>P. pusillus</i> , <i>Hydrilla verticillata</i> , lettuce, <i>Elodea canadensis</i> , <i>E. nuttallii</i> , <i>Hydrilla verticillata</i> , <i>Myriophyllum spicatum</i> , <i>Zannichellia palustris</i>	Shoot formation	Frank & Dechoretz 1980 Ashton et al. 1985 <sup>b</sup> Yeo 1980 <sup>b</sup> Yeo 1984
<i>E. equisetoides</i>	L*	Unknown	Lettuce	Growth	Wooten & Elakovich 1991 <sup>b</sup>
<i>E. flavescens</i>	L*	Unknown	Lettuce	Growth	Wooten & Elakovich 1991 <sup>b</sup>
<i>E. geniculata</i>	F	Unknown	<i>Hydrilla verticillata</i>	Growth	Sutton 1986 <sup>b</sup>
<i>E. interstincta</i>	L*	Unknown	<i>Lemna paucicostata</i> , <i>Hydrilla verticillata</i>	Growth	Sutton & Portier 1989
<i>E. montana</i>	L*	Unknown	Duckweed, lettuce	Growth	Wooten & Elakovich 1991 <sup>b</sup>
<i>E. obtusa</i>	L*	Unknown	Duckweed, lettuce	Growth	Elakovich & Wooten 1989

<sup>a</sup> L = laboratory study, F = field study, L/F = both, \* only extract tested.<sup>b</sup> *sensu* Elakovich & Wooten 1995.



Table 1.1 Continued

Genus/species	Evidence <sup>a</sup>	Identified compound(s)	Active against	Mode of action	References
<i>E. quadrangulata</i>	L*	Unknown	Duckweed, lettuce	Growth	Wooten & Elakovich 1991 <sup>b</sup>
<i>Eleocharis</i> spp.	L	Dihydroactinidiolide	Other macrophytes	Germination, root growth	Ashton et al. 1985 <sup>b</sup> Stevens & Merrill 1980 Wooten & Elakovich 1991 <sup>b</sup>
<i>E. tuberculosa</i>	L	Unknown	Lettuce	Growth	Wooten & Elakovich 1991 <sup>b</sup>
<b><i>Elodea</i></b>					
<i>E. canadensis</i>	L, F, L*	Unknown	Cyanobacteria, chlorophytes <i>Nitzschia palea</i>	Growth, photosynthesis	Erhard & Gross, <i>unpublished</i> Hasler & Jones 1949 Wium-Andersen 1987
<i>E. nuttallii</i>	L	Unknown	Cyanobacteria, chlorophytes	Growth	Erhard & Gross <i>unpublished</i>
<b><i>Equisetum</i></b>					
<i>E. fluviatile</i>	L	Unknown	<i>Pragmites australis</i>	Growth	Szczepański 1977 <sup>b</sup>
<i>E. limosum</i>	L	Unknown	<i>Pragmites communis</i>	Growth	Szczepańska 1971
<i>E. palustris</i>	L	Unknown	<i>Pragmites australis</i> , <i>Typha latifolia</i>	growth	Szczepański 1977 <sup>b</sup>
<i>Hydrilla verticillata</i>	F, L, L*	Unknown	<i>Ceratophyllum</i> spp., duckweed, lettuce	Shoot length, growth	Kulshretha & Gopal 1983 Elakovich & Wooten 1989
<i>Hydrocharis morus-ranae</i>	L/F	Unknown	<i>Aldrovanda vesiculosa</i>	Growth	Kaminski 1987

<sup>a</sup> L = laboratory study, F = field study, L/F = both, \* only extract tested.<sup>b</sup> *sensu* Elakovich & Wooten 1995.

Table 1.1 Continued

Genus/species	Evidence <sup>a</sup>	Identified compound(s)	Active against	Mode of action	References
<b><i>Juncus</i></b>					
<i>J. acutus</i>	L*	Phenanthrenoids	<i>Selenastrum capricornutum</i>	Growth	Della Greca et al. 2003
<i>J. effusus</i>	L	Phenylpropane, glycerides, unknown, dihydrophenanthrene, tetrahydropyrene	<i>Selenastrum capricornutum</i> , autotoxicity, algicidal activity	Growth, seedling growth	Della Greca et al. 1996 Della Greca et al. 1998 Della Greca et al. 2001b Ervin & Wetzel 2000
<i>J. repens</i>	L*	Unknown	Duckweed, lettuce	Growth	Elakovich & Wooten 1989
<i>Lemna gibba</i>			<i>Spirodela polyrrhiza</i> , <i>Wolffia arrhiza</i>		Wolek 1974 <sup>b</sup>
<i>Limnophila sessiliflora</i>	L	Unknown	Cyanobacteria	Growth	Nakai et al. 1999
<i>Limnobium spongia</i>	L*	Unknown	Duckweed, lettuce	Growth	Elakovich & Wooten 1989
<b><i>Myriophyllum</i></b>					
<i>M. alterniflorum</i>	L	Unidentified polyphenols	Cyanobacteria, chlorophytes	Growth	Gross, <i>unpublished</i>
<i>M. aquaticum</i>	L*	Unknown	Duckweed, lettuce, <i>Hydrilla verticillata</i>	Growth	Elakovich & Wooten 1989 Jones 1993 <sup>b</sup>
<i>M. brasiliense</i>	L	Tellimagrandin II (eugenin), phenolic compounds	Cyanobacteria	Growth	Saito et al. 1989
<i>M. heterophyllum</i>	L	Unidentified polyphenols	Cyanobacteria, chlorophytes	Growth	Gross, <i>unpublished</i>

<sup>a</sup> L = laboratory study, F = field study, L/F = both, \* only extract tested.<sup>b</sup> *sensu* Elakovich & Wooten 1995.

Table 1.1 Continued

Genus/species	Evidence <sup>a</sup>	Identified compound(s)	Active against	Mode of action	References
<i>M. spicatum</i>	L/L*	Tellimagrandin II, hydrolysable tannins, unknown, polyphenols	Phytoplankton, <i>Najas marina</i> , cyanobacteria, green algae, natural phytoplankton	Exoenzymes (alkaline phosphatase), Photosystem II Growth	Agami & Waisel 1985 Elakovich & Wooten 1989 Fitzgerald 1969 Gross & Sütfield 1994 Gross et al. 1996 Jasser 1995 Körner & Nicklisch 2002 Leu 2001 Leu et al. 2002 Nakai et al. 1996, 1999, 2000, 2001 Planas et al. 1981 Aliotta et al. 1992
<i>M. verticillatum</i>	L	Phenylpropanoid-glucosides	Cyanobacteria, chlorophytes	Growth	
<b><i>Najas</i></b>					
<i>N. guadalupensis</i>	L*	Unknown	Duckweed, lettuce, <i>Hydrilla verticillata</i>	Growth	Elakovich & Wooten 1989 Jones 1993 <sup>b</sup>
<i>N. marina</i> ssp. <i>intermedia</i>	L	Unknown	Cyanobacteria	Growth	Gross et al. 2003b
<i>Nelumbo lutea</i>	L*	Unknown	<i>Hydrilla verticillata</i>	Growth	Jones 1993 <sup>b</sup>
<b><i>Nitella</i></b>					
<i>Nitella</i> sp.	L*	Dithiolane	<i>Nitzschia palea</i> (diatom)	Photosynthesis	Wium-Andersen 1987
<i>Nitella gracilis</i>	L*	Unknown	Cyanobacteria	Growth	Berger & Schagerl 2004

<sup>a</sup> L = laboratory study, F = field study, L/F = both, \* only extract tested.<sup>b</sup> *sensu* Elakovich & Wooten 1995.

Table 1.1 Continued

Genus/species	Evidence <sup>a</sup>	Identified compound(s)	Active against	Mode of action	References
<i>Nitella opaca</i>	L*	Unknown	Cyanobacteria	Growth	Berger & Schagerl 2004
<i>Nitellopsis obtusa</i>	L*	Unknown	Cyanobacteria	Growth	Berger & Schagerl 2004
<i>Nuphar lutea</i>	L	Unknown/ 6,6'-dihydroxythiobinupharidine	Duckweed, lettuce	Killing, frond number, radical growth	Elakovich & Wooten 1991 Elakovich & Yang 1996
<i>Nymphaea odorata</i>	L*	Unknown	Duckweed, lettuce, <i>Hydrilla verticillata</i> , <i>Myriophyllum spicatum</i>	Growth	Elakovich & Wooten 1989 Jones 1993 <sup>b</sup>
<i>Nymphoides cordata</i>	L*	Unknown	Duckweed, lettuce	Growth	Elakovich & Wooten 1989
<i>Phragmites australis</i>	L	Unknown	<i>Carex elata</i>	Growth in coexistence cultures	Szczepański 1977 <sup>b</sup>
<i>Pistia stratiotes</i>	L*	Phenolic compounds, fatty acid derivatives, sterols	Algae, cyanobacteria	Growth	Aliotta et al. 1991
<i>Pontederia lanceolata</i>	L*	Unknown	<i>Hydrilla verticillata</i>	Growth	Jones 1993 <sup>b</sup>
<b>Potamogeton</b>					
<i>P. amplifolius</i>	F	Unknown	<i>Vallisneria americana</i>	Growth	Titus & Stephens 1983
<i>P. crispus</i>	L	Unknown	Cyanobacteria	Growth	Nakai et al. 1999
<i>P. foliosus</i>	L*	Unknown	Duckweed, lettuce	Growth	Elakovich & Wooten 1989
<i>P. illinoensis</i>	L*	Unknown	<i>Lemna paucicostata</i>	Growth	Sutton & Portier 1989
<i>P. lucens</i>	L*	Unknown	Cyanobacteria	Total biomass decline	Jasser 1995

<sup>a</sup> L = laboratory study, F = field study, L/F = both, \* only extract tested.<sup>b</sup> *sensu* Elakovich & Wooten 1995.

Table 1.1 Continued

Genus/species	Evidence <sup>a</sup>	Identified compound(s)	Active against	Mode of action	References
<i>P. natans</i>	L*	Lactone diterpenes, furano diterpenes	Cyanobacteria, algae	Unknown	Cangiano et al. 2001 Cangiano et al. 2002 Della Greca et al. 2001a Usenko et al. 2002
<i>P. nodosus</i>	L*	Unknown	<i>Hydrilla verticillata</i>	Growth	Jones 1993 <sup>b</sup>
<i>P. oxyphyllus</i>	L	Unknown	Cyanobacteria	Growth	Nakai et al. 1999
<b><i>Sagittaria</i></b>					
<i>S. graminea</i>	F	Unknown	<i>Hydrilla verticillata</i>	Growth	Sutton 1986 <sup>b</sup>
<i>S. lancifolia</i>	L*	Unknown	<i>Hydrilla verticillata</i> <i>Myriophyllum spicatum</i>	Growth	Jones 1993 <sup>b</sup>
<i>S. pygmaea</i>			Rice		Lee & Guh 1982 <sup>b</sup>
<i>S. subulata</i>			<i>Potamogeton</i>		Oborn et al. 1954
<i>Schoenoplectus lacustris</i>	L	Unknown	<i>Equisetum limosum</i> , <i>Pragmites communis</i> , <i>Potamogeton australis</i>	Growth in coexistence cultures	Szczepańska 1971 Szczepański 1977
<i>Sparganium americanum</i>	L, L*	Leachate, water extract, unknown	Duckweed, lettuce	Growth	Cheng & Riemer 1988 Elakovich & Wooten 1989
<i>Spirodela polyrrhiza</i>			<i>Lemna gibba</i> , <i>L. minor</i> , <i>Wolffia arrhiza</i>	Growth	Wolek 1974 <sup>b</sup>

<sup>a</sup> L = laboratory study, F = field study, L/F = both, \* only extract tested.

<sup>b</sup> *sensu* Elakovich & Wooten 1995.

Table 1.1 Continued

Genus/species	Evidence <sup>a</sup>	Identified compound(s)	Active against	Mode of action	References
<i>Stratiotes aloides</i>	L, F, L*	Unknown/phenolic acids	Phytoplankton, filamentous algae, <i>Scenedesmus obliquus</i> , cyano-bacteria and green algae, <i>Nitzschia palea</i> , <i>Aldrovanda vesiculosa</i>	Nutrient interference, growth, colony formation, photosynthesis	Brammer 1979 Brammer & Wetzel 1984 Jasser 1995 Kaminski 1987 Mulderij et al. 2005ab, Chapters 4 & 5 Usenko et al. 2002 Wium-Andersen 1987
<b><i>Typha</i></b>					
<i>T. domingensis</i>	L*	Linoleic & linolenic acid, caffeic, p-coumaric and gallic acid, extracts and 2-chloro-phenol and salicyl-aldehyde	<i>Salvinia minima</i>	Growth	Gallardo et al. 1998ab Gallardo et al. 1999 Gallardo-Williams et al. 2002
<i>T. latifolia</i>	L	Unknown	<i>Typha angustifolia</i> , <i>T. latifolia</i> , lettuce, radish, tomato, cucumber, <i>Anabaena flos-aqua</i> , <i>Chlorella vulgaris</i> , <i>Phragmites communis</i> , <i>Equisetum fluviatile</i> , <i>Pragmites australis</i> ,	Autotoxicity	Aliotta et al. 1990 Bonasera et al. 1979 <sup>b</sup> Della Greca et al. 1990 Grace 1983 McNaughton 1968 Szczepańska 1971 Szczepański 1977

<sup>a</sup> L = laboratory study, F = field study, L/F = both, \* only extract tested.<sup>b</sup> *sensu* Elakovich & Wooten 1995.

Table 1.1 Continued

Genus/species	Evidence <sup>a</sup>	Identified compound(s)	Active against	Mode of action	References
<b><i>Vallisneria</i></b>					
<i>V. censeserrulata</i>	L	Unknown	Cyanobacteria	Growth	Nakai et al. 1999
<i>V. americana</i>	L, L*	Leachate, water extract, unknown	Duckweed, lettuce, <i>Hydrilla verticillata</i> , <i>Myriophyllum spicatum</i>	Germination Growth	Cheng & Riemer 1988, 1989 Elakovich & Wooten 1989 Jones 1993 <sup>b</sup>
<i>Wolffia arrhiza</i>			<i>Lemna gibba</i> , <i>L. minor</i> , <i>Spirodela polyrrhiza</i>		Wolek 1974 <sup>b</sup>
<i>Zostera marina</i>	L*	Unknown	Phytoplankton (8 species)	Growth	Harrison & Chan 1980

<sup>a</sup> L = laboratory study, F = field study, L/F = both, \* only extract tested.

<sup>b</sup> *sensu* Elakovich & Wooten 1995.





Center stage:

The crucial role of macrophytes in regulating trophic  
interactions in shallow lake wetlands

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### Acknowledgements

## Center stage

### 2.1 Introduction and objectives

Hydrophilic/water-loving, macrophytes characterize wetland ecosystems, indicating prerequisite conditions of hydric soils and sufficient hydrology. The presence of macrophytes is a key descriptor in multiple wetland definitions (Lewis 2001a) and macrophytes may be further used to actually describe particular types of wetlands, such as cattail marshes. Macrophytes contribute significant biomass to these wetland systems and represent a critical component of wetland biogeochemistry as primary producers and drivers of organic matter cycling within aquatic systems. In this chapter, we argue that macrophytes occupy the center of trophic interactions in shallow lakes, influencing outcomes through structural, behavioural and chemical interactions. We define shallow lakes as permanently flooded wetlands that often contain submerged or floating macrophytes and that may be surrounded by emergent vegetation (i.e. marshy habitat). Shallow remains a relative term in limnology circles, but typically is less than 3-m average depth such that macrophytes can fill a substantial portion of the water column, and stratification is neither predictable nor long term. Such systems may be termed lakes, ponds or wetlands depending on their size and the ecological context. Macrophytes may regulate trophic interactions in ephemeral systems without permanent inundation.

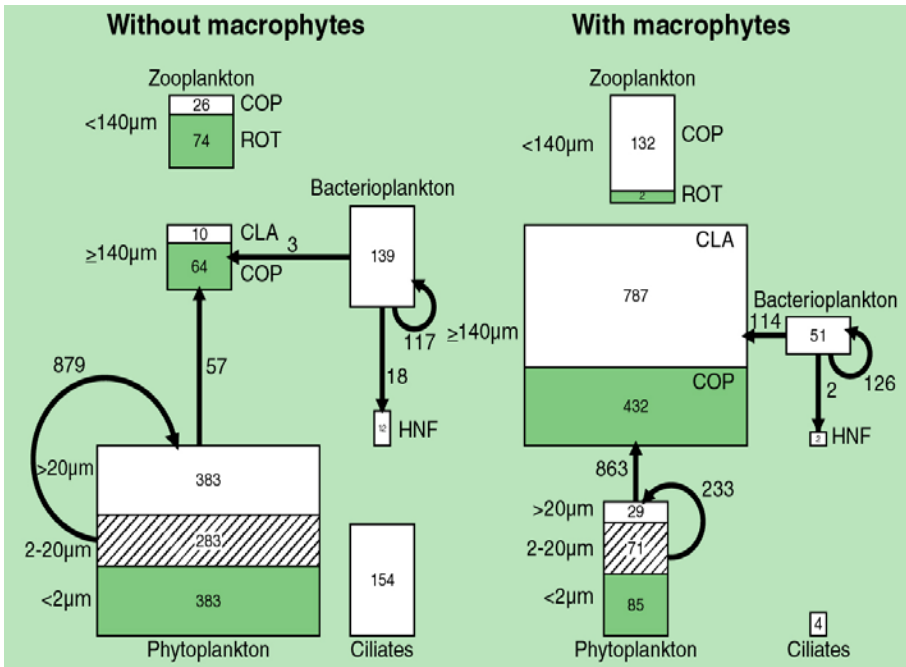
## 2.2 Central position of aquatic vegetation

Terrestrial ecologists historically have focussed on vegetation. Early aquatic studies, however, also noted the dominance of plants in aquatic systems. For example, in *The Lake as a Microcosm*, Forbes (1925) derided areas ‘clogged with weeds,’ but then elaborated on how these weeds (*Chara*, *Potamogeton*, *Ceratophyllum*) swarmed with life including fish, invertebrates, zooplankton and algae. Lindeman (1942) included pondweeds as an important component of lake food webs and Teal (1962) went a step further and quantified the energy flow between trophic levels in a salt marsh. Thus, historical precedent exists for focusing attention on macrophytes as part of the aquatic community. Carpenter & Lodge (1986) were the first to emphasize ecosystem processes, and summarized the impacts of submerged macrophytes on abiotic and biotic components of aquatic systems. With a similar aim, Jeppesen et al. (1998a) published a comprehensive book documenting the structuring role of submerged macrophytes in lakes. We extend their discussion of trophic interactions and also consider other macrophyte architectures (i.e. floating-leaved, emergent and freely-floating).

Reinforced by multiple mechanisms, macrophytes promote water clarity and help stabilize a clearwater state (Jeppesen 1998, Scheffer 1998) and enhance biodiversity (Declerck et al. 2005). Several of those feedbacks depend on structural or chemical effects that, in turn, influence behaviours of multiple trophic levels. To evaluate our progress in understanding the role of macrophytes in wetland systems since Jeppesen et al. (1998a), we performed an extensive literature search (1997 - 2004) regarding aquatic macrophytes. With four search engines (Web of Science, Science Direct (Elsevier), BioOne and Kluwer-Online) our inquiry discovered 126 papers that involved trophic interactions with macrophytes.

The vast majority (74%) of recent studies focused on behavioural interactions with macrophytes, with an additional 13% exploring this within a chemical context. The remaining 13% focused on single structural-chemical interactions largely between macrophytes and phytoplankton. Macrophytes, fish and zooplankton composed the most common suite of organisms studied together (12%), although 35% of the studies involved three groups and 56% only examined a single interaction. Thirteen studies (10%) examined more complex food webs in shallow wetland systems; however, only four of these studies (3%) examined five trophic levels. Surprisingly, similar numbers of single-interaction studies involved both fish and invertebrates, dominated by studies including fish (44%). Only 10% of the studies included an

additional primary producer (periphyton or phytoplankton) besides macrophytes. These complex trophic connections that occur with macrophytes dramatically alter the carbon flow through the system (Fig. 2.1). With this context, we used the literature survey to identify four key themes regarding the regulation of trophic interactions by macrophytes.



**Figure 2.1** The dramatic alteration of energy flow in the presence of macrophytes, originally published in Jeppesen et al. (2002). This illustrates the cascading trophic interactions within enclosures placed in the littoral zone of a shallow Danish lake (Stigsholm). Shown are the carbon flows between trophic levels in enclosures with and without submerged macrophytes for the first diel periods where day and night sampling occurred. Boxes represent biomass ( $\mu\text{g C L}^{-1}$ ) of different trophic components and the arrows measure flux per day. COP = copepods, ROT = rotifers, CLA = cladocerans, HNF = heterotrophic nanoflagellates. Broken box lines indicate that phytoplankton production and zooplankton grazing are community estimates for all groups within the boxes. Note that zooplankton grazing by far exceeded phytoplankton production in the macrophyte state, suggesting extremely high grazing on phytoplankton and likely benthic facilitation of the zooplankton as well. The latter may, in turn, ensure a steady high abundance of zooplankton and thus high grazing on phytoplankton when zooplankton migrate to the open water at night.

### 2.2.1 Zooplankton depend on macrophytes as habitats

While many historical studies noted the abundance of macroinvertebrates among macrophytes (e.g., for damselflies see Lombardo 1997, for snails see Brönmark & Vermaat 1998), fewer studies recognized the role of macrophytes as providing refuge for zooplankton. Limnologists generally restrict their discussion of refuges for zooplankton to the hypolimnetic refuge provided by deep lakes for large-bodied zooplankton undergoing diel vertical migration (i.e. *Daphnia*). However, littoral-associated cladocerans (i.e. *Alona*, *Chydorus*, *Simocephalus*, *Ceriodaphnia*) commonly occur among macrophytes in large lacustrine wetlands (Hann & Zrum 1997) and small glacial kettleholes (Gaiser & Lang 1998). Recent studies from shallow lake and wetland systems demonstrated that macrophyte presence can bolster pelagic zooplankton, as large-bodied species such as *Daphnia* migrate horizontally into macrophytes to seek refuge from predators (Burks et al. 2002).

Zooplankton dependence on macrophyte beds acts as a positive feedback mechanism that helps to maintain clearwater conditions in lakes, especially in eutrophic systems with a high macrophyte biomass (Jeppesen et al. 1999b, although see Blindow et al. 2000). Multiple scenarios exist, however, for the relationship between zooplankton size (at the individual or populations scale) and macrophyte density. One possibility is a non-linear relationship based on the assumptions that increased prey size leads to increased vulnerability (Brooks & Dodson 1965) and that increased structural complexity makes foraging more difficult for some predators in a non-linear way (Jeppesen et al. 1998a, Burks et al. 2001a). This refuge scenario suggests that some threshold of macrophytes is required, which is corroborated by a large mesocosm experiment showing that smaller rotifers and cyclopoid copepods comprised the zooplankton in plant-free controls while large-bodied cladocerans dominated enclosures with plants, when the plant density exceeded a certain threshold (Jeppesen et al. 1998a, 2002, Fig. 2.1).

With horizontal migration, the extent of entry into macrophytes by zooplankton varies depending on the costs of inhabiting macrophytes. These costs include competition with other zooplankton, food scarcity, chemical inhibition, or adverse abiotic conditions (Burks et al. 2002). Such costs might be responsible for the horizontal patchiness that occurred in Rybak & Weglenska's (2003) study, where sparse beds showed more limnetic species of Cladocera, while dense beds held more littoral species. Intense predation pressure from macrophyte-associated invertebrates (Burks et al. 2001b) also may factor into the non-linear relationship between macrophyte density and zooplankton size and contribute to patterns commonly observed in the field. Invertebrate density across vertical and horizontal dimen-

sions varies among macrophytes. Substantial surface area of submerged macrophytes likely supports higher abundances of macroinvertebrates than floating or emergent plants (Cattaneo et al. 1998). Marklund et al. (2001) found higher densities of invertebrate grazers in the layers closer to the sediment than near the surface of *Chara* beds. Their results also showed higher densities at the macrophytes' edge versus the innermost parts for some species. The latter corroborates earlier results by Lauridsen et al. (1996). Thus, for zooplankton, macrophyte beds may form a horizontal transition zone in predation, from invertebrate predation inshore to fish predation offshore (Tolonen et al. 2001).

The utility of macrophytes as refuge by zooplankton is also likely to decline with increasing turbidity. While several studies document the use of submerged and floating plants as a refuge, the ability of emergent plants to provide refuge for zooplankton remains questionable. Nurminen & Horppila (2002) investigated the importance of emergent macrophytes as refugia for littoral cladocerans in lakes of differing trophic status. No tendency toward horizontal migration into emergent macrophytes occurred in a clearwater system. In the turbid system, Nurminen & Horppila (2002) found reverse migration by large-bodied filter feeders (*Limnospina frontosa* and *Sida crystallina*), possibly in response to high densities of small fish among the vegetation. This agrees well with a recent study of changes along a chlorophyll *a* (turbidity) gradient in the proportion of fish near shore versus in the open water. Numerous Danish lakes showed an increasing share of the most abundant fish species in the littoral as turbidity increased (Jeppesen et al. *in press*). On the other hand, anti-predator behaviour of juvenile fish, such as using macrophytes for refuge, may occur less frequently in turbid water, where hunting by sight-dependent predator fish is less successful (Abrahams & Kattenfeld 1997). Under clearwater conditions, juvenile pike (*Esox lucius*) spent more time foraging among complex structures compared with an even distribution between complexity and open water under turbid conditions (Skov et al. 2002). In a survey of 33 inter-connected shallow ponds, Cottenie et al. (2001) found that the zooplankton community structure depended strongly on the predation intensity and macrophyte cover and that the clearwater state coincided with macrophytes, piscivorous fish and large *Daphnia* species.

Besides the influence of turbidity, the ability of macrophytes to serve as a refuge for zooplankton also declines when macrophytes play host to other predators, suggesting avoidance of macrophytes by zooplankton. If one considers its root network, water hyacinth (*Eichhornia crassipes*) may possess a comparable surface area to that of submerged plants. However, *Eichhornia* does not support a similar high diversity of invertebrates, including zooplankton (Meerhoff et al. 2003). Lower colonization of macrophytes than predicted may be due to (1) chemical deterrence (Burks et al. 2000, 2001a), (2) differences in water chemistry from the floating plant canopy or (3) high fish predation (Meerhoff et al. 2003 and *unpublished data*). However, the few studies on freely-floating plants make such generalizations difficult and more research is needed to understand if this architectural type differs in function from submerged or emergent macrophytes.

Despite any potential differences, high fish predation can occur outside all types of macrophyte architectures. Romare et al. (2003), for example, found that roach (*Rutilus rutilus*) and perch (*Perca fluviatilis*) congregated outside dense emergent vegetation in a small Danish lake, thus likely eliminating the advantage of horizontal migration for cladocerans. This negative relationship may exist with the carnivorous macrophyte bladderwort, *Utricularia* (Guisande 2000, Englund & Harms 2003), depending on zooplankton size. Furthermore, larval dragonflies also pose a serious predation threat to zooplankton undergoing diel horizontal migration, particularly at low macrophyte densities (Burks et al. 2001b). Alternatively, littoral fish may reinforce the refuge effect for zooplankton if they prefer macroinvertebrates (Sagrario & Balseiro 2003). Overall, macrophytes act as effective refuges for zooplankton or planktivorous fish when the diversity or the density is sufficient enough to depress predation rates of planktivores (Priyadarshana et al. 2001) or piscivores (i.e. largemouth bass, *Micropterus salmoides*, Valley & Bremigan 2002). Further experiments and field studies that investigate patterns of zooplankton size, macrophyte density and composition will help to solve the ambiguity associated with these multiple scenarios and lend further insight into how carbon cycling occurs across multiple trophic interactions (Fig. 2.1).

### 2.2.2 Chemical ecology spans trophic levels

Biochemical interactions between (submerged) macrophytes and competing primary producers, ('allelopathy' *sensu* Molisch 1937, Chapter 1), often appear in the literature, but at the same time ignite much controversy. Allelopathy is unlikely to act as a determining factor of a clear versus turbid state, but it certainly influences the interactions between the main opponents. Multiple laboratory studies docu-



ment inhibitory effects of extracts, exudates or intact macrophytes on algae (summarized e.g., in Gross 2003a, for recent example from freely-floating plants, see Mulderij et al. 2005ab, Chapters 4 & 5). Most studies show differential sensitivity among algal groups, with cyanobacteria often being the most sensitive and chlorophytes and diatoms being less susceptible (e.g., Gross et al. 1996, but see Mulderij et al. 2005b, Chapter 5). Macroalgae, such as charophytes, also employ allelopathic chemicals in their interactions with other primary producers (Van Donk & Van de Bund 2002, Mulderij et al. 2003, Chapter 3). Relative to other macrophyte growth forms, the likelihood of allelopathic interactions increases in dense stands of submerged macrophytes where more stagnant water prevails. Based on our literature survey, we provide a ranked list of macrophyte species that we thought best represented allelopathic species (Table 2.1).

**Table 2.1** Ranking the allelopathic potential of aquatic macrophytes (based on evidence presented in studies cited by Wiium-Andersen 1987, Gopal & Goel 1993, Gross 2003a). Evidence was considered high when different authors reported the same or similar observations and/or when proof for the release of active compounds occurred. The \* indicates that evidence was not as strong as for “high” species. Note that not all *Chara* species show indications of allelopathic properties.

Allelopathic potential	Species	Common name
High	<i>Myriophyllum spicatum</i>	Eurasian water milfoil
–	<i>Chara globularis</i> , <i>Ch. aspera</i> , <i>Ch. contraria</i>	Stonewort or charophyte
–	<i>Ceratophyllum demersum</i>	Coontail
–	<i>Stratiotes aloides</i>	Water soldier
–	–	–
Medium/High*	<i>Elodea</i> spp	Waterweed, water pest
–	<i>Eleocharis acicularis</i>	Spike rush
–	<i>Myriophyllum brasiliense</i>	Parrotfeather, Brazilian milfoil
–	<i>Myriophyllum verticillatum</i>	Whorl–leaf watermilfoil
–	–	–
Medium	<i>Berula erecta</i> ( <i>Sium erectum</i> )	Water parsnip
–	<i>Eichhornia crassipes</i>	Water hyacinth
–	<i>Egeria densa</i>	Common waterweed
–	<i>Najas marina</i> ssp. <i>intermedia</i>	Spiny naiad
–	<i>Nuphar lutea</i>	Yellow water lily
–	–	–
Low	<i>Potamogeton</i> spp.	Pondweeds

Knowledge of chemical ecology in freshwater systems lags behind marine and terrestrial systems (Burks & Lodge 2002), but we see substantial progress. Our literature survey indicated that 25% of the recent studies included an aspect of chemical influence, suggesting that more investigations of this nature are underway. The practicality of both realistic laboratory and *in situ* experiments will increase as we identify more compounds. For now, we noticed particularly the variety of ways in which chemical cues or allelopathic compounds alter trophic interactions. The impacts often depend on species identity and commonly involved macroinvertebrates.

Some macrophyte species showed higher tendencies to be allelopathic than others (Table 2.1). For example, Körner & Nicklisch (2002) demonstrated that *Myriophyllum spicatum* and *Ceratophyllum demersum* both inhibited the photosystem II activity of phytoplankton, but found no allelopathic effect of *Potamogeton pectinatus*. Based on the absence of plant fragments in fish guts, Nurminen et al. (2003) also implied selective avoidance of *M. spicatum* and *C. demersum* by rudd (*Scardinius erythrophthalmus*). Macrophytes may influence both behavioural and chemical interactions. For example, water incubated with the macrophyte *Stratiotes aloides* (i.e. Water soldier) demonstrated a highly significant inhibitory effect on the green alga *Scenedesmus obliquus* (Mulderij et al. 2005a, Chapter 4), the cyanobacterium *Microcystis*, and the eustigmatophyte *Nannochloropsis* (Mulderij et al. 2005b, Chapter 5). Furthermore, the presence of *Stratiotes aloides*, especially young plants, increased colony formation by *S. obliquus*. With the formation of colonies by *S. obliquus*, the sinking rates of this alga increased, leading to improved water clarity and light conditions for *S. aloides* (Mulderij et al. 2005a, Chapter 4). In the reverse direction, cyanobacterial compounds can have detrimental impacts on aquatic macrophytes. For example, Microcystin-LR showed allelopathic properties against *M. spicatum* and *C. demersum* (Pflugmacher 2002).

The deterrent chemical nature of some macrophytes may strongly regulate the biological community. One study suggests that aquatic plants repellent to grass carp (*Ctenopharyngodon idella*) could serve as areas of amphibian rehabilitation (Murphy et al. 2002). Endangered odonate larvae (*Aeshna viridis*) also preferred *S. aloides* as it also served as a refuge from perch predation (Rantala et al. 2004). Other work connects herbivores and predators through the chemical nature of macrophytes. For example, *Hyallela azteca* (Amphipoda) readily graze on roots of the emergent macrophyte, *Berula erecta* (Water parsnip), even though these roots may be chemically defended (Rowell & Blinn 2003). Consequently, these amphipods

exhibited lower susceptibility to fish predation. Although common in marine literature, this study provided the first experimental evidence of reduced predation on a freshwater herbivore that consumed a chemically defended plant (Rowell & Blinn 2003).

Of all the aquatic macrophytes, one species, *Myriophyllum spicatum*, Eurasian watermilfoil (EWM), clearly stood out as chemically the most active species (Table 2.1) because of multiple interactions that occur with herbivores and competing phototrophs. On the whole lake scale, macroinvertebrate biomass on dominant plant species in a lake may decline as the percentage of EWM cover increases (Cheruvilil et al. 2001). Furthermore, Lindén & Lehtiniemi (2005) found that chemicals excreted by dense stands of EWM resulted in a high mortality (73 - 89%) of the mysids *Neomysis integer* and *Praunus felxuosus*, and lowered the feeding efficiencies of 3-spined sticklebacks (*Gasterosteus aculeatus*). At a smaller scale, *Acentria* (Lepidoptera) exhibited higher feeding rates on *Myriophyllum* than on *Elodea* (Gross et al. 2001). In choice experiments, *Acentria* larvae clearly preferred EWM. By selectively removing apical tissue with the highest concentrations of allelopathically active compounds (Choi et al. 2002), herbivory by *Acentria* might weaken the allelopathic strength of this species, indirectly altering competitive interactions and the plant's susceptibility to bacterial infection. Overall, the magnitude of herbivory on EWM may (1) shape competitive interactions between submerged macrophytes, (2) change invertebrate biomass, and perhaps (3) alter ecosystem processes such as primary production (Gross 2003a).

Remarkably, many invasive species, such as *M. spicatum* in Northern America or *Elodea* spp. in Europe (Gross et al. 1996, Erhard & Gross 2005), act as allelopathically active species (Table 2.1). Significant differences in allelopathic activity between native and invasive specimens remain uncertain for aquatic systems, although Bais et al. (2003) documented such an occurrence with invasive, spotted knapweed (*Centaurea maculosa*). In addition, the extent to which allelopathic interactions add significantly to successful invasion also merits further attention. The concentration of active compounds in EWM may differ between native and invaded ranges of plants and may vary strongly depending on environmental conditions (Gross 2003b). Therefore, the role of macrophytes as chemical signalers under field conditions strongly depends on (1) the macrophyte species present, (2) the density or spatial heterogeneity of their patches, (3) the velocity of breakdown of active compounds and possibly (4) the nutrient state of both donor (macrophyte) and target organisms (e.g., phytoplankton).

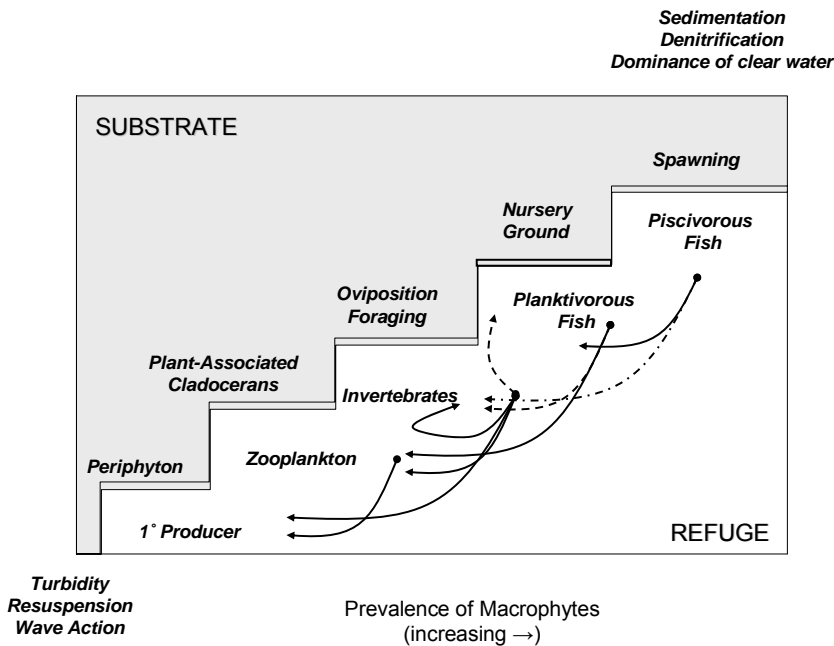
The magnitude of the deterrent effect of macrophytes on trophic interactions may also differ among species and in the presence of other predatory cues. For example, *Daphnia* actively avoided submerged macrophytes in the absence of fish, but overcame this repellency and sought refuge when fish occurred (Burks et al. 2001a), despite the possible costs of reduced zooplankton growth (Burks et al. 2000). In the absence of fish, Van de Meutter et al. (2004a) found an additional, although weak, avoidance of macrophytes that housed *Ischnura elegans* (10% less within vegetation). Unfortunately, the predictability of macrophyte avoidance by *Daphnia* varies, perhaps depending on the clone, the presence of conspecific cues or the presence of other predators. In a similar study, Van de Meutter et al. (2004b) found no horizontal migration response toward the open water in the presence of *Ischnura elegans* alone. However, when *Ischnura* co-occurred with *Chaoborus*, daphnids showed an increased avoidance of plants. Van de Meutter et al. (2004b) attributed this to an increased risk of predation in the vegetation. If strong enough, chemical cues from littoral predators may contribute to the already repellent nature of macrophytes and override the tendency for zooplankton to seek refuge from fish, suggesting a hierarchal nature of chemical cues.

### 2.2.3 *Impacts of grazer-epiphyton interactions with macrophytes*

Although the term periphyton encompasses the whole community of organisms that colonize surfaces, we use epiphyton to stress the competitive interactions between algae and macrophytes. Macrophytes can serve as a habitat, refuge from predators, or can act as a substrate where potential food sources can colonize (Fig. 2.2). Most epiphytes belong to the classes Bacillariophyta (diatoms) and Chlorophyta (green algae), but nuisance, grazing resistant forms, such as filamentous blue-greens (Cyanobacteria) and *Cladophora*, can occur when the community is grazed by invertebrates which are selective in their feeding (Jones et al. 2000). Snail densities on macrophytes depend, in part, on plant palatability, periphyton density, and algal quality and growth form (Brönmark & Vermaat 1998, Lombardo & Cooke 2002, Jones et al. 2000). Besides snails, insects including caddisflies, mayflies, beetles, and corixids, along with several crustaceans (e.g., amphipods, cladocerans and ostracods) consume ample amounts of epiphyton (Jones et al. 1998).

We often recognize the importance of top-down control of phytoplankton by zooplankton grazing and its central role in maintaining the clearwater state (Jeppesen et al. 1998b). The distinction between the origin and maintenance of alternative equilibria is, however, often not made, and it may be that phytoplankton plays a less important role in precipitating the shift from turbid to clear water. Macro-

phytes lead to increased sedimentation, through reduced water movement, which results in an influx of particles to the sediment. Consequently, there is less phytoplankton available to zooplankton in macrophytes, thus increasing the importance of epiphyton. Traditional approaches centered on the response of epiphyton to increased nutrient availability (Phillips et al. 1978), but recent work has provided insight into how higher trophic levels affect the interaction between periphyton and plants, and accordingly shallow lake function.

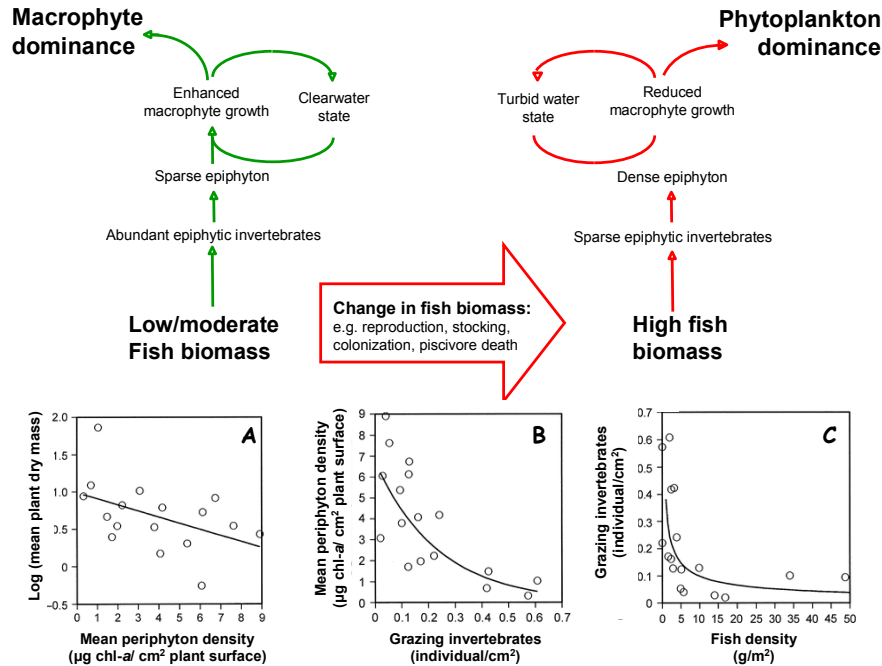


**Figure 2.2** Multi-faceted dual roles of macrophytes as 'refuge' and 'substrate' across trophic levels. Steps suggest that the refuge potential of macrophytes increases with increasing macrophyte density after a threshold is established (i.e. the plateaus). Arrows show the predator-prey relationships that necessitate the need for macrophyte refuge. Solid lines illustrate most predicted prey choice (origin with predator, arrow points to prey). Dotted lines illustrate prey choices that could occur with ontogenetic diet shifts. Three arrows originate from invertebrates because of the versatility of functional groups (i.e. predators, grazers, filterers). Increasing refuge provided by macrophytes leads to sedimentation and to the dominance of clearwater while the absence of macrophytes increases turbidity, promotes wave action and resuspension.

Shading by periphyton shortened the clearwater phase associated with optimum growth conditions for submerged plants (e.g., *P. pectinatus*) in Lake Müggelsee, Germany (Roberts et al. 2003). Nutrients alone, however, do not appear to determine whether epiphyton can reach sufficient densities to negatively impact macrophytes. Experimental work suggests that other mechanisms are important for the loss of macrophytes (Jones et al. 2000, Williams et al. 2002). These experiments, previous work (Brönmark & Vermaat 1998), and a survey of 17 macrophyte-dominated lakes in the UK (Jones & Sayer 2003) have led to a proposal that the strength of a trophic cascade in the littoral zone determines the fate of macrophytes (Fig. 2.3). We agree with a recent review (Vadeboncoeur et al. 2002) that benthic organisms, including epi- or periphyton and grazers, form integral links in lake food webs and deserve more emphasis in studies of trophic interaction.

In eutrophic to hypertrophic lakes, the macrophyte biomass exhibits a negative relationship with epiphyton growth. The density of epiphyton, in turn, depends on the density of grazing macroinvertebrates, but not nutrients (Fig. 2.3, Jones & Sayer 2003). A 20-fold increase in nutrient loading had no effect on the abundance of epiphyton growing on the surface of *Elodea nuttallii* in artificial ponds, whereas epiphyton density negatively correlated with increased grazers (Jones et al. 2002). In experimental ponds constructed to simulate reservoir wetlands, Balci & Kennedy (2003) also found that macroinvertebrate abundance correlated well with epiphyton biomass, although epiphyton biomass varied between exotic EWM and native water stargrass, *Heteranthera dubia* (Balci & Kennedy 2003).

Fish biomass also serves as a reliable predictor for the density of invertebrates and accordingly epiphyton (Jones & Sayer 2003). Williams et al. (2002) showed that epiphyton chlorophyll *a* in experimental mesocosms increased significantly with increasing fish biomass (0 - 700 kg ha<sup>-1</sup>). Interestingly, Jones & Waldron (2003) found that the proportion of periphyton in the diets of zooplankton increased with fish density, possibly as a result of increased horizontal migration. For these reasons, we highlight the strong potential for epiphyton-grazer interactions with macrophytes, mediated through interactions with fish, to facilitate shifts between turbid and clear states (Section 2.2.4).



**Figure 2.3** Illustration of a littoral trophic cascade that could mediate the shift from turbid to clear water conditions in shallow lakes. The graphs were originally published in Jones & Sayer (2003). In 17 British shallow lakes, plant biomass was negatively correlated with mean periphyton density (**A**;  $R^2 = 0.240$ ,  $P = 0.046$ ). Periphyton density was then strongly negatively related to the density of grazing invertebrates (**B**;  $R^2 = 0.714$ ,  $P < 0.0001$ ). Lastly, the density of grazing invertebrates declined significantly with increasing fish biomass (**C**;  $R^2 = 0.395$ ,  $P = 0.007$ ). Overall, this suggests that a high fish abundance would eliminate epiphyton grazers, allowing epiphyton to accumulate on plant surfaces. Consequently, macrophyte loss would occur due to light limitation and the absence of macrophytes would allow phytoplankton and a turbid state to dominate. Note that the biomass of plankti-benthivorous fish increases with increasing nutrient levels. Thus, the red scenario to the right is the most typical in eutrophic lakes unless high eutrophication leads to fish kills.

Colonization by epiphytes generally places macrophytes at a substantial disadvantage, due to increased competition for light, nutrients, and carbon (Jones et al. 2000, Williams et al. 2002). By continuously grooming macrophyte hosts of unwanted algae and sediments, grazers may help to maintain littoral communities (James et al. 2000). The periphyton removal rate generally increases allometrically with grazer biomass (Jones et al. 1998). Most grazing studies focus on snails (Gastropoda, Brönmark & Vermaat 1998) because they are among the largest and most visible grazers on aquatic plants and can remove substantial biomass. While it may be advantageous for macrophytes to host snails, Jones et al. (2000) suggest that it is unlikely that macrophytes alter the community composition of epiphytes to increase their attractiveness to snails, although Brönmark's (1985) study showed how macrophyte exudates (dissolved organic matter excreted from *Ceratophyllum demersum*) attracted periphyton grazers (i.e. the snail *Lymnaea peregra*).

Direct consumption of macrophytes also alters trophic interactions. Direct consumption of macrophytes by waterfowl was demonstrated by Van Donk & Otte (1996). Further, some large invertebrates, including the invasive channeled apple-snail *Pomacea canaliculata* (Lach et al. 2000) or crayfish (Lodge et al. 1994), may consume or destroy macrophytes copiously, decreasing the habitat available for colonization. *Procambarus clarkii*, for example, reduced the percentage macrophyte cover in a mesotrophic, Spanish, shallow lake from 97% to below 10% in less than three years (Rodríguez et al. 2003). However, species-specific impacts occur as Hessen et al. (2004) demonstrated that the crayfish *Astacus astacus* failed to control the rapid growth of *Elodea*. In addition, macrophytes differ in their palatability to snails and other invertebrates (Elger & Willby 2003), therefore changing plant species composition through selective herbivory (Elger et al. 2002).

The large size and omnivorous nature of crayfish differentiate them from other macroinvertebrates, underlining their importance in littoral habitats (Lodge et al. 1994). With a demonstrated preference for herbivory over carnivory and for native over exotic macrophytes (i.e. *Eichhornia*), *P. clarkii* could be responsible for the extirpation of native aquatic macrophytes in Lake Naivasha, Kenya (Smart et al. 2002). In small Michigan ponds, vascular macrophytes failed to establish in the presence of crayfish; cyanobacteria started to dominate; and two epiphyton herbivores (i.e. tadpoles and snails) did not occur with crayfish (Dorn & Wojdak 2004). Beyond just consuming macrophytes, Dorn & Wojdak's (2004) study showed that crayfish preyed heavily on fish eggs, reducing fish recruitment (Fig. 2.2) and (indirectly) enhancing zooplankton biomass. Crayfish also influence life history traits



and habitat choice of snails. Increased abundance led to the aggregation of snails on food-poor macrophytes and reduced snail growth (Lewis 2001b). Crayfish can also structure the abundance and size distribution of thin-shelled snails through size-selective predation, although crayfish effects on snail size distribution may be less pronounced in complex habitats such as macrophyte beds (Nyström & Pérez 1998). Collectively, these results indicate that crayfish can have dramatic direct and indirect impacts on littoral pond communities via feeding links with multiple trophic levels and non-trophic activities.

#### 2.2.4 *Prevalence of fish influence in shallow lakes*

Tight coupling makes it challenging to discuss the role of macrophytes in shallow lakes without routinely acknowledging the prevalence of fish in the littoral. Multiple interactions highlighted in our literature survey involved fish. Particular littoral fish communities exist in both temperate (Weaver et al. 1997, Lewin et al. 2004) and tropical systems (Vono & Barbosa 2001, Jeppesen et al. 2005). Such fish spend their time predominately within macrophyte beds, although the proportion of that time may change with ontogenetic shifts (Persson & Crowder 1998). For example, juvenile pike (*Esox lucius*) inhabit littoral vegetation but as they grow, pike associate with open water along the edges of macrophyte beds (Bry 1996) or less complex structure(s) of emergent macrophytes (i.e. *Typha*, Skov & Berg 1999).

Apart from ontogenetic habitat shifts, the time spent in the littoral by fish also varies on a diel and a seasonal scale (Romare et al. 2003). For instance, many juvenile littoral fish perform diel horizontal migrations into the open water at night (e.g., Jacobsen & Perrow 1998, Shoup et al. 2003). Limited knowledge exists regarding winter behaviour in shallow lake fish, but littoral structure might act as a winter refuge, possibly resulting in a lower ratio of zooplankton to phytoplankton as compared to summer (Jeppesen et al. 2004). This suggests a lower potential for zooplankton grazing. Habitat use of macrophytes by lower taxa strongly depends on the presence and species identity of littoral fish. As suggested earlier, the water clarity of the system also influences interactions between macrophytes and fish. For example, Jacobsen et al. (2004) showed that roach hide under water lilies during daytime in a clearwater lake inhabited by piscivorous bird species. Therefore, floating-leaved macrophytes might provide an effective cover for fish if the predation threat comes from above.

Macrophytes also serve as substrate for spawning (Fig. 2.2, Winfield 2004). Pike prefer a short dense structure, even flooded grasslands, for spawning. As for several other species, eggs require structure on which to adhere. Some fish (e.g., *Gasterosteus aculeatus*) even use vegetation for nest building during spawning (Kraak et al. 2000). Littoral fish density and identity, however, fail to tell the whole story. The structural complexity of some macrophytes negatively impacts the foraging rate of some fish (Warfe & Barmuta 2004). Predation risk and competition can influence the distribution of fish species and age or size structure. For example, small bluegills (*Lepomis*) spent more time in artificial macrophytes during day than at night, when predators occur (Shoup et al. 2003), while large bluegill (> 200 mm), in contrast, used open water, emergent and submerged vegetation similarly during the day (Paukert & Willis 2002). No single factor controls habitat use of macrophytes by fish although macrophyte density, predation risk, and prey availability may be most influential.

Macrophytes also affect the trophic structure and interactions by enhancing fish kills under ice in lakes with long-lasting ice-cover. Canadian studies have shown higher oxygen depletion under ice in shallow lakes with abundant macrophyte biomass (Meding & Jackson 2001). This may, in turn, affect the entire trophic dynamic and water clarity quite substantially in such lakes (Bayley & Prather 2003). Grazing by fish may also alter the biomass and species composition of submerged macrophytes (Van Donk & Otte 1996). Furthermore, some fish may not intentionally consume macrophytes, but instead inflict damage at a critical developmental stage when the fish forage for macroinvertebrates (Körner & Dugdale 2003). Despite these known impacts, the complexity of interactions associated with macrophytes and fish seems limitless and warrant further study.

## 2.3 In the wings: research areas worthy of attention

Our synthesis of the role of macrophytes in regulating trophic interactions suggests the need for more information to answer several key questions.

### 2.3.1 *Predictability of macrophyte function in trophic interactions across a climatic gradient*

Most studies of aquatic macrophytes have been confined to North temperate lakes (Jeppesen et al. 2003), while little information exists about warm, temperate to tropical lakes (Jeppesen et al. 2005). We might expect that a higher temperature and favourable growth conditions during winter promote growth of macrophytes (Rooney & Kalff 2003, Liboriussen et al. 2005). We must also consider that floating plants and floating-leaved plants are more prominent in the tropics and subtropics, but these architectures have not been studied as well as submerged plants. In dry areas, a lower water table might also promote macrophyte growth (Blindow et al. 1993, Beklioglu et al. *submitted*). However, some macrophyte species, such as *Elodea*, may also be broadly resilient to small increases associated with warming (McKee et al. 2002). Recent enclosure studies along a climatic gradient in Europe suggested a less positive role of macrophytes on water clarity in warmer versus colder temperate lakes (Moss et al. 2004). Bachmann et al. (2002) drew similar conclusions from cross-comparison of data from Florida lakes. One explanation may be that increased omnivory and a shift to small fish size in warm lakes also increase the use of macrophytes as habitat for fish, making the plants a less useful refuge for zooplankton and macroinvertebrate grazers (Fig. 2.3, Meerhoff et al. 2003, Blanco et al. 2003, Jeppesen et al. 2005). In the tropics, life cycles of some fish species are completely coupled to plants (Sazima & Zamprogno 1985, Agonstinho et al. 2003). More comparative studies of submerged, freely-floating, floating-leaved and emergent plant communities are needed to understand how exactly architecture influences the dual roles of macrophytes (Fig. 2.2). We advise more studies to consider this temporal scale and to explore how macrophyte age and ontogeny of other organisms (fish, zooplankton, invertebrates) affects the use of macrophytes as substrates, habitats, or refuge. We especially encourage more studies along climatic gradients to gain needed insight into how climate affects the role of macrophytes in lakes. Such studies are also needed to provide knowledge of how global warming may affect plants and their role in lake ecosystems.

### 2.3.2 *Relative importance of chemical ecology across trophic levels*

As others often propose, we also stress the need for collaboration between chemists and ecologists to identify allelopathic compounds of aquatic macrophytes. Such identification would produce incredible potential for experiments that provide definitive answers to testable hypotheses. Experimental evidence for *in situ* allelopathic activity on epiphyte density is challenging because of natural heterogeneity due to wave action, grazers, and macrophyte structure. Thus, no general proof for lower epiphyte densities on allelopathically active macrophytes exists (Wium-Andersen 1987, Gross et al. 2003a), but different classes of compounds may differentially affect primary producers. For example, after investigating the allelopathic properties of *Ceratophyllum demersum* and *Najas marina*, Gross et al. (2003b) suggested that water-soluble allelochemicals may inhibit phytoplankton, whereas lipophilic allelochemicals may act, for example against epiphytes through direct cell-cell contact. Although they are difficult to conduct, we argue for more field studies that examine allelopathy. For example, we could benefit from more detailed knowledge on how changes in dissolved organic matter (i.e. concentration, composition, or source) or turbidity alter interactions inside and outside macrophyte beds.

### 2.3.3 *Disproportional impacts of certain invertebrates and exotic species*

Fish undoubtedly drive interactions in pelagic communities (Jeppesen 1998). In littoral communities, however, macroinvertebrates (acting as grazers or predators) may be nearly as important in regulating trophic interactions among macrophytes. We recommend that studies continue to focus on invertebrates, especially large individuals, such as snails and crayfish. Our literature review also indicated that epiphyton (in combination with grazers) and benthic-pelagic interactions may play a significant role in pushing systems toward a clear or turbid state. We recommend more field experiments that explore this possibility and its relation to lake/wetland management. Lastly, we encourage wetland scientists and ecologists to examine how macrophytes may facilitate the introduction of exotic species and the implications of macrophytes themselves as exotic species. The introduction of exotic species has the potential to alter primary production, nutrient cycling as well as native biodiversity. Furthermore, climate warming may further facilitate the invasiveness of exotic species.

## **2.4 Returning to center stage: macrophytes are common players in trophic interactions**

Any wetland textbook will enumerate the many ways in which macrophytes alter biogeochemical cycles. Recognition of the importance of macrophytes in regulating ecosystem processes is not novel (Carpenter & Lodge 1986). The take home message of our chapter, however, focused on examining macrophytes at the level of the community and emphasizing the multi-faceted interactions that result. Macrophytes compete with other primary producers, serve as 'prey' for herbivores, function as habitat or substrate for multiple trophic levels, and may serve as vectors for exotic species. Our chapter broadens the discussion from Jeppesen et al. (1998a) by speculating about how floating-leaved, freely-floating, and emergent macrophytes may structure interactions beyond what is known for submerged macrophytes. We also discuss how regulation of such interactions could differ between temperate and tropical systems. Regardless of locality, we strongly suggest that the presence of aquatic macrophytes drives interactions within the rest of the food web and determines which trophic interactions play a role in influencing shifts between alternative states.

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# Laboratory experiments



Phytoplankton batch cultures in the laboratory of the NIOO-KNAW, Centre for Limnology.

Picture: G. Mulderij





Differential sensitivity of green algae to allelopathic  
substances from *Chara*

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## Acknowledgements

# Differential sensitivity

## 3.1 Abstract

Three short-term laboratory experiments were conducted to investigate allelopathic effects of a mixture of *Chara globularis* var. *globularis* Thuillier and *Chara contraria* var. *contraria* A. Braun ex Kützing on three different green algae. Single phytoplankton species were exposed to filtered water originating from charophyte cultures. Phytoplankton growth was monitored by determination of chlorophyll concentrations in batch cultures. Changes in chlorophyll concentration during the experiments was analysed with a logistic growth model, resulting in an estimate of the exponential growth rate and duration of the lag phase of the single green algae.

The results indicate allelopathic effects of *Chara* on the growth of the green algae *Selenastrum capricornutum* and *Chlorella minutissima*, but *Scenedesmus obliquus* did not seem to be affected. The exponential growth rate of *S. capricornutum* decreased 7% in the presence of water from a charophyte culture, while the growth rate of *C. minutissima* decreased with 3%.

The allelopathic effect of *Chara* did not increase when the green alga *C. minutissima* was P-limited. The effect of *Chara* was different when young sprouts were used. With young sprouts the duration of the lag phase of *C. minutissima* was extended (25%), whilst for old plants the growth rate of the green alga decreased.

Although the inhibitory effect of charophytes on specific phytoplankton species is rather small, the differential sensitivity of phytoplankton species to *Chara* exudates might influence the composition and biomass of phytoplankton communities in the field.

## 3.2 Introduction

Until the 1950's most shallow lakes in The Netherlands were characterized by clear water with rich aquatic vegetation (e.g., Simons & Nat 1996). Today many lakes are highly eutrophic and turbid due to increased nutrient loading from agricultural, industrial and domestic sources (Smolders & Roelofs 1993, Jeppesen 1998).

In those shallow lakes that shifted from a clear to a turbid state of the water (Scheffer et al. 1993a, Jeppesen 1998, Van Donk 1998), most submerged macrophytes disappeared (Blindow 1992ab, Coops & Doef 1996). Intense eutrophication resulted in a gradual increase of phytoplankton biomass and epiphytic layers that cover macrophytes (Moss 1976, Phillips et al. 1978). Poor light availability due to shading by epiphytes and phytoplankton was suggested to be the main reason for the disappearance of these macrophytes (Phillips et al. 1978).

The presence of submerged macrophytes is of great importance for the biological structure and water quality of shallow lakes (Moss 1990, Jeppesen et al. 1998b). Submerged macrophytes are believed to play a key role in sustaining the clear water state by mechanisms like competing with algae for nutrients (Ozimek et al. 1990, Van Donk et al. 1993) and light (Pokorný et al. 1984, Ozimek et al. 1990), enhancing denitrification (Weisner et al. 1994), or providing a refuge for zooplankton (Jeppesen et al. 1998a, Burks et al. 2001a), as reviewed by Van Donk & Van de Bund (2002).

Finally, submerged macrophytes may also directly reduce phytoplankton and periphyton growth by releasing allelopathic substances (Anthoni et al. 1980, Wium-Andersen et al. 1982, Jasser 1995, Gross et al. 1996). Allelopathic substances isolated from different macrophytes are suggested to have inhibitory effects on phytoplankton growth. In Lake Swartvlei (South Africa), Howard-Williams (1978) reported stands of *Chara globularis* with very few epiphytes, while dense epiphytic algal growth occurred on *Potamogeton pectinatus*. Low epiphyte densities on charophytes could be an indication of allelochemical excretion by these macrophytes (Anthoni et al. 1980). Blindow (1987), however, recorded higher epiphyton densities on three charophytes than on *P. pectinatus* in a lake in southern Sweden.

Further, Crawford (1977, 1979) recorded a suppression of phytoplankton growth in prairie ponds by introduction of *Chara vulgaris*. Dithiolane and trithiane, two sulphuric compounds isolated from *C. globularis* have been shown to inhibit the photosynthesis of the epiphytic diatom *Nitzschia palea* Kützing and also natural phytoplankton communities from a eutrophic lake and a small macrophyte dominated pond (Wium-Andersen et al. 1982, Wium-Andersen 1987).

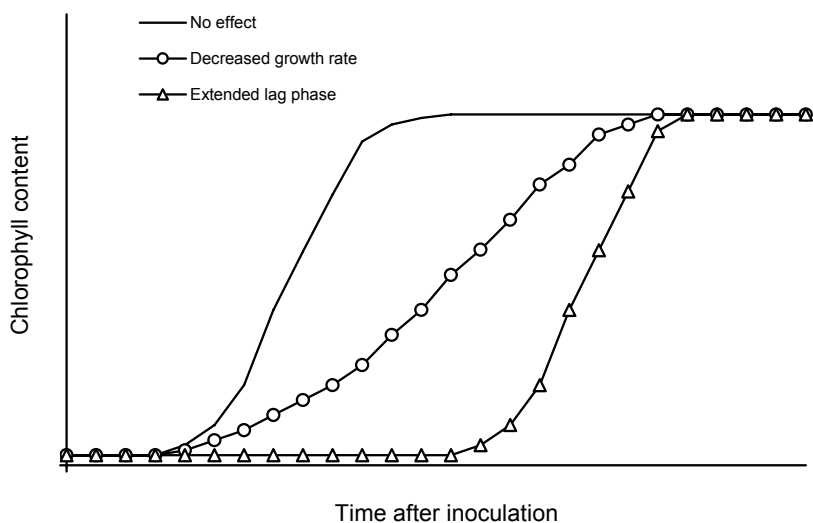
Also a number of almost epiphyte-free macrophytes species were recorded in Danish waters: *Chara*, *Nitella*, *Stratiotes*, *Elodea*, *Berula* and *Ceratophyllum* (Wium-Andersen 1987, Wium-Andersen et al. 1982). Several other studies suggested allelopathic effects of *Ceratophyllum demersum* (Jasser 1995, Mjelde & Faafeng 1997) or its extracts (Wium-Andersen et al. 1983, Jasser 1995) on the growth of phytoplankton. Similar results were recorded for *Myriophyllum spicatum* (Godmaire & Planas 1986, Planas et al. 1981, Jasser 1995, Gross et al. 1996) and some other macrophytes (Fitzgerald 1969).

Gopal & Goel (1993) reviewed current knowledge on competition and allelopathy in aquatic plant communities. They stated that the available information is still too fragmentary and many studies are required to explain the role of allelopathy and competition in aquatic communities. Van Donk & Van de Bund (2002) recently reviewed current knowledge on the impact of submerged macrophytes with special emphasis on the role of charophytes and allelopathy. They concluded that, in most studies on allelopathy, it was difficult to distinguish allelopathic effects from e.g., nutrient and/or light deficiency and stated that future research should concentrate on intact macrophytes and new methods in which competitive interactions (light and nutrient competition) can be excluded.

As described above, charophytes (or stoneworts) often play an important role in aquatic ecosystems (Hootsmans & Vermaat 1991, Meijer & Hosper 1997, Van den Berg 1999) because they are typically rapid colonizers (Simons et al. 1994, Beltman & Allegrini 1997). They are often the first to emerge after lake restoration by bio-manipulation (Van Donk et al. 1990, Meijer et al. 1995, Meijer & Hosper 1997). Compared with other aquatic macrophytes, charophyte meadows probably have an especially strong positive effect on water transparency (Crawford 1979, Scheffer et al. 1994, Van den Berg et al. 1998ab), but Vermaat et al. (2000) stressed that, *Alisma gramineum*, might even be more effective in enhancing the water transparency by sediment trapping.

Whereas the investigations mentioned above suggested allelopathic activity in charophytes, Forsberg et al. (1990), on the contrary, stated that allelopathy by *Chara* was absent *in situ*. Among others, Wium-Andersen (1987) stressed that it is not enough to identify an active substance in an aquatic macrophyte. It is even more important to demonstrate that this compound is actually released in sufficient amounts to affect organisms nearby (Sand-Jensen & Borum 1991).

The present paper describes the response of three green algae (Chlorophyta, Chlorococcales) to cell-free filtrates derived from a mixed culture of two charophyte species. Unlike most other investigations, the present study excludes the competitive interactions nutrient and light competition. The hypothesis of this study is based on findings in toxicity tests (Lage et al. 1996, Okay & Gaines 1996) and other studies on the allelopathic effect of macrophytes on phytoplankton (Blindow & Hootsmans 1991, Jasser 1995, Gross et al. 1996). Like with toxins, the effect of allelopathic substances may be expressed in an increased duration of the lag phase and/or a decline of the exponential growth rate (Fig. 3.1). The aim of this study was to show an allelopathic effect of *Chara* on the lag phase and/or exponential growth rate of three green algae.



**Figure 3.1** Hypothetical curves of differential allelopathic effects of *Chara* on phytoplankton. The possible responses of phytoplankton are indicated in the legend.

The general hypothesis was tested in the first two experiments that were carried out on the three green algae under optimal light, temperature, and nutrient conditions. The third experiment focussed on the role of phosphorus limitation of phytoplankton upon the extent of the allelopathic effect of *Chara* on *Chlorella minutissima*. The allelopathic effect of charophytes on phytoplankton is expected to be more pronounced when phytoplankton is nutrient limited (Reigosa et al. 1999). Fitzgerald (1969) studied the effect of *C. demersum* and *M. spicatum* on epiphytes and phytoplankton and observed a growth inhibiting effect of the macrophytes under N-limited conditions, while no effect was found under P-limited or nutrient sufficient conditions. N-limited cultures of these aquatic weeds remained free of contaminating algae or epiphytes.

Since the second and the third experiment were carried out with charophytes of a different age, it was possible to investigate the differences in allelopathic activity between young and older *Chara* plants. Although these experiments were not conducted simultaneously, the results might give an idea about the effect of charophyte age on the allelopathic activity of these plants. Wolfe & Rice (1979) already suggested that the concentration and/or composition of allelopathic substances may change with time. However, they studied allelopathic effects among algae.

### 3.3 Materials and methods

#### 3.3.1 Macrophyte cultures

In the first two experiments (21 - 28 November and 5 - 12 December 2000), a mixed culture of *Chara globularis* var. *globularis* Thuillier and *Chara contraria* var. *contraria* A. Braun ex Kützing was used. Two aquaria (60 L) were filled with sediment (3 cm) originating from a *Chara* stand in Dubbroek (6°04'60''E, 51°21'00''N), a pond in the south of The Netherlands, close to Venlo. When the sediment was collected, *C. globularis* var. *globularis* and *C. contraria* var. *contraria* were present in the pond. Simultaneously with the collection of sediment, these charophytes were collected. After introduction of the sediment, the aquaria were filled with an artificial macrophyte medium (Table 3.1), modified after Smolders (1995) and Roelofs (1991). The concentration of each element in this synthetically composed medium was comparable to concentrations recorded in Dutch *Chara* stands (De Lyon & Roelofs 1986). Trace elements were not present in the synthetic medium, but it was assumed that charophytes could withdraw these from the sediment. Light was provided by a 16-hour photoperiod at 140  $\mu\text{mol m}^{-2}\text{s}^{-1}$  (Philips HLRG 400W; light intensity mea-

sured upon the water surface). The temperature of the aquaria was maintained at 18°C. In one of the aquaria, charophytes from Dubbroek were introduced in a density comparable to a Dutch field situation (*Chara* treatment). The aquarium without macrophytes served as control. The charophytes, used in the first two experiments, emerged in the field and were transferred into the laboratory on October 26, 2000. Before the start of the first experiment these plants were cultivated in the laboratory for about a month.

The third experiment (1 - 9 March 2001) was also conducted with a mixed culture of *C. globularis* var. *globularis* and *C. contraria* var. *contraria*. Two aquaria (15 L) were filled with sediment (Dubbroek, 3 cm) containing oospores of the charophytes. Under the laboratory conditions mentioned above (18°C, 16 hours photoperiod, 140  $\mu\text{mol m}^{-2}\text{s}^{-1}$ ), sporelings of these stoneworts emerged from the sediment and covered the whole aquarium within five weeks. The sprouts were much younger than those used in the first two experiments. The artificial macrophyte medium (Table 3.1) was also used to refill the aquaria when a part of the water volume was used for experiments.

**Table 3.1.** Composition of modified WC medium and macrophyte medium.

Modified WC medium				Macrophyte medium	
Major nutrients (mg L <sup>-1</sup> )		Trace elements (mg L <sup>-1</sup> )		Major nutrients (mg L <sup>-1</sup> )	
NaNO <sub>3</sub>	85.01	Na <sub>2</sub> EDTA . 2H <sub>2</sub> O	4.36	NaHCO <sub>3</sub>	84.01
MgSO <sub>4</sub> . 7H <sub>2</sub> O	36.97	FeCl <sub>3</sub> . 6H <sub>2</sub> O	1.00	CaCl <sub>2</sub> . 2H <sub>2</sub> O	147.04
CaCl <sub>2</sub> . 2H <sub>2</sub> O	36.76	MnCl <sub>2</sub> . 4H <sub>2</sub> O	0.18	MgCl <sub>2</sub> . 6H <sub>2</sub> O	61.00
Na <sub>2</sub> SiO <sub>3</sub> . 9H <sub>2</sub> O	28.42	CuSO <sub>4</sub> . 5H <sub>2</sub> O	0.001	Na <sub>2</sub> SO <sub>4</sub>	7.1
H <sub>3</sub> BO <sub>3</sub>	24.00	ZnSO <sub>4</sub> . 7H <sub>2</sub> O	0.022	KNO <sub>3</sub>	1.01
NaHCO <sub>3</sub>	12.60	Na <sub>2</sub> MoO <sub>4</sub> . 2H <sub>2</sub> O	0.022	K <sub>2</sub> SO <sub>4</sub>	8.72
K <sub>2</sub> HPO <sub>4</sub>	8.71	CoCl <sub>2</sub> . 6H <sub>2</sub> O	0.012	K <sub>2</sub> HPO <sub>4</sub>	8.71
		NH <sub>4</sub> VO <sub>3</sub>	0.0011		
		Na <sub>2</sub> SeO <sub>3</sub> . 5H <sub>2</sub> O	0.0016		
TES*	85.00				

\* TES = N-Tris(hydroxymethyl)-methyl-2-aminoethane-sulphonic acid (C<sub>6</sub>H<sub>13</sub>NO<sub>6</sub>S); Sigma T-1375



### 3.3.2 Phytoplankton cultures

Prior to the initiation of the experiments, short term batch cultures of single phytoplankton species (*Scenedesmus obliquus*, Max-Planck-Institute for Limnology, Plön, Germany; *Selenastrum capricornutum*, NIVA-CHL 1; *Chlorella minutissima*, CCAP 211/52) were grown in 300 mL Erlenmeyer flasks filled with 200 mL modified (Lürling 1999) WC medium (Guillard & Lorenzen 1972, Table 3.1) and placed in an incubator (100 rpm, 20°C, 16 hour photoperiod, 81  $\mu\text{mol m}^{-2}\text{s}^{-1}$ , Philips TLD 30W/965). These exponentially growing batch cultures were used for inoculation of cultures at the initiation of the experiments.

To culture P-limited *C. minutissima*, a P-sufficient *C. minutissima* cells were washed twice by centrifuging the culture (6 min., 2300 rpm) and resuspension of the pellet in fresh phosphorus-free WC-medium. Thereafter, these *C. minutissima* cells were grown on phosphorus-free WC-medium for 5 days to obtain a P-limited culture. The phosphorus content of the P-limited cells was eleven times lower than the *C. minutissima* cells in the P-sufficient culture (0.09 versus 1.00% P of the dry weight). At the end of the experiment with the P-limited and the P-sufficient *C. minutissima* cultures (*Exp. III*) the P-concentration in the cells of both cultures was equal, at an average P-level of  $1.06 \pm 0.09\%$  P of the dry weight (ANOVA,  $p < 0.05$ ).

### 3.3.3 Experimental set-up

To initiate the experiments, 3 L culture medium from each aquarium (with and without charophytes) was filtered (Whatman GF/F,  $\varnothing$  47 mm). Both types of medium received concentrated nutrient solutions according to the modified WC-medium (Table 3.1), to prevent possible nutrient limitation. Thereafter, each homogeneously mixed medium was divided among 8 Erlenmeyers flasks (500 mL). In total, 16 Erlenmeyer flasks were filled with 350 mL medium (8 Controls & 8 *Chara* treatments).

In the first experiment (*Exp. I*), *S. obliquus* was inoculated in medium with (*Chara* treatment) and in medium without (Control) *Chara* exudates (Table 3.2). Both treatments were performed with two different initial algae cell densities ( $n = 4$ , Table 3.3). In the second experiment (*Exp. II*), the two media (control & *Chara*) were inoculated with two different green algae species: *C. minutissima* and *S. capricornutum* (Table 3.2). The inoculum size in *Exp. II*, expressed in biovolume ( $1.02 \text{ mm}^3\text{L}^{-1}$ ), was equal to the biovolume of the high initial cell density in *Exp. I* (Table 3.3) to enable comparison of *Exp. I* and *II*. In the third experiment (*Exp. III*), the allelopathic effect of young *Chara* sprouts on P-sufficient and P-limited *C. minutissima* was investiga-

ted (Table 3.2). The biovolume of P-sufficient *C. minutissima* cells was equalled to the biovolume of *S. obliquus* cells inoculated in *Exp. I* (low cell density, Table 3.3), to enable comparison between *Exp. I* and *III*. The cell density of P-limited *C. minutissima*, inoculated in *Exp. III*, was equal to the cell density of P-sufficient cells (Table 3.3).

**Table 3.2** Summary of phytoplankton species, *Chara* treatments (– = without; + = with *Chara* water) and the difference in treatment within experiment I, II and III.

Experiment no.	Phytoplankton species	Chara treatment	Treatment within experiment
I	<i>S. obliquus</i>	–	Low initial cell density
I	<i>S. obliquus</i>	+ old plants	Low initial cell density
I	<i>S. obliquus</i>	–	High initial cell density
I	<i>S. obliquus</i>	+ old plants	High initial cell density
II	<i>S. capricornutum</i>	–	–
II	<i>S. capricornutum</i>	+ old plants	–
II	<i>C. minutissima</i>	–	–
II	<i>C. minutissima</i> ,	+ old plants	–
III	<i>C. minutissima</i>	–	P-sufficient
III	<i>C. minutissima</i> ,	+ young sprouts	P-sufficient
III	<i>C. minutissima</i>	–	P-limited
III	<i>C. minutissima</i>	+ young sprouts	P-limited

**Table 3.3** Inoculated amounts of phytoplankton in the experiments (*Exp. I, II* and *III*).

Exp. No.	Phytoplankton species	Biovolume (mm <sup>3</sup> L <sup>-1</sup> )	Cell density (no. of cells L <sup>-1</sup> )
I	<i>S. obliquus</i> , MPI	1.02 10 <sup>-1</sup> / 1.02	1.00 10 <sup>6</sup> / 1.00 10 <sup>7</sup>
II	<i>C. minutissima</i> , CCAP 211/52	1.02	3.43 10 <sup>8</sup>
II	<i>S. capricornutum</i> , NIVA-CHL 1	1.02	2.10 10 <sup>8</sup>
III	<i>C. minutissima</i> , P <sup>+</sup> CCAP 211/52	1.02 10 <sup>-1</sup>	3.24 10 <sup>7</sup>
III	<i>C. minutissima</i> , P <sup>-</sup> CCAP 211/52	5.07 10 <sup>-2</sup>	3.24 10 <sup>7</sup>

### 3.3.4 Sampling and analysis

Immediately after inoculation, a 50-mL sample was taken from each batch culture (remaining volume: 300 mL). After sampling, all Erlenmeyer flasks were placed in an incubator (100 rpm, 20°C, 16 h photoperiod, 112  $\mu\text{mol m}^{-2}\text{s}^{-1}$ ; Philips TLD 30W/965). The chlorophyll content ( $\mu\text{g L}^{-1}$ ) of the samples was determined with a phyto-PAM phytoplankton analyzer (Walz, Mess- & Regeltechnik GmbH, Effeltrich, Germany).

Further, a 50-mL sample was taken from each Erlenmeyer flask, every 12 h, to determine the chlorophyll content. The sampled volume (50 mL) was replaced with fresh medium. 'Fresh medium' was either new control- or new *Chara* water, filtered (Whatman GF/F, Ø 47 mm) and spiked with nutrients according to modified WC-medium (Table 3.1). Fresh medium was added to achieve a semi-continuous addition of *Chara* exudates/allelopathic substances to the algal cultures. The whole investigation period lasted for 7 - 11 days.

### 3.3.5 Data-analysis and statistics

The chlorophyll measurements of *Exp. I*, *II* and *III* were analysed with a logistic growth model. The logistic growth curve was fitted through the observations by minimizing the residual sum of squares calculated from Ln-transformed observed and predicted data. The following logistic equation was applied:

$$A_t = \frac{K}{1 + q * e^{-r(t-T)}} \quad (\text{if } t > T)$$

Here  $A_t$  is the chlorophyll concentration at time  $t$ ,  $K$  is the asymptotic maximum value for  $A_t$ ,  $q$  is an integration constant determining  $A_t$  at time zero ( $q = K/(A_0 - 1)$ ),  $r$  is the exponential growth rate, and  $T$  is the duration of the lag phase. This formula was applied for the exponential and the stationary growth phase ( $t > T$ ). The lag phase ( $T$ ) was defined as the period prior to the exponential growth phase, in which cell numbers remain nearly stationary (Fogg & Thake 1987). The chlorophyll concentration during the lag phase ( $t \leq T$ ) was estimated with the equation:

$$A_t = A_0 \quad (\text{if } t \leq T)$$

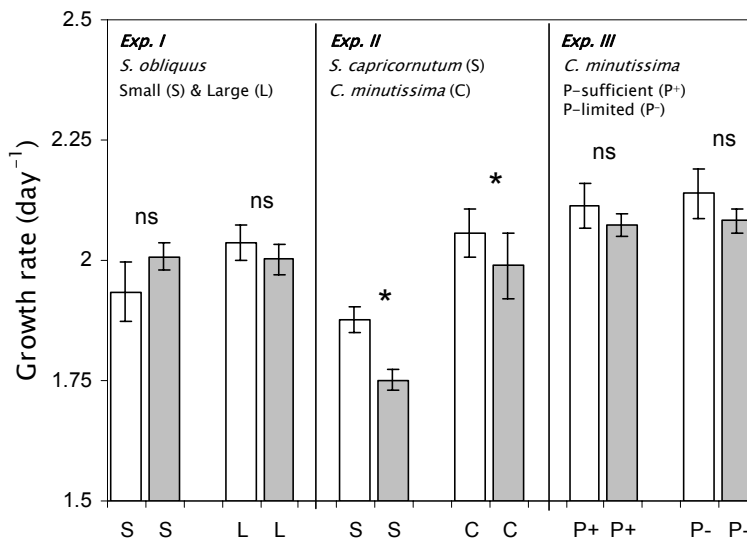
Fitting both equations simultaneously to the chlorophyll data of each batch culture (by numerical optimization) resulted in an estimate for the duration of the lag phase ( $T$ ), exponential growth rate ( $r$ ), integration constant ( $q$ ), and the maximum chlorophyll concentration ( $K$ ). In the present paper, the effect of charophyte exudates on the lag phase ( $T$ ) and exponential growth rate ( $r$ ) of three green algae was investigated. The estimated growth rates were corrected for dilution due to refilling with fresh medium (100 mL per 300 mL culture per day) by increasing the estimated values with  $0.33 \text{ day}^{-1}$ .

Effects of *Chara* exudates on the lag phase ( $T$ ) and exponential growth rate ( $r$ ), as well as the influence of charophyte age (*Exp. II* versus *Exp. III*) and P-limitation of phytoplankton (*Exp. III*) on the extent of the allelopathic effect were assessed by means of ANOVA ( $\alpha = 0.05$ , Fowler et al. 1998, Sokal & Rohlf 1995). Prior to assessment by ANOVA, the estimated values for the parameters mentioned above were tested for normality (Kolmogorov-Smirnov test,  $\alpha = 0.05$ ) and homoscedasticity ( $F_{\max}$  test,  $\alpha = 0.05$ ). All data followed a normal distribution and all variances were homogenous. If the ANOVA revealed a significant interaction, a Tukey test ( $\alpha = 0.05$ ) was performed to group homogeneous means (Fowler et al. 1998). All tests were performed in SPSS version 7.5 (Dytham 1999).

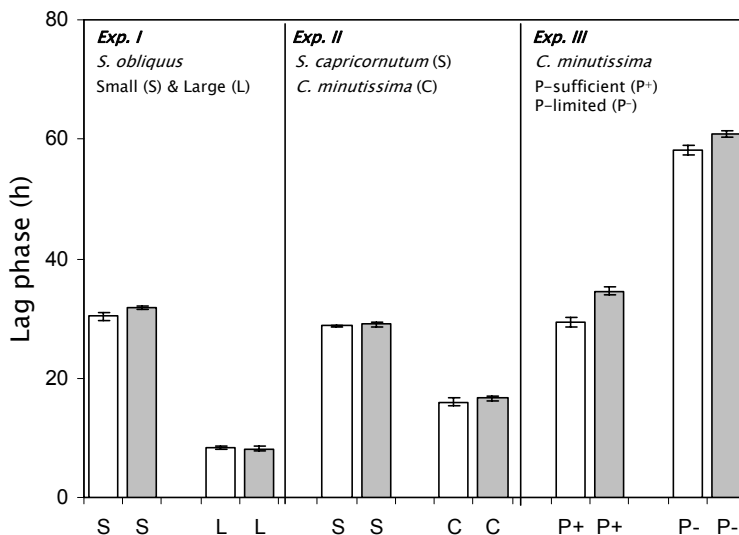
### 3.4 Results

#### 3.4.1 *Chara* and *S. obliquus* (Exp. I)

No effect of the mixed culture of *Chara globularis* var. *globularis* and *C. contraria* var. *contraria* was found on the exponential growth rate ( $r$ ) and the duration of the lag phase ( $T$ ) of *S. obliquus* (Figs. 3.2 & 3.3). The size of the inoculum (Table 3.3) did not affect the exponential growth rate of *S. obliquus* ( $p > 0.05$ ; Fig. 3.2). The average exponential growth rate of all treatments was  $2.0 \text{ day}^{-1}$  ( $n = 16$ ). Inoculation of  $1.02 \times 10^{-1} \text{ mm}^3 \text{ L}^{-1}$  *S. obliquus* culture, resulted in a significantly shorter lag phase (Fig. 3.3). The lag phase decreased with 73% from circa 30 to 8 hours.



**Figure 3.2** Exponential growth rates ( $\text{day}^{-1}$ ) of *S. obliquus* (Exp. I), *S. capricornutum* (Exp. II, S) and *C. minutissima* (Exp. II, C; Exp. III) in the control (white bars) and *Chara* treatments (green bars). 'Small' (Exp. I, S) indicates an inoculated biovolume of  $1.02 \times 10^{-1} \text{ mm}^3 \text{ L}^{-1}$  *S. obliquus* cells, whereas 'Large' (Exp. I, L) stands for an inoculum of  $1.02 \text{ mm}^3 \text{ L}^{-1}$  (Table 3.3). The third experiment (Exp. III) was carried out with P-sufficient (P+) and P-limited (P-) *C. minutissima* cells. The number of replicates within one treatment was four, except for the *Chlorella* treatments in Exp. II ( $n = 3$ ). The individual bars represent average values with error bars (1 SE). The significance of the difference between the control and *Chara* treatments is indicated (\* = significant,  $p < 0.05$ ; 'ns' = non-significant).



**Figure 3.3** Lag phase (hours) of *S. obliquus* (Exp. I), *S. capricornutum* (Exp. II, S) and *C. minutissima* (Exp. II, C; Exp. III) in the control (white bars) and *Chara* treatments (green bars). 'Small' (Exp. I, S) indicates an inoculated biovolume of  $1.02 \times 10^{-1} \text{ mm}^3 \text{ L}^{-1}$  *S. obliquus* cells, whereas 'Large' (Exp. I, L) indicates  $1.02 \text{ mm}^3 \text{ L}^{-1}$  (Table 3.3). The third experiment (Exp. III) was carried out with P-sufficient (P+) and P-limited (P-) *C. minutissima* cells. The number of replicates within one treatment was four, except for the *Chlorella* treatments in Exp. II ( $n = 3$ ). The individual bars represent average values with error bars (1 SE). The significance of the difference between the controls and *Chara* treatments (two-way ANOVA,  $p < 0.05$ ) is indicated (\* = significant; 'ns' = non-significant).

### 3.4.2 Chara, Chlorella and Selenastrum (Exp. II)

The exponential growth rates of *C. minutissima* and *S. capricornutum* were significantly lower in the presence of water from the *C. globularis*/*C. contraria* culture ( $p < 0.05$ ). The growth rate of *C. minutissima* decreased from  $2.05$  to  $1.98 \text{ day}^{-1}$  (3%), whereas it decreased from  $1.88$  to  $1.75 \text{ day}^{-1}$  (7%) for *S. capricornutum* (Fig. 3.2). P+

The length of the lag phase of both green algae was not significantly influenced by the presence of *Chara* water. The lag phase of *S. capricornutum* was almost 29 h, whereas the lag phase of *C. minutissima* in this experiment was significantly shorter with 16 h ( $p < 0.05$ ; Fig. 3.3).

### 3.4.3 Young *Chara* sprouts, P-limited and P-sufficient *Chlorella* (Exp. III)

At the initiation of the experiment, P-limited *C. minutissima* was transferred into a nutrient sufficient medium, what probably enabled these algae to grow as fast as the algae in the non-limited culture. The exponential growth rate of P-limited *C. minutissima* cells was equal (Fig. 3.2) to that of P-sufficient cells, with an average value of  $2.1 \text{ day}^{-1}$  ( $n = 16$ ). In this experiment, the presence of *Chara* water did not have a significant effect on the exponential growth rate, although there seemed to be a tendency towards lower values in the presence of *Chara* water.

The duration of the lag phase of P-limited *C. minutissima* cells was significantly longer than that of non-limited *C. minutissima* cells ( $p < 0.05$ ; Fig. 3.3). Without P-limitation, the lag phase observed in the controls, was almost 28 h, whilst it increased (25%) to almost 35 h in the presence of *Chara* water. In case of P-limitation, the estimates for the duration of the lag phase were, respectively, 58 and 61 h (5% increase). The difference in the duration of the lag phase between control and *Chara* treatments was only significant for P-sufficient *C. minutissima* ( $p < 0.05$ ; Fig. 3.3). The extent of the allelopathic effect was less apparent under P-limitation (26 % versus 5 % extension of the lag phase).

### 3.4.4 Exp. II versus Exp. III: Effect of charophyte age

The presence of *Chara* exudates showed significant effects on the exponential growth rate of P-sufficient *C. minutissima* in Exp. II, but not in Exp. III (the experiment with the younger *Chara* sprouts, Fig. 3.2).

The lag phase of P-sufficient *C. minutissima* in Exp. II was significantly shorter than the lag phase in Exp. III ( $p < 0.05$ ; Fig. 3.3). Like for *S. obliquus* in Exp. I, the difference in length of the lag phase between the *C. minutissima* cultures in Exp. II and Exp. III can probably be explained by the initial inoculum size (Droop 1966, Eberly 1967, Table 3.3). The duration of the lag phase decreases with increasing inoculum size. Analysis of the estimated values for the duration of the lag phase ( $T$ ) of P-sufficient *C. minutissima* also revealed significant differences between the treatments (control and *Chara*). The extent of the allelopathic effect (elongated lag phase,  $T$ , in the presence of *Chara* water) was stronger in Exp. III than in Exp. II, as indicated by the significant interaction in the ANOVA ( $p < 0.05$ ; Fig. 3.3). Multiple comparisons by means of Tukey testing ( $T_{\text{statistics}} = 2.68$ ), indicated a significant difference between the control and *Chara* treatments in Exp. III ( $T = 7.09$ ) but not for Exp. II ( $T = 0.62$ ).

### 3.5 Discussion

As in the experiments of Blindow & Hootsmans (1991), not all the charophyte/phytoplankton combinations showed convincing allelopathic inhibition. In the present study, a significant growth inhibition was found for *Chlorella minutissima* and *Selenastrum capricornutum* (Exp. II), while no significant effects were indicated for *Scenedesmus obliquus* (Exp. I). The exponential growth rate of *C. minutissima* was lowered by 3% (Exp. II), whereas it decreased with 7% in *S. capricornutum* (Exp. II). For the green alga *C. minutissima*, the duration of the lag phase was extended in the presence of water from a culture of young *Chara* sprouts (Exp. III).

Comparing the present results with the hypothetical curves (Fig. 3.1), we conclude that each of the three suggested effects (no effect, extended lag phase, and decreased growth rate) seem to be possible responses of phytoplankton to allelopathic substances excreted by the mixed culture of *Chara globularis* and *C. contraria*. Moreover, the results agree with our hypothesis and indicate allelopathic interactions between charophytes and several planktonic algae.

The exponential growth rate values in the controls estimated for the three different green algae were in accordance with those found in other investigations (*Scenedesmus*: Reynolds 1984, Hälling-Sørensen et al. 1996, *Chlorella*: Shi et al. 1999, 2000, and *Selenastrum*: Mayer et al. 1997, Grade et al. 2000).

Whereas our study showed no effect of a mixed culture of two charophytes (*Chara globularis* var. *globularis* and *C. contraria* var. *contraria*) on the growth of *S. obliquus*, Blindow & Hootsmans (1991) found 10% inhibition in a similar combination: *C. globularis* and *Scenedesmus communis* (Table 3.4) which was comparable with the 7% growth inhibition on *S. capricornutum* in our study. Other charophytes in the same study, however, did not show inhibitory effects. Howard-Williams (1978) reported low epiphytic algal densities on *C. globularis* and Blindow (1987) observed high densities on *C. tomentosa*. Low epiphyton densities on *C. globularis* are in line with the hypothesized allelopathic activity of this aquatic macrophyte.

The absence of an allelopathic effect on *S. obliquus* (Exp. I) might be explained by the composition of the charophyte culture. The mixing of *C. globularis* and *C. contraria* may have diluted the allelopathic effect of *C. globularis* suggested by Blindow & Hootsmans (1991).



Another possibility could be the differential sensitivity among *Scenedesmus* species. Blindow & Hootsmans (1991) used *S. communis* instead of *S. obliquus* as a test organism. Several other studies with different macrophytes (Table 3.4) demonstrated that *Scenedesmus* was not inhibited. This suggests that not all *Scenedesmus* species are sensitive to allelopathic substances from macrophytes. For the other two algae species used in the present study, growth inhibition as well as no allelopathic effects were found in other investigations (Table 3.4).

Additionally, there could be a variation over time in the allelopathic activity of macrophytes. In the study of Blindow & Hootsmans (1991), *C. globularis* did not always show an effect on *S. communis*.

**Table 3.4** Results from studies on the allelopathic effect of macrophytes on some green algae, similar to the ones used in the present study (+ = growth inhibition; – = no allelopathic inhibition).

Macrophyte	Phytoplankton	Effect	Author(s)
<i>Nitzschia frustulum</i> *	<i>Chlorella vulgaris</i>	+	Rice (1984)
Emergent and submerged macrophytes including <i>Myriophyllum spicatum</i>	<i>Selenastrum capricornutum</i>	+	Weeks (1988)
<i>Chara globularis</i>	<i>Scenedesmus communis</i>	+	Blindow & Hootsmans 1991
<i>Brasenia schreberi</i> (floating leaved macrophyte)	<i>Chlorella pyrenoidosa</i>	+	Elakovich & Wooten 1987
<i>Ceratophyllum demersum</i>	<i>Scenedesmus sp.</i>		Kogan et al. 1972
	<i>Chlorella sp.</i>	–	
<i>Chara tomentosa</i> , <i>C. hispida</i> , & <i>C. delicatula</i>	<i>Ankistrodesmus bibraianus</i> , <i>Scenedesmus communis</i>	–	Blindow & Hootsmans 1991
<i>Chara globularis</i>	<i>Ankistrodesmus bibraianus</i>	–	Blindow & Hootsmans 1991
<i>C. demersum</i> & <i>M. spicatum</i>	<i>Scenedesmus sp.</i> , <i>Chlorella sp.</i>	–	Jasser 1995

\* no macrophyte but diatom.

The difference in allelopathic effects among strains of *C. globularis* could be a fourth possibility for the fact that our results did not show an allelopathic effect on *S. obliquus*. Proctor (1971, 1975) studied the extent of reproductive isolation of *C. globularis* and concluded that there is a widespread genetic isolation between the different populations: *C. globularis* is highly variable and consists of one or more biological species. The allelopathic activity among these species may, therefore, vary as well.

The results from the present study also show that the duration of the lag phase of *S. obliquus* and P-sufficient *C. minutissima* appeared to depend on the number of cells in the inoculum at the start of the experiment. The length of the lag phase varies inversely with inoculum size (Droop 1966, Eberly 1967).

Next, P-limitation of *C. minutissima* did not increase the extent of the allelopathic effect on this species. This agrees with results from Fitzgerald (1969) who stated that allelopathic effects occurred when macrophytes and phytoplankton were grown on N-limited medium, while they were absent under normal conditions or under P-limitation.

Moreover, comparison of the results from *Exp. II* and *III* suggests that there is an effect of the charophyte age upon the extent of the allelopathic effect of *Chara*. This corresponds with the findings of Hootsmans & Blindow (1991) who observed that the allelopathic activity of *P. pectinatus* diminished over the course of the season. In our study, the difference between the lag phase of *C. minutissima* in the control and *Chara* treatments (*Exp. II* and *Exp. III*) was significantly larger in the presence of young charophyte sprouts (*Exp. III*), while the effect of *Chara* water on the growth rate of phytoplankton was larger in the experiment with older charophytes (*Exp. II*). Unlike *Exp. II*, the effect of *Chara* water on the exponential growth rate of *C. minutissima* in *Exp. III* was not significant. Nevertheless, there seemed to be a trend towards a lower growth rate in the presence of *Chara* water. Young charophyte sprouts seem to enhance the duration of the lag phase, whereas older charophytes decrease the exponential growth rate of the algae. However, to elucidate the effect of charophyte age, more experiments should be carried out simultaneously with old and young sprouts.

Our experimental evidence confirms the possible existence of allelopathic interactions between charophytes and planktonic green algae. Charophytes had a differential impact on the three green algae. This suggests that charophytes may influence the phytoplankton composition by selective inhibition of some phytoplankton species. Full assessment of the strength of the interactions as a stabilizing feedback requires field experiments and more laboratory experiments with other phytoplankton species/groups and more charophyte species. As indicated by the present results, new experiments should also focus on the use of charophytes in different growth stages. The presently established list of active *Chara* species and sensitive phytoplankton taxa is short, which calls for caution in statements on *in situ* relevance (Crawford 1977, 1979, Forsberg et al. 1990).

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# 4

Allelopathic growth inhibition and colony formation of  
the green alga *Scenedesmus obliquus* by the aquatic  
macrophyte *Stratiotes aloides*

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## Acknowledgements

## Growth inhibition and colony formation

### 4.1 Abstract

Laboratory experiments were conducted to elucidate the allelopathic effects of exudates from the aquatic macrophyte, *Stratiotes aloides*, on the growth and morphology of the green alga *Scenedesmus obliquus*. Both water originating from a *S. aloides* culture and water that had not been in contact with *S. aloides* was simultaneously inoculated with *S. obliquus* cells.

In all experiments we observed a highly significant inhibitory effect of *S. aloides* exudates on the green alga. The duration of the lag phase as well as the initial bio-volume doubling time of *S. obliquus* was extended. The growth rate of the green alga was only significantly inhibited by the presence of exudates from young *Stratiotes* plants.

Furthermore, the mean particle volume (MPV) of *S. obliquus* increased significantly in the presence of *S. aloides* water in all three experiments. Microscopic analyses confirmed that *S. obliquus* forms (more) colonies when exposed to water from a *S. aloides* culture. Colonies of phytoplankton have higher sinking rates than single phytoplankton cells of the same species. These colonies may therefore disappear faster from the upper water layers, resulting in a reduced competition for light between phytoplankton and macrophytes.

## 4.2 Introduction

Aquatic macrophytes may produce toxic substances to defend themselves against herbivory (Ostrofsky & Zettler 1986, Lodge 1991, Newman et al. 1996). The production and secretion of metabolites by aquatic macrophytes could also be an effective defence strategy against other phototrophic organisms (e.g., epiphyton and phytoplankton) in the competition for light and nutrients (Wium-Andersen et al. 1982, Gopal & Goel 1993, Elakovich & Wooten 1995). This phenomenon is called *allelopathy* and it was originally defined as *biochemical interactions (both positive and negative) among primary producers as well as between primary producers and micro-organisms* (Molisch 1937). In this study, however, we will only discuss the inhibitory biochemical interactions between macrophytes and phytoplankton.

Among other mechanisms, the secretion of allelopathic compounds by aquatic macrophytes may play an important role in stabilizing the clear water state of aquatic ecosystems (Scheffer et al. 1993a). Charophytes are often believed to inhibit phytoplankton growth. The allelopathic activity of an extensively growing *Chara* population, for example, was considered to be the cause of an unexpected low level of phytoplankton primary production in a brackish water area (Steeman-Nielsen 1937). The transparency of water surrounding charophytes is often extremely high (Van den Berg 1999) and the density of phytoplankton in the proximity of charophytes is very low (Crawford 1979, Anthoni et al. 1980, Wium-Andersen et al. 1982).

Many other macrophytes seem to possess allelopathic activity as well (Hasler & Jones 1949, Jasser 1995, Gross et al. 1996). Allelopathic activities of aquatic macrophytes on phytoplankton have recently been reviewed by Gross (2003a) and Van Donk & Van de Bund (2002). As already reported in Wolfe & Rice (1979), these two reviews again pointed out that the concentration or composition of allelopathic substances or both, may change with time within the same macrophyte species. This may be caused by the influence of nitrogen or light limitation on the biosynthesis of allelopathic substances, but it may also be related to the age of the macrophyte. The concentration of allelopathic substances in extracts of charophytes has been reported to decrease from summer to autumn (Blindow & Hootsmans 1991). Further, Mulderij et al. (2003, Chapter 3) observed varying degrees of allelopathic activities within one macrophyte species when charophytes of different ages were used.



Chemical defence (anti-herbivore and allelopathic interactions) of submerged macrophytes has recently received more attention through the chemical characterization of substances in macrophyte exudates (Gross & Süttfeld 1994, Newman et al. 1996, Bolser et al. 1998, Gross 1999, Nakai et al. 2000, Kubanek et al. 2000) and it seems that they belong to rather different chemical classes (Anthoni et al. 1980, Wium-Andersson et al. 1983, Gross 1999, Nakai et al. 2001 and Chapter 1). Gross & Süttfeld (1994) and Nakai et al. (2000), for example, have shown that polyphenols released from *Myriophyllum spicatum* into the surrounding water, strongly inhibited the growth of cyanobacteria. In our study, the allelopathic activity of another aquatic macrophyte, *Stratiotes aloides* L. (Hydrocharitaceae) was investigated. *S. aloides* (Water soldier), is a dioecious, perennial aquatic macrophyte (Bloemendaal & Roelofs 1988) that propagates freely by stolons as well as seeds. An important part of its life cycle is submerged (Chapter 1, Fig. 1.6). The macrophyte becomes buoyant in spring as a result of increased photosynthesis and remains floating on the water surface until autumn (De Geus-Kruyt & Segal 1973). It used to be abundant in Dutch canals, broads and ditches (Higler 1977), but the abundance declined dramatically due to changes in the hydrology of aquatic ecosystems (Roelofs 1991, Smolders et al. 1996) causing severe eutrophication, iron limitation, sulphide and ammonium toxicity. The presence of *S. aloides*, nowadays, is often restricted to nature reserves where this species is still very important because, like charophytes, it is a typically rapid colonizer. *S. aloides* is a vigorous-growing plant that will tend to outcompete many plants when grown in small ecosystems.

Compared with other submerged macrophytes the role of *S. aloides* in the inhibition of phytoplankton is still poorly investigated. Brammer (1979) was one of the few who studied the exclusion of phytoplankton in the proximity of *S. aloides*. The interaction between *S. aloides* and phytoplankton was investigated in two similar lakes (Sweden and Poland) that were dominated by this macrophyte. The availability of potassium or sodium rather than the presence of an inhibiting substance from *S. aloides*, however, appeared to be the most influential growth-limiting factor for phytoplankton. Due to the persistent potassium uptake by *Stratiotes* and the lower *in situ* concentrations of this cation, a potassium deficit is more likely to occur than a sodium deficit among dense *S. aloides* stands (Brammer & Wetzel 1984). Besides the potassium depletion, also co-precipitation of phosphates with calcium has been considered as an important mechanism inhibiting the growth of phytoplankton in the proximity of *S. aloides* (Brammer 1979, Brammer & Wetzel 1984). Bloemendaal & Roelofs (1988) observed higher ammonium and phosphate concentrations in water samples taken in the proximity (in the rosettes) of *S. aloides* than in

water samples taken further away from the rosettes. These findings point to allelopathic activity of *S. aloides* plants rather than to nutrient limitation as determining factor in the limitation of phytoplankton growth. Nutrient analyses of water samples, taken between the rosettes and at some distance from the rosettes, showed that there was a concentration gradient and the concentration within the *S. aloides* rosettes was higher for Si, but similar for Cl, NH<sub>4</sub>, K, Ca, Mg and CO<sub>2</sub> (Mulderij et al. *submitted*). Only concentrations of orthophosphate and total phosphorus were slightly lower within the rosettes (Mulderij et al. *submitted*). The concentrations were, however, not low enough to cause severe limitation of phytoplankton growth.

The inhibitory effect of *S. aloides* on phytoplankton growth was studied in the laboratory using the green alga *S. obliquus*. It is among the most common phytoplankton genera in freshwater ecosystems and has a worldwide distribution (Trainor 1998). It is therefore expected to occur in similar habitats as *S. aloides*: mesotrophic to slightly eutrophic ecosystems.

From a morphological point of view, *Scenedesmus* is an extremely variable genus (Trainor 1991). There are unicellular and colonial species that may possess spines or not. Even within the same species, belonging to the genus *Scenedesmus*, the morphology is highly variable. The species may be represented by several ecomorphs (Trainor & Egan 1990). The formation of colonies is one of the possible switches between ecomorphs. The first studies on the morphological variability of *Scenedesmus* focussed on their relationship with the physico-chemical environment, while, thereafter, the focus shifted towards the biological environment. Hessen & Van Donk (1993) discovered that chemical substances released from a cladoceran grazer *Daphnia*, induced colony formation in the green alga *Scenedesmus subspicatus*. These findings have been confirmed for other *Scenedesmus* species (Lampert et al. 1994, Verschoor 2005). These induced changes provide grazing resistance against small zooplankton and can be interpreted as an adaptive anti-herbivore strategy.

Colony formation did not seem to lower the growth rate, but the sedimentation rate of *Scenedesmus coenobia* was higher than the sedimentation rates of unicellular ecomorphs (Lüring & Van Donk 2000). Also, according to Reynolds (1984) large colonies must have higher sinking rates. Higher sedimentation rates of phytoplankton lead to a faster removal of these species from the upper layers of the water column. If aquatic macrophytes, such as *S. aloides*, could secrete compounds that induce colony formation in *Scenedesmus* they would enhance latter's elimination rates from the water column and gain competitive advantage in the competition for light and nutrients at the water surface.

We, therefore, investigated if allelopathic substances secreted by *S. aloides* can reduce the growth rate (1) and induce colony formation (2) in *Scenedesmus* sp. Most studies on allelopathic or 'antagonistic' interactions between macrophytes and phytoplankton investigate coexistence cultures of macrophytes and the algae. In such a set-up it is very difficult to determine whether nutrient or light competition or the secretion of allelopathic substances is the dominant factor limiting phytoplankton growth. In the present study an experimental set-up that excluded the competition for light and nutrients between the macrophyte and phytoplankton was designed by separating the macrophyte and phytoplankton cultures.

## 4.3 Materials and methods

### 4.3.1 Macrophyte cultures

The first two experiments with *Stratiotes aloides* L. were conducted in March (*Exp. I*) and July (*Exp. II*) 2001. We used plants from a laboratory culture, which was started in winter (November 2000) with plants from a stream in Tienhoven (52° 10' 0N 5° 4' 60E), a small village close to Utrecht, The Netherlands. In the laboratory the macrophytes were grown in 500-L aquaria at a density (ca. 10 g FM/L) comparable with a moderate density in the field. The aquaria received 10 cm sediment originating from the same stream in Tienhoven and were filled with copper-free tap water. The water in the aquaria was refreshed fortnightly. The aquaria were illuminated ( $30 \mu\text{mol m}^{-2}\text{s}^{-1}$ ) for 16 h per day and the water temperature was maintained at 18°C. The macrophytes used in the first experiment were 4 months younger than those in the second. A third experiment (*Exp. III*) was carried out in October 2003 using a new laboratory culture of *S. aloides* established in July 2003. The plants were taken from the same *S. aloides* stand in Tienhoven and were cultured under similar conditions as during the first two experiments.

**Table 4.1** Composition (final concentrations) of algal growth medium (modified WC medium, Guillard & Lorentzen 1972, Lüring 1999) and the synthetic macrophyte medium (modified by Roelofs 1991 and Smolders et al. 1996). Nutrients were dissolved in de-ionized water.

<b>Modified WC medium</b>			
Major nutrients (mg L <sup>-1</sup> )		Trace elements (mg L <sup>-1</sup> )	
NaNO <sub>3</sub>	85.01	Na <sub>2</sub> EDTA . 2H <sub>2</sub> O	4.36
MgSO <sub>4</sub> . 7H <sub>2</sub> O	36.97	FeCl <sub>3</sub> . 6H <sub>2</sub> O	1.00
CaCl <sub>2</sub> . 2H <sub>2</sub> O	36.76	MnCl <sub>2</sub> . 4H <sub>2</sub> O	0.18
Na <sub>2</sub> SiO <sub>3</sub> . 9H <sub>2</sub> O	28.42	CuSO <sub>4</sub> . 5H <sub>2</sub> O	0.001
H <sub>3</sub> BO <sub>3</sub>	24.00	ZnSO <sub>4</sub> . 7H <sub>2</sub> O	0.022
NaHCO <sub>3</sub>	12.60	Na <sub>2</sub> MoO <sub>4</sub> . 2H <sub>2</sub> O	0.022
K <sub>2</sub> HPO <sub>4</sub>	8.71	CoCl <sub>2</sub> . 6H <sub>2</sub> O	0.012
		NH <sub>4</sub> VO <sub>3</sub>	0.0011
TES*	85.00	Na <sub>2</sub> SeO <sub>3</sub> . 5H <sub>2</sub> O	0.0016
<b>Macrophyte medium</b>			
Major nutrients (mg L <sup>-1</sup> )		Trace elements (mg L <sup>-1</sup> )	
NaHCO <sub>3</sub>	84.01	Na <sub>2</sub> EDTA . 2H <sub>2</sub> O	4.36
CaCl <sub>2</sub> . 2H <sub>2</sub> O	147.04	FeCl <sub>3</sub> . 6H <sub>2</sub> O	1.00
MgCl <sub>2</sub> . 6H <sub>2</sub> O	61.00	MnCl <sub>2</sub> . 4H <sub>2</sub> O	0.18
Na <sub>2</sub> SO <sub>4</sub>	7.1	CuSO <sub>4</sub> . 5H <sub>2</sub> O	0.001
KNO <sub>3</sub>	1.01	ZnSO <sub>4</sub> . 7H <sub>2</sub> O	0.022
K <sub>2</sub> SO <sub>4</sub>	8.72	Na <sub>2</sub> MoO <sub>4</sub> . 2H <sub>2</sub> O	0.022
K <sub>2</sub> HPO <sub>4</sub>	8.71	CoCl <sub>2</sub> . 6H <sub>2</sub> O	0.012
		NH <sub>4</sub> VO <sub>3</sub>	0.0011
		Na <sub>2</sub> SeO <sub>3</sub> . 5H <sub>2</sub> O	0.0016

TES = N-Tris(hydroxymethyl)-methyl-2-aminoethane-sulphonic acid (C<sub>6</sub>H<sub>15</sub>NO<sub>6</sub>S); Sigma T-1375

### 4.3.2 *Phytoplankton cultures*

Prior to the experiments, *Scenedesmus obliquus* (Turpin) Kützing (Max-Planck-Institute for Limnology, Plön, Germany) was grown in 300 mL Erlenmeyer flasks filled with 200 mL WC medium (Guillard & Lorenzen 1972) modified after Lürling (1999; Table 4.1) and placed in an incubator (100 rpm, 20°C, 16 h photoperiod: ca. 95  $\mu\text{mol m}^{-2}\text{s}^{-1}$ , Philips TLD 30W/965). These short-term batch cultures were established from a continuous culture of *S. obliquus*. Although the batch cultures were not axenic, the bacterial densities measured using a Coulter® Counter Multisizer II (Beckman Coulter, Fullerton, USA) were too low to influence algal growth. Small aliquots of these exponentially growing batch cultures were used for the inoculation of the batch cultures at the start of the experiments.

### 4.3.3 *Experimental set-up*

A week before the start of each experiment the plants were carefully rinsed with tap water to remove adhering phytoplankton, zooplankton and epiphytes. Thereafter the plants were transferred to a sediment-free 60-L aquarium (same macrophyte density) filled with synthetic macrophyte medium (Table 4.1). The nutrient concentrations in this macrophyte medium were similar to nutrient levels found in *S. aloides* stands in The Netherlands. The aquaria were illuminated (30  $\mu\text{mol m}^{-2}\text{s}^{-1}$ ) for 16 h per day and the temperature was maintained at 18°C.

For the first two experiments (*Exp. I & II*) ‘*Stratiotes* water’ was taken from the *Stratiotes* culture in the 60-L aquarium and filtered over Whatman GF/F filters ( $\varnothing$  47 mm). The synthetic macrophyte medium served as a control and was further treated the same way as ‘*Stratiotes* water’. After filtration, nutrients were added in excess to both control medium and *Stratiotes* medium, in concentrations that were the same as in WC medium (Guillard & Lorenzen 1972) modified by Lürling (1999, Table 4.1). For both control and *Stratiotes* medium 100 mL Erlenmeyer flasks were filled with 75 mL medium and inoculated with a small amount of the dense and exponentially growing *S. obliquus* batch culture. The initial biovolume of *Scenedesmus* in all batch cultures was circa  $10^5 \mu\text{m}^3 \text{mL}^{-1}$ . In *Exp. I*, both the control and the *Stratiotes* treatments were replicated 12 times, while the treatments in *Exp. II* were replicated 6 times. Immediately after inoculation, a 5-mL sample was taken from each Erlenmeyer flask to determine the initial biovolume and particle density of the *S. obliquus* cultures using the Coulter® Counter (see 4.3.2 *Phytoplankton cultures*). The increase in biovolume in all cultures was determined daily, for about one week until all cultures of *S. obliquus* had reached the stationary growth phase.

The culture conditions in these experiments were similar to those for the cultures used as inoculum (100 rpm, 20°C, 16 h light: 8 h dark, 95  $\mu\text{mol m}^{-2}\text{s}^{-1}$ ).

We conducted a third experiment (*Exp. III*) to investigate the role of *S. aloides* exudates on the growth and colony formation of *S. obliquus*. Because we observed an increased number of *S. obliquus* colonies in the treatments with *Stratiotes* water in the first two experiments, we optimized the experimental set-up of *Exp. I* and *II*. Instead of using one aquarium with *S. aloides* and one aquarium without *S. aloides*, like in the first two experiments, we duplicated both treatments in *Exp. III*. This avoids pseudo-replication and decreases the chance of observing an artefact (e.g., ‘aquarium-effect’). From each of the four 1-L aquaria (2 *Stratiotes*, 2 controls) we established four batch cultures of *S. obliquus* (quadruplicates). Theoretically, also the presence of bacteria in the macrophyte medium could have caused inhibitory effects on the growth of *S. obliquus* in the first two experiments. To prevent such an effect in the third experiment, we used 0.2  $\mu\text{m}$  cellulose acetate membrane filters ( $\varnothing$  47 mm, Schleicher & Schuell) instead of the Whatman GF/F filters ( $\varnothing$  47 mm), to enable removal of bacteria from the culture media (control and *Stratiotes*). To ensure that the observed increase in mean particle volume (MPV) of *S. obliquus* is really an increase in the number of cells per coenobium and not just an increase in cell size (e.g., as a response to nutrient depletion), we took samples for microscopic analysis (every day) by fixing five mL sample of each batch culture with Lugol’s solution, and the samples were analysed with an inverted microscope (Leitz Fluovert FS) at a magnification of 400. The number of single cells as well as the number of colonies (2- to 8-celled coenobia) was recorded until at least 300 coenobia were counted per sample. Furthermore, the MPV of *S. obliquus* was determined with a Casy® 1 Counter (Schärfe System, Reutlingen, Germany). The light intensity used in *Exp. III* was somewhat lower than in the first experiments (light intensity: 81  $\mu\text{mol m}^{-2}\text{s}^{-1}$ ) due to a renewal of the lamps in the incubator. All other experimental conditions were similar to the conditions in *Exp. I* and *II*.

#### 4.3.4 Data-analysis and statistics

The biovolume data of *S. obliquus* were analysed with an exponential growth model that included both the lag phase and the exponential growth phase of the alga. The model was fitted through the biovolume data by minimizing the residual sum of squares calculated from Ln-transformed observed and predicted data. The following equation was used to model the exponential growth phase.

$$A_t = A_0 * e^{r(t-T)} \quad (\text{if } t > T)$$

$A_t$  is the biovolume at time  $t$ ,  $A_0$  is the biovolume at the start of the experiment ( $t = 0$ ),  $r$  is the exponential growth rate of the green alga *S. obliquus*, and  $T$  is the duration of the lag phase. This equation was only applied for the exponential growth phase ( $t > T$ ) of *S. obliquus*. The lag phase ( $T$ ) is defined as the period prior to the exponential growth phase, in which cell numbers remain nearly stationary (Fogg & Thake 1987). The biovolume during the lag phase ( $t \leq T$ ) was estimated with the equation:

$$A_t = A_0 \quad (\text{if } t \leq T)$$

Simultaneous fitting of these two equations to the biovolume data of each Erlenmeyer flask by numerical optimization provided an estimate for the initial biovolume of *S. obliquus* ( $A_0$ ), the duration of the lag phase ( $T$ ), and the exponential growth rate ( $r$ ). From the exponential growth rate the biovolume doubling time ( $G$ ) of *S. obliquus* was calculated (Fogg & Thake 1987).

$$G = \frac{\ln 2}{r} \quad (\text{for } t > T)$$

This equation is, however, only valid in the exponential growth phase of *Scenedesmus*. Since *S. obliquus* showed a lag phase, the duration of the lag phase should be included to calculate the time needed for the *initial* doubling (from  $A_0$  to  $2A_0$ ), an estimate of the *initial* biovolume doubling time,  $G^*$ :

$$G^* = \frac{\ln 2}{r} + T$$

Similarly, Mulderij et al. (2003, Chapter 3) observed differential sensitivity of phytoplankton to substances from charophytes. In some species the lag phase ( $T$ ) was elongated, while in others the growth rate ( $r$ ) decreased. An allelopathic effect on either  $T$  or  $r$  is reflected in an increase of  $G^*$ . In both cases phytoplankton needs more time to increase the biovolume to a certain level, so with one measure we tested two processes.

Additionally, the effect of *S. aloides* water on the morphology of *S. obliquus* was investigated. To obtain a useful parameter for the investigation of changes in the morphology of *S. obliquus* the mean particle volume (MPV) was derived from the total biovolume and the total particle density:

$$\text{MPV} = \text{total biovolume} / \text{total particle density}$$

The influence of *Stratiotes* water on the initial biovolume doubling time ( $G^*$ ) and mean particle volume (MPV) of *S. obliquus* was assessed by means of ANOVA ( $\alpha = 0.05$ , Fowler et al. 1998, Sokal & Rohlf 1995). Prior to this assessment, the estimated values for  $G^*$  and the MPV were tested for normality (Kolmogorov-Smirnov test,  $\alpha = 0.05$ ) and homoscedasticity ( $F_{\max}$  test,  $\alpha = 0.05$ ). To meet the two assumptions for assessment of ANOVA (normality and homoscedasticity), the MPV data of *Exp. I* and *II* were log-transformed, while for the  $G^*$  data it was only necessary to transform the data of *Exp. I* to square root values. All data then followed a normal distribution, while all variances were homoscedastic. When the ANOVA revealed significant effects, a Tukey test ( $\alpha = 0.05$ ) was applied to group homogeneous means. Statistical analysis (ANOVA) of the data of *Exp. III* revealed no significant difference between the duplicates within one treatment (e.g., the two control aquaria). Therefore, the data of the two separate aquaria were pooled before the final statistical analyses. All analyses were performed in SPSS version 7.5.

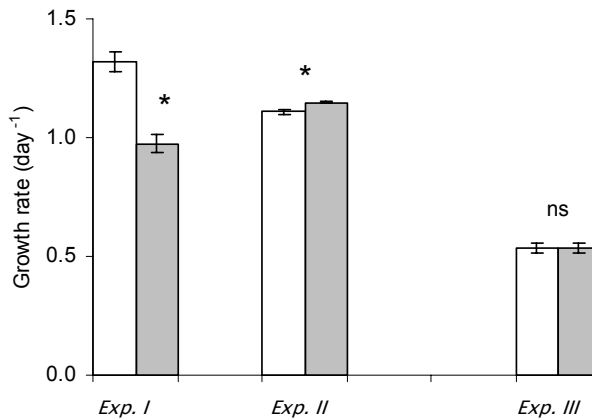


## 4.4 Results

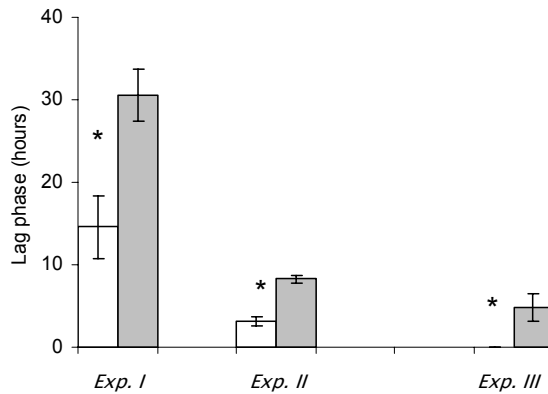
### 4.4.1 Experiment I and II

From the exponential growth model we estimated the growth rate ( $r$ ) and the lag phase ( $T$ ) of *S. obliquus*. In *Exp. I* with the younger *S. aloides* plants, the growth rate was significantly ( $p < 0.001$ ) lower in the presence of *Stratiotes* water (Fig. 4.1). Surprisingly, in *Exp. II*, the growth rate of *S. obliquus* was slightly but significantly ( $p = 0.005$ ) higher in the *Stratiotes* treatment than in the control (Fig. 4.1). In both experiments the lag phase was significantly ( $p < 0.001$ ) longer when the green alga was cultured in the presence of *Stratiotes* water (Fig. 4.2).

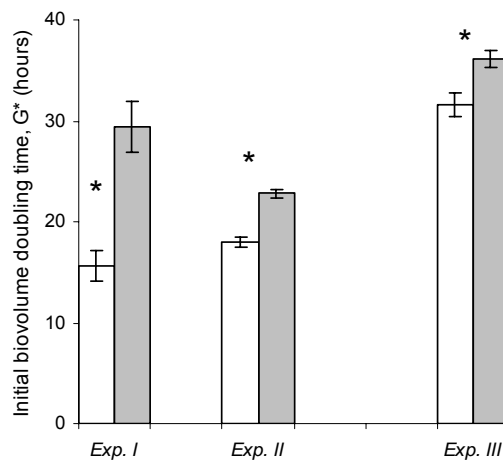
The initial biovolume doubling time ( $G^*$ ) of the *S. obliquus* cultures was derived from the estimates for  $r$  and  $T$ . Both in *Exp. I* and *Exp. II*  $G^*$  increased significantly ( $p < 0.001$ ) in the presence of water from the *S. aloides* culture (Fig. 4.3): 80% in *Exp. I* and 27% in *Exp. II*. Thus, the difference in the initial biovolume doubling time between the cultures exposed to control water and to *Stratiotes* water was much larger in *Exp. I*.



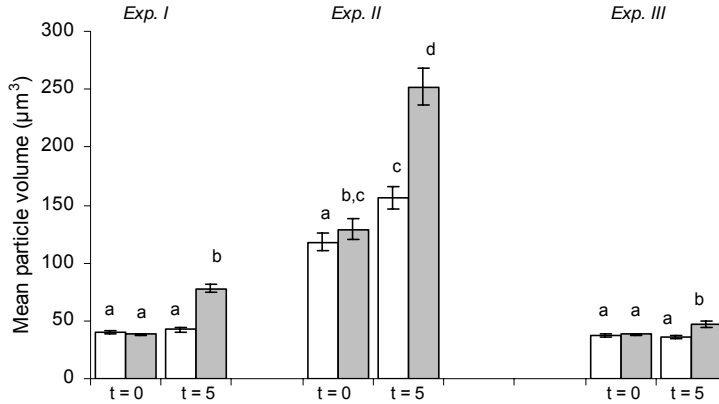
**Figure 4.1** Estimated exponential growth rate  $r$ , of *S. obliquus* cultures in the presence (green bars) or absence (white bars) of *Stratiotes* water. The results from all three experiments are depicted (*Exp. I*, *Exp. II* & *Exp. III*). Data represent average values (*Exp. I* :  $n = 12$ ; *Exp. II* :  $n = 6$ , *Exp. III* :  $n = 8$ ) with error bars (1 SE). Significant differences between control and *Stratiotes* are indicated with \* ( $p < 0.05$ ), while non-significant differences are indicated with 'ns'.



**Figure 4.2** Estimated duration of the lag phase  $T$ , of *S. obliquus* cultures in the presence (green bars) or absence (white bars) of *Stratiotes* water. The results from all three experiments are depicted (*Exp. I*, *Exp. II* & *Exp. III*). Data represent average values (*Exp. I*:  $n = 12$ ; *Exp. II*:  $n = 6$ , *Exp. III*:  $n = 8$ ) with error bars (1 SE). Significant differences between control and *Stratiotes* are indicated with \* ( $p < 0.05$ ). The average value for the lag phase of the control in the third experiment was 0.



**Figure 4.3** Calculated values of the initial biovolume doubling time  $G^*$ , for cultures of the green alga *Scenedesmus obliquus* grown in the presence (green bars) or absence (white bars) of *Stratiotes* water. The results from all three experiments are depicted (*Exp. I*, *Exp. II* and *Exp. III*). Data represent average values (*Exp. I*:  $n = 12$ ; *Exp. II*:  $n = 6$ , *Exp. III*:  $n = 8$ ) with error bars (1 SE). Significant differences between *Stratiotes* and controls are indicated with \* ( $p < 0.05$ ).

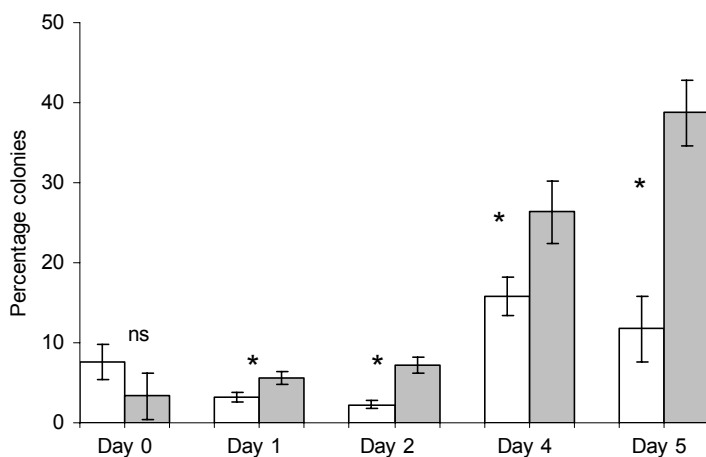


**Figure 4.4** Mean particle volumes (MPV) calculated for cultures of the green alga *S. obliquus* at the start ( $t = 0$  days) and end ( $t = 5$  days) of *Exp. I*, *II* and *III*. The bars represent the mean particle volume of *S. obliquus* cultures grown in the presence (green bars) or absence (white bars) of *Stratiotes* water. The individual bars indicate average values (*Exp. I*:  $n = 12$ ; *Exp. II*:  $n = 6$ , *Exp. III*:  $n = 8$ ) with error bars (1 SE). Based on multiple comparisons within one experiment using a Tukey test ( $\alpha = 0.05$ ), groups with homogeneous means are indicated with letters (a – d).

The mean particle volume (MPV) of *S. obliquus* at the beginning of these two experiments was equal for both control and *Stratiotes* treatments (Fig. 4.4), but increased significantly ( $p < 0.001$ ) towards the end of the experiments in the *Stratiotes* treatments. In *Exp. I* the MPV increased 80% in the presence of *Stratiotes* water, while it increased 60% in *Exp. II*. Although these increases in MPV of *S. obliquus* in the presence of *Stratiotes* water were similar ( $p = 0.990$ ), the initial MPVs differed significantly ( $p < 0.001$ ) between the experiments (40 versus  $124 \mu\text{m}^3$ ). Moreover, there was also a slight (but significant) difference in the initial MPV for the control and the *Stratiotes* treatment in *Exp. II*. Nevertheless, the difference between the treatments increased over the course of the experiment.

#### 4.4.2 Experiment III

In *Exp. III* the effect of *S. aloides* exudates on the growth rate of *S. obliquus* did not significantly differ ( $p = 0.903$ ) between the treatments (Fig. 4.1). In contrast, the lag phase of *S. obliquus* was significantly ( $p = 0.011$ ) longer than in the control (Fig. 4.2). Moreover, the cultures exposed to *Stratiotes* water had a significantly ( $p = 0.009$ ) longer initial biovolume doubling time than cultures of the control (Fig. 4.3). The MPV of *S. obliquus* cultures in *Stratiotes* water increased significantly ( $p < 0.001$ ) from ca.  $38 \mu\text{m}^3$  at day 0 to  $47 \mu\text{m}^3$  at day 5 (14%), while the MPV in the control did not change significantly. Microscopic analysis showed a significant increase in the percentage colonies in the treatments with *Stratiotes* water (Fig. 4.5). Already from the first day after inoculation of the (mainly) unicellular *S. obliquus* cultures, significantly more 2- to 8-celled coenobia were observed in the presence of *S. aloides* exudates. The difference with the controls became larger towards the end of the experiment (Fig. 4.5), i.e. at the end there were ca. 40% colonies in the *Stratiotes* treatments and only ca. 10% colonies in the controls.



**Figure 4.5** Percentage of cells present as colonies in cultures of *S. obliquus* grown in the presence (green bars) or absence (white bars) of *Stratiotes* water. The individual bars indicate average values ( $n = 8$ ) with error bars (1 SE). Based on multiple comparisons using a Tukey test ( $p < 0.05$ ), significant differences between control and *Stratiotes* treatments are indicated with \*. Non-significant differences are indicated with 'ns'. Samples of Day 3 for microscopic analysis were lost.

## 4.5 Discussion

The present study demonstrates that addition of water from the *S. aloides* culture had a significant effect on the exponential growth rate of *S. obliquus*. The initial biovolume doubling time in *S. obliquus* cultures increased between 14 and 80% compared to the control. This implies that the growth of *Scenedesmus* was moderately to severely inhibited by the presence of *S. aloides* exudates. The exponential growth rate and the duration of the lag phase of *S. obliquus* show that the inhibitory effects on this green alga are mainly expressed in an extension of the lag phase. Only in the first experiment, where we used exudates from young macrophytes, did we observe a significant inhibition of the exponential growth rate of *S. obliquus*. The lag phase, however, was extended in all three experiments, i.e. there was an inhibitory effect.

Inhibition of phytoplankton by exudates from macrophytes has been observed by many workers (e.g., Planas et al. 1981, Jasser 1995, Mjelde & Faafeng 1997). The extent of the allelopathic inhibition of *Stratiotes* water in our study (14 to 80%) is similar or higher than inhibitory effects reported for other macrophytes (Kogan & Chinnova 1972, Blindow & Hootsmans 1991, Mulderij et al. 2003, Chapter 3). We observed that an initial addition of water from the *S. aloides* culture to phytoplankton batch culture was enough to induce a strong, allelopathic inhibition. In a similar experiment studying the allelopathic effects of charophytes on phytoplankton (Chapter 2), it was necessary to add water from a charophyte culture semi-continuously to phytoplankton batch cultures to cause discernible growth inhibition of phytoplankton. Thus, chemical cues from *S. aloides* compared with those from charophytes appear to be both more stable and/or more potent.

Contrary to the observations of Mulderij et al. (2003, Chapter 3), Hootsmans & Blindow (1991) had reported an inhibitory effect of charophyte exudates on *Scenedesmus* upon initial addition. These authors, however, used *Scenedesmus communis* and *Chara globularis*. They observed no growth inhibition on *S. communis* when using exudates from several other *Chara* species (*C. tomentosa*, *C. hispida*, *C. delicatula*). Identification of allelopathic substances originating from *S. aloides* as well as many other macrophytes will shed further light on the differences reported in the literature, and should therefore be given a high priority in future studies.

Although *Exp. I* (March 2001) and *Exp. II* (July 2001) were conducted with macrophytes from the same laboratory culture, the  $G^*$  data show that the effect of *Stratiotes* water was stronger in *Exp. I*, where the macrophytes were younger. These results support observations of Wolfe & Rice (1979), and the works reviewed by Gross (2003a) and Van Donk & Van de Bund (2002). Both the concentration and composition of allelopathic substances released by the macrophytes can change with time.

In *Exp. II* the macrophytes were four months older than those in *Exp. I*. Therefore, it appears that younger *S. aloides* plants have a stronger allelopathic effect on the growth of *S. obliquus* than older plants. From the viewpoint of survival it may be an important strategy for young plants to produce more potent allelochemicals, to be able to successfully compete for light and nutrients with other autotrophs while growing upwards to reach the water surface. Older *S. aloides* plants that already have reached the water surface can considerably circumvent light limitation by floating on the water surface. Such plants can afford to invest in the production of turions and seeds. Since we did not replicate the age-effect in our experiments it is, however, not possible to statistically test whether exudates from younger plants are really more potent than those from the older ones. The effect of macrophyte age upon the allelopathic inhibition of phytoplankton can be best studied in parallel experiments using a series of plants of different ages but originating from the same clone.

In all experiments the mean particle volume (MPV) of *S. obliquus* significantly increased in the presence of *Stratiotes* water. In *Exp. III* we confirmed the increase in number of cells per coenobium. These findings compare well with several others reported in literature (Hessen & Van Donk 1993, Lampert et al. 1994, Lürling & Van Donk 2000). Although the studies cited were confined mainly to the effect of chemical cues from zooplankton, the response observed for *S. obliquus* was similar to that observed by us: an increased MPV in the presence of chemical cues, due to formation of colonies by *S. obliquus*.

Increased MPV or colony formation of this green alga will enhance the sedimentation rate and thereby also the disappearance rate of *S. obliquus* from the water column. So, colony formation by *S. obliquus* can be an important algal defence strategy against zooplankton grazing, but it can also be a disadvantage for this alga in the competition for light with macrophytes. *S. obliquus* may sink faster to the deeper water layers, thereby changing the relative composition of phytoplankton in the upper water layers. It still needs to be investigated whether induction of colony formation can also be found in other phytoplankton species.

The MPV values of *S. obliquus* at the start of *Exp. I* and *III* differed from those of *Exp. II*. This was more likely due to difference in the moment of sampling than any (physiological) changes in the cultures. Since we used a daily photoperiod of 16 h for incubation *S. obliquus* cells started to synchronize, i.e. the cell division started just before the start of photoperiod and the cell growth took place in the light period until darkness (Fogg & Thake 1987). The time interval between the start of the photoperiod and the moment of sampling mainly determines the size of *Scenedesmus* cells. The longer this period is the larger the algal cells will be at the moment of sampling. The timing of the sampling within each experiment was, however, similar and this most likely ensured that at all sampling events within one experiment the cells had similar growth history. In *Exp. II*, however, the time that the cultures were daily exposed to light before sampling was longer. This discrepancy in the sampling procedure is most likely the cause of the larger initial MPV values for *S. obliquus* in *Exp. II*. Since our aim was to examine the effect of exudates from *S. aloides* on growth rates of *S. obliquus* rather than on the MPV of *S. obliquus* cells we did not pay attention to the timing of sampling after the commencement of the photoperiod. For *Exp. III*, however, we used the same photoperiod history before sampling as in *Exp. I*. This explains the similarity of MPVs in *Exp. I* and *Exp. III*. One could, nevertheless, argue that the physiology of *S. obliquus* cultures may have changed between the two experiments. This, however, does not appear to have been the case because in both experiments the batch cultures were started using an inoculum from the same continuous culture.

In all three experiments the MPV of *S. obliquus* significantly increased in the presence of *Stratiotes* water. Only in the last experiment did we carry out a microscopic analysis to confirm the results from the MPV data of the other two experiments. Within one day after inoculation of the unicellular *S. obliquus* cells, we observed a significantly higher percentage 2 - 8 celled coenobia in the *Stratiotes* treatments than in the controls. This supports our line of reasoning that colony formation of *S. obliquus* was caused by exudates from *S. aloides*.

In conclusion, we found a significant allelopathic effect of *S. aloides* on the growth of *S. obliquus*. Furthermore, exudates from *S. aloides* induced colony formation in *S. obliquus*, promoting the sedimentation of this alga from the water column. Both growth inhibition and induction of sedimentation of phytoplankton appear to be evolutionary strategies adapted by macrophytes as *S. aloides* to suppress the phytoplankton for avoiding competition for common resources such as nutrients and light. In this respect the young macrophytes seem to have the strongest effect on the initial biovolume doubling time of *S. obliquus*. If macrophytes were to produce more allelopathic substances later in the growing season (autumn) when they are older, it may be difficult for the macrophyte to compete with phytoplankton for resources. This argument is based on the fact that phytoplankton may, in the absence of such a competition early in the season, reach much higher densities later in the season and would, therefore, be much more difficult to be outcompeted.

The present study helps to reduce the gap in our knowledge about allelopathic effects of macrophytes on phytoplankton. Our conclusions are, however, based on laboratory experiments. To gain more insight into the role of *S. aloides* in Dutch pools and ditches, and to understand the ecological importance of allelopathic interactions, together with its repercussions on resource competition, it is indispensable to conduct field or enclosure experiments that focus on the effect of exudates from macrophytes, such as *S. aloides*, on the whole phytoplankton community in the different seasons. Lastly, we need to simultaneously focus on identifying metabolites secreted by macrophytes to investigate the chemical composition of substances that can cause such strong inhibitory effects on phytoplankton.



## Acknowledgements

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# 5

Inhibition of phytoplankton by allelopathic substances from  
*Stratiotes aloides*

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## Allelopathic growth inhibition

### 5.1 Abstract

The allelopathic potential of exudates from the aquatic macrophyte *Stratiotes aloides* on the growth of phytoplankton was investigated. A selection of phytoplankton species, occurring in habitats similar to that of *S. aloides*, was used: two cyanobacterial strains (toxic and non-toxic *Microcystis aeruginosa*), one green alga (*Scenedesmus obliquus*) and one eustigmatophyte (*Nannochloropsis limnetica*).

The results indicate allelopathic effects of *S. aloides* on phytoplankton in six of the eight cases, expressed in an extended duration of the initial biovolume doubling time. The overall inhibitory effect (8 - 51%) was strain-specific for the two cyanobacteria. We also studied the effect of light on the sensitivity of phytoplankton to exudates from *S. aloides*. Light influenced the response of *S. obliquus* only. The inhibitory effect of *S. aloides* exudates on the growth of this green alga was stronger at  $35 \mu\text{mol m}^{-2}\text{s}^{-1}$  than at  $105 \mu\text{mol m}^{-2}\text{s}^{-1}$ .

We conclude that *S. aloides* has allelopathic effects on phytoplankton, and that light can, but does not always determine the extent of the allelopathic inhibition. In our experiments, the sensitivity of cyanobacteria to *S. aloides* exudates was not higher than for other phytoplankton strains, but within cyanobacteria, the toxic strain was more sensitive than the non-toxic one.

## 5.2 Introduction

The presence of submerged macrophytes is considered to be of great importance for maintaining the clear water state in shallow lakes (e.g., Jeppesen et al. 1998a). Submerged macrophytes play different roles in shallow aquatic ecosystems. They fix substantial amounts of nutrients in their biomass (e.g., Kufel & Ozimek 1994, Van Donk & Van de Bund 2002) and thus compete for these nutrients with other autotrophic organisms, such as phytoplankton, and limit the latter's growth so that the water transparency improves. Macrophytes may also promote clear water by providing a refuge for zooplankton (Jeppesen et al. 1998b) and by the excretion of allelopathic substances that inhibit phytoplankton growth (Gross 2003a).

Molisch (1937) originally defined allelopathy as both stimulatory and inhibitory biochemical interactions between classes of plants (including micro-organisms). Most recent studies, however, have focussed on the inhibitory effect of allelopathic substances (Gross 2003a). Allelopathy may be a useful strategy for macrophytes against other phototrophic organisms in aquatic food webs. There are several reported cases of allelopathic activity of macrophytes e.g., *Chara* (Wium-Andersen et al. 1982, Blindow & Hootsmans 1991, Mulderij et al. 2003, Chapter 3), *Ceratophyllum* (Jasser 1995, Mjelde & Faafeng 1997) and *Myriophyllum* (Jasser 1995, Gross et al. 1996), resulting in changes in phytoplankton biomass, phytoplankton composition or both.

In the present study we focussed on allelopathic effects of the macrophyte *Stratiotes aloides* on several phytoplankton species. *S. aloides* is one of the characteristic species in the waters of peaty lowlands in The Netherlands. It is a dioecious, perennial aquatic macrophyte that shows vigorous clonal branching by means of tillers and turions and may form very dense stands (Smolders et al. 1996). In spring, the plants rise to the water surface (Smolders et al. 2003), where they float, flower and produce new tillers and turions. In autumn, all plants (with tillers and turions) sink to the sediment, where they stay until next spring (see Chapter 1, Fig. 1.6). For growth and reproduction, *S. aloides* needs moderately high nutrient concentrations and is, therefore, absent in oligotrophic waters (Smolders et al. 2003).

Brammer (1979) and Brammer & Wetzel (1984) observed that *S. aloides* markedly changes the *in situ* concentrations of  $K^+$ ,  $Na^+$  and  $Ca^{2+}$ . Also lake water phosphorus and nitrogen levels decreased, suggesting that the dominant *S. aloides* might withdraw nutrients not only from the sediment, but also from the surrounding water (Brammer 1979). Co-precipitation of phosphorus with calcium on *S. aloides* leaves was also discussed as alternative explanation for clear water around the *S. aloides* plants (Brammer 1979, Brammer & Wetzel 1984). However, rather than allelopathy, competition for essential nutrients and changes in the ionic composition of the water seemed to be more likely explanations for the observed low phytoplankton densities (Brammer 1979, Brammer & Wetzel 1984).

Waterbodies dominated by *S. aloides* are, however, usually very clear despite the moderately high nutrient concentrations. Therefore, nutrient limitation may not be the critical growth limiting factor for phytoplankton. Jasser (1995) showed that extracts of *S. aloides* significantly inhibited three cyanobacteria. This study does not rule out competition for nutrients, but it implies another possible mechanism. It suggests that allelopathic substances were present in the extracts.

The contradictory conclusions of the two previous studies illustrate the difficulty of demonstrating unequivocally the importance of allelopathy in natural aquatic ecosystems. It is often hard to distinguish allelopathy (a chemical cue from a donor organism that affects the fitness of a target organism) from resource competition. Therefore, most studies on allelopathic interactions are conducted in laboratories, where experimental conditions can be controlled. Additionally, most laboratory studies on allelopathic interactions between aquatic macrophytes and phytoplankton are performed with extracts of aquatic macrophytes (e.g., Nakai et al. 1996) or substances isolated from plant tissue (Wium-Andersen et al. 1982, Gross & Sütfeld 1994). This allows exclusion of direct nutrient competition between two competing organisms. These studies can, however, only show the presence of allelopathic substances in macrophyte tissue.

To effectively inhibit phytoplankton growth by allelopathic substances, macrophytes need to produce allelopathic substances in the first place, but they also need to excrete them in the vicinity of phytoplankton (Wium-Andersen 1987). Unfortunately, observations of this extracellular release are rare (Gross et al. 1996, Nakai et al. 2000). Investigations of the effect of allelopathic substances from intact macrophytes on phytoplankton growth are often confounded by factors like competition for light and nutrients, both of which can limit phytoplankton growth (Jasser 1994,

Brammer 1979). Light limited growth of phytoplankton occurs frequently in nature (Huisman & Weissing 1994). Under light limitation, and also due to multiple other stress factors, allelopathic effects on phytoplankton are expected to become stronger (Reigosa et al. 1999).

In addition, the response of phytoplankton to allelopathic substances from macrophytes is likely to be species-specific. Jasser (1994) found cyanobacteria to be more sensitive to allelochemicals from *Ceratophyllum* than green algae, which were unaffected. In contrast, the study of Wium-Andersen et al. (1982) showed that the growth response of cyanobacteria to allelochemicals did not differ from that of other phytoplankton groups.

In our approach four phytoplankton strains and two different light intensities were used. Nutrient limitation as growth inhibiting factor for phytoplankton was excluded. We tested the following hypotheses relating to the importance of allelopathic substances:

1. Culture water from a *S. aloides* culture inhibits phytoplankton growth.
2. The extent of this inhibitory effect caused by exudates from *S. aloides* is dependent on the light intensity used for the phytoplankton cultures.
3. Cyanobacteria are more sensitive to allelopathic substances from *S. aloides* than other phytoplankton species (a) and, within cyanobacteria, toxic strains are more sensitive to these substances than non-toxic strains (b).



## 5.3 Materials and methods

### 5.3.1 *Macrophyte cultures*

*Stratiotes aloides* L. used in this study was derived from a laboratory culture established in November 2000 using plants originating from a ditch in Tienhoven (52° 10' 0N 5° 4' 60E), near Utrecht, The Netherlands. The macrophytes were grown in 500-L aquaria with copper-free tap water and ca. 10 cm sediment originating from the same site in Tienhoven. Every second week the aquaria were refilled with fresh copper-free tap water. The plants were exposed to a 16:8 h light : dark cycle (30  $\mu\text{mol m}^{-2}\text{s}^{-1}$ ) at 18°C.

One week before the start of the experiment, seven *S. aloides* plants (diameter ca. 20 cm) were carefully rinsed with tap water (to remove epiphyton and zooplankton), and transferred to a 60-L aquarium with synthetic macrophyte medium (Mulderij et al. 2005a, Chapter 4, Table 4.1). The concentrations of major elements (e.g., N, P, S, K, Na, Ca, Mg) resembled those recorded in natural *S. aloides* stands in the Netherlands (De Lyon & Roelofs 1986). Trace elements that were not present in this synthetic medium, were added in concentrations similar to those in modified WC medium (Mulderij et al. 2003, Chapter 3). As a control, a similar aquarium (60 L) filled with synthetic macrophyte medium and trace elements, but without *S. aloides* was used. The growth conditions were 140  $\mu\text{mol m}^{-2}\text{s}^{-1}$  (16 : 8 h light : dark cycle) and 18°C. At the time of the experiment, the plants were mainly submerged, only a minor part of the leaves emerged from the water surface.

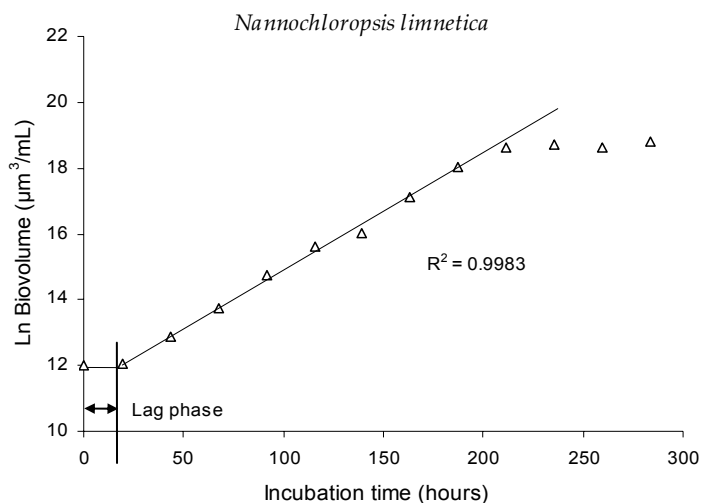
### 5.3.2 *Phytoplankton cultures*

We used three phytoplankton species: two *Microcystis aeruginosa* strains (non-toxic NIVA-CYA 43 and toxic NIVA-CYA 140: 5091  $\mu\text{g L}^{-1}$  Microcystin-LR), one eustigmatophyte (*Nannochloropsis limnetica* Krienitz 1998/3) and one green alga (*Scenedesmus obliquus* Turpin (Kützing), CCAP 276/3A). These four strains occur regularly in meso- to eutrophic habitats (Chorus & Bartram 1999, Krienitz et al. 2000, Naselli-Flores & Barone 2000), and may therefore interact with *S. aloides* as this macrophyte also occurs in meso- to eutrophic ecosystems.

Prior to the laboratory experiment, each phytoplankton species was grown in batch culture in 300-mL Erlenmeyer flasks containing 200 mL modified WC medium (buffered with TES, Mulderij et al. 2003, Chapter 3), at 21°C, 16 : 8 h light : dark cycle and shaken at 100 rpm. The exponentially growing cells were used as inoculum at the start of an experiment. The two cyanobacteria were pre-cultured at 35  $\mu\text{mol m}^{-2}\text{s}^{-1}$  (Philips TLD 30W/965), and the other two phytoplankton species (*S. obliquus* and *N. limnetica*) were precultured at 105  $\mu\text{mol m}^{-2}\text{s}^{-1}$  (Philips TLD 30W/965).

### 5.3.3 Experimental set-up

In the present study, two 60-L aquaria were used. One was filled with *S. aloides* (at moderate natural biomass: ca. 10 g FM/L) and growth medium (*Stratiotes* treatment) and the other was filled with growth medium only, i.e. without plants (control treatment). Both aquaria were incubated for one week before incubation water was taken and filtered separately over Whatman GF/F filters ( $\varnothing$  47 mm). Nutrients were added in excess to avoid nutrient limitation during the experiment. The nutrient concentrations in both media (control and *Stratiotes*) were similar to concentrations in modified WC medium. Nutrient concentrations at the end of the experiment were never limiting. After nutrient addition, the Erlenmeyer flasks (300 mL) were filled with 200 mL control water or *Stratiotes* water. Thereafter, a small aliquot of one of the four dense, exponentially growing phytoplankton batch cultures was added to the flasks (initial biovolume: 0.13 mm<sup>3</sup> L<sup>-1</sup>). The four phytoplankton species were inoculated separately in control as well as in *Stratiotes* medium. The biovolumes of the four phytoplankton species were determined with a Coulter Counter (Coulter® Multisizer II, Beckman Coulter, Fullerton, USA) every 24 h for 13 days until all phytoplankton cultures reached a stationary growth phase. To investigate the effect of light on the allelopathic inhibition of phytoplankton growth, 50 % of the cultures of each phytoplankton species was exposed to 35  $\mu\text{mol m}^{-2}\text{s}^{-1}$ , and 50 % to 105  $\mu\text{mol m}^{-2}\text{s}^{-1}$ . Therefore, the experimental set-up consisted of 16 treatments (four phytoplankton species, two medium types, two light intensities). Each combination of species, medium and light was replicated five times. A randomized block design was used to distribute the batch cultures in the incubators (21°C, 16:8 h light : dark cycle and shaken at 100 rpm).



**Figure 5.1** Example of a growth curve of the eustigmatophyte *Nannochloropsis limnetica* cultured at  $105 \mu\text{mol m}^{-2}\text{s}^{-1}$  in the presence of *S. aloides* exudates. The model (solid line) was fitted to the observations (triangles) by minimizing the residual sum of squares derived from the Ln-transformed data. The last four observations of the time series were not included in the model fit, because of a transition to the stationary growth phase. Note the 15-hour duration of the lag phase.

#### 5.3.4 Data-analysis and statistics

The phytoplankton biovolume data were analysed with an exponential growth model (Mulderij et al. 2005a, Chapter 4) that was fitted to the data by minimizing the residual sum of squares derived from the Ln-transformed observed and predicted data (Fig. 5.1). The growth of typical phytoplankton batch cultures goes through several phases: the lag phase, the exponential phase, the stationary phase and finally the death phase (Fogg & Thake 1987). For analysis of the inhibitory effect of *S. aloides* on phytoplankton growth, the stationary and death phase are of little interest because during these phases phytoplankton growth may also be limited by factors other than allelopathy (e.g., by light or nutrients). A visual inspection of the plotted log-transformed biovolume showed a clear transition between the exponential and the stationary growth phase (Fig. 5.1) and the period of interest was determined on basis of these graphs.

The lag phase of phytoplankton cultures is defined as the period prior to the exponential growth phase, during which cell numbers remain nearly constant (Fogg & Thake 1987). Because the observed lag phases were in the same order as (or even smaller than) the sampling interval, they could not be estimated from the observations directly. Instead, we followed the approach advocated by Fogg & Thake (1987) of taking the intersection of the linear curve through the log-transformed biovolumes during the exponential growth phase and a horizontal line through the log-transformed initial biovolume (Fogg & Thake 1987: Fig. 2.4). The calculated lag phases should thus be interpreted as the time interval between the onset of the experiment and the moment that the phytoplankton species attain an exponential growth rate. Obviously, this method precludes conclusions on whether the biovolume remains truly constant during the lag phase, or alternatively that some growth already happens during the lag phase. The graphical method of Fogg & Thake (1987) can be represented in the following model for phytoplankton biovolume  $A_t$  at time  $t$ . This model has three parameters: the initial biovolume  $A_0$ , the duration of the lag phase ( $T$ ) and the growth rate during the exponential growth phase ( $r$ ):

$$A_t = A_0 \quad (\text{if } t < T)$$

$$A_t = A_0 * e^{r(t-T)} \quad (\text{if } t > T)$$

where  $A_t$  is the biovolume at time,  $t$ . From the exponential growth rate, the biovolume doubling time ( $G$ ) of the four phytoplankton species was calculated (Fogg & Thake 1987).

$$G = \frac{\text{Ln } 2}{r} \quad (\text{if } t > T)$$

This equation is only valid in the exponential growth phase of the phytoplankton species. Since the tested phytoplankton species show a lag phase, the duration of this lag phase should be included in the equation to calculate the time needed for the *initial* doubling (from  $A_0$  to  $2A_0$ ). The *initial* biovolume doubling time,  $G^*$ , is then defined as follows:

$$G^* = \frac{\ln 2}{r} + T$$

The predictions of the model fitted well to the observed data ( $R^2$  values above 0.97 in 95% of the cases, see also Fig. 5.1). The influence of *S. aloides* water and the additional effect of light on  $T$ ,  $r$  and  $G^*$  of the four phytoplankton strains was assessed by two- and three-way ANOVA (Sokal & Rohlf 1995, Fowler et al. 1998).

Prior to assessment by ANOVA, all data were tested for normality (Kolmogorov-Smirnov test,  $\alpha = 0.05$ ) and homoscedasticity ( $F_{\max}$  test,  $\alpha = 0.05$ ). All data followed a normal distribution but the variances were not always homogeneously distributed. The latter was the case for the data of the cyanobacterial strain NIVA-CYA 140. Therefore all data of this strain were log-transformed. Unfortunately, transformation of the lag phase data did not remove heteroscedasticity and, therefore, an  $\alpha$ -value of 0.01 was used where normally an  $\alpha$ -value of 0.05 is used as the criterion for significance (Fowler-Walker & Connell 2002). Tukey tests ( $\alpha = 0.05$ ) were used to group homogeneous means (Fowler et al. 1998).

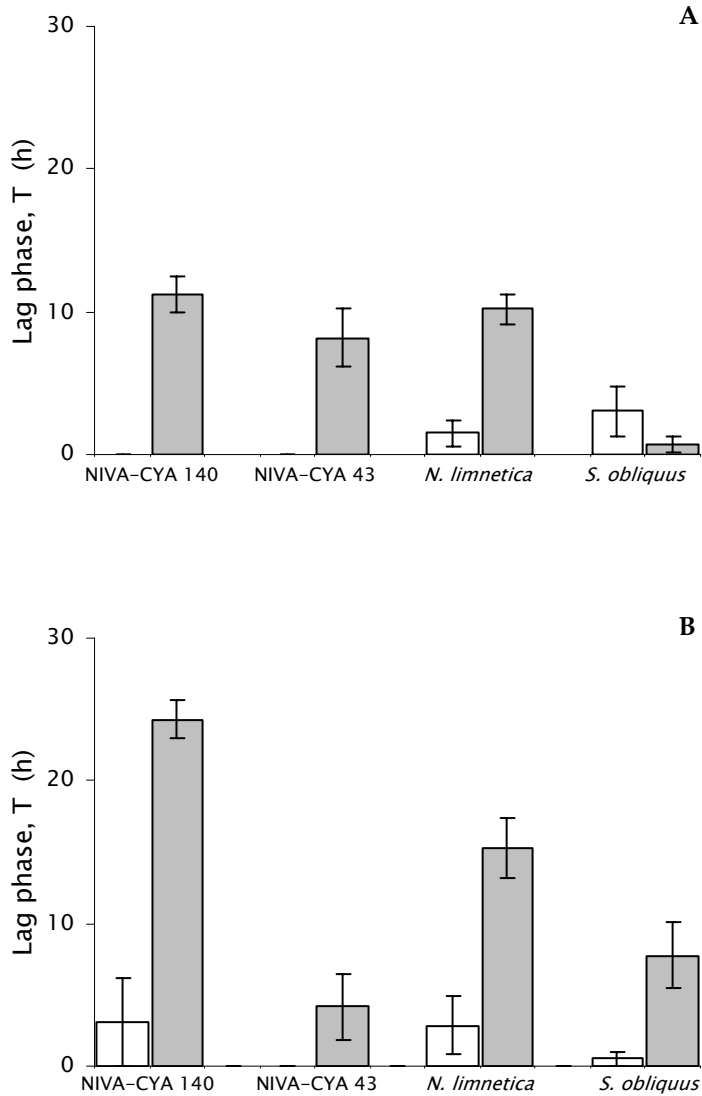
## 5.4 Results

The lag phase was longer in the presence of *Stratiotes* water than in the control (Fig. 5.2,  $p < 0.01$ ) except for (i) *S. obliquus* when incubated at  $105 \mu\text{mol m}^{-2}\text{s}^{-1}$  and (ii) the non-toxic *Microcystis* NIVA-CYA 43 incubated at  $35 \mu\text{mol m}^{-2}\text{s}^{-1}$ . The interactive effect of *Stratiotes* water and light on the lag phase was significant only for *Scenedesmus* ( $p < 0.01$ ). The effect of *Stratiotes* water on the lag phase of this green alga was stronger at  $35 \mu\text{mol m}^{-2}\text{s}^{-1}$  than at  $105 \mu\text{mol m}^{-2}\text{s}^{-1}$ . Due to heteroscedasticity the same interactive effect for NIVA-CYA 140 could not be judged significant ( $p = 0.015$ , see *Data-analysis and statistics*).

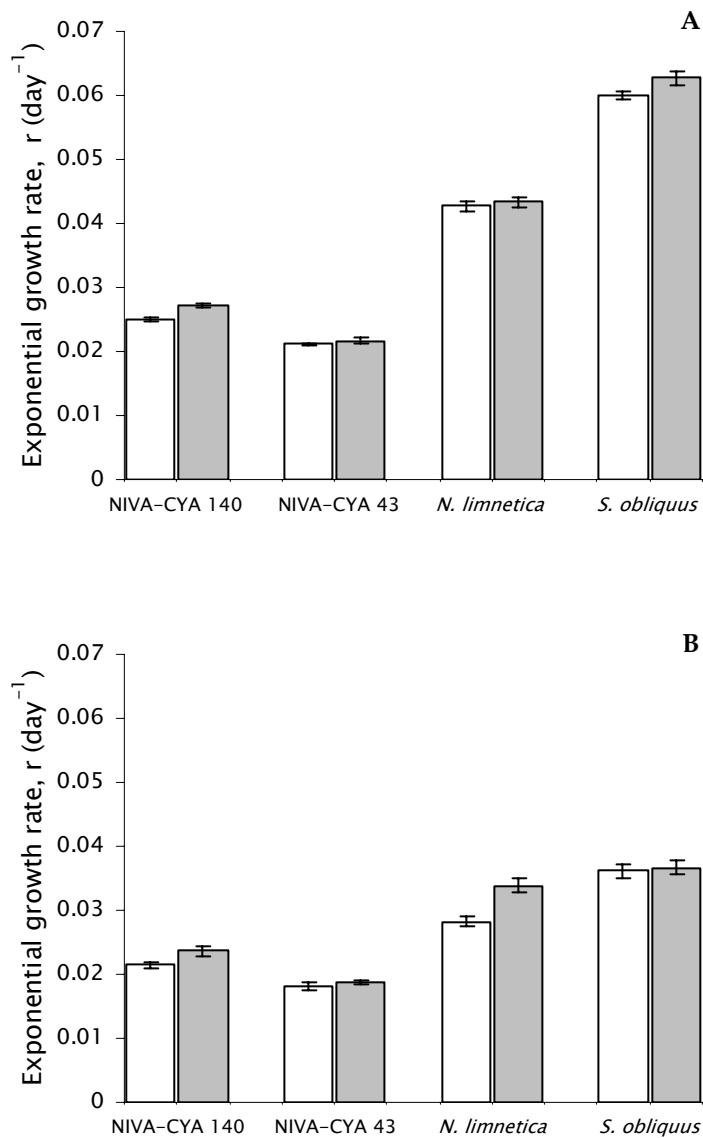
In most cases, the exponential growth rate was not significantly inhibited by the presence of *Stratiotes* water (Fig. 5.3). On the contrary, a slight stimulation ( $p < 0.001$ ) for the toxic *Microcystis* strain (NIVA-CYA 140) at both light intensities and for *N. limnetica* at  $35 \mu\text{mol m}^{-2}\text{s}^{-1}$  was observed.

The overall response of the different phytoplankton species to *Stratiotes* water (effect on  $G^*$ , Fig. 5.4) ranged from 8 to 51 % inhibition, with the strongest inhibition for the toxic *Microcystis* strain cultured at  $35 \mu\text{mol m}^{-2}\text{s}^{-1}$ . The response of cyanobacteria was not stronger than that of the other phytoplankton species (Fig. 5.4). Additionally, the effect of light on the allelopathic growth inhibition of phytoplankton was generally not different between cyanobacteria and the other species. Moreover, the interactive effect of *Stratiotes* water and light on the initial bio-volume doubling time of all tested species was not significant.

Comparisons between the two cyanobacterial strains showed that the initial bio-volume doubling time of the two *Microcystis* strains was elongated by *Stratiotes* water ( $p < 0.001$ ), but shortened ( $p < 0.001$ ) by higher light intensities. The response of the toxic *Microcystis*, NIVA-CYA 140, was stronger ( $p < 0.05$ ) than the response of the non-toxic *Microcystis* NIVA-CYA 43, as indicated by the significant interactive effect ( $p < 0.017$ ) between *Stratiotes* water and cyanobacterial strain.

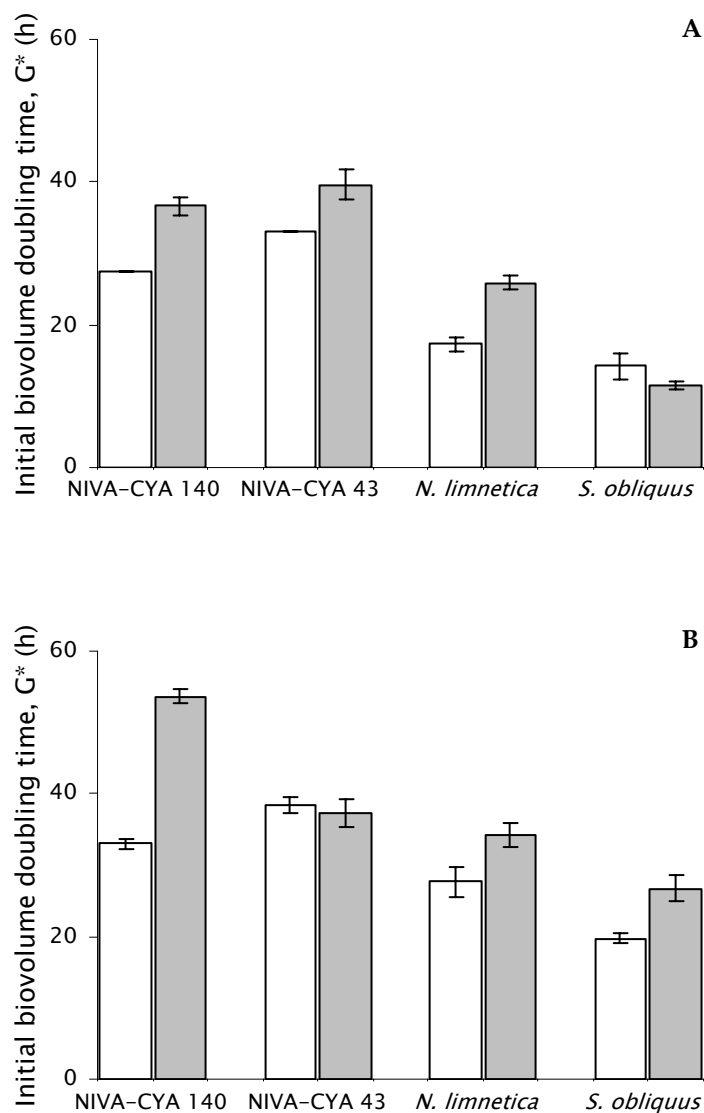


**Figure 5.2.** Duration of the lag phase,  $T$ , of the four phytoplankton cultures (NIVA-CYA 140 = toxic *Microcystis*, NIVA-CYA 43 = non-toxic *Microcystis*; *N. limnetica* = *Nannochloropsis limnetica*; *S. obliquus* = *Scenedesmus obliquus*) grown at light intensities of 105 (A) and 35  $\mu\text{mol m}^{-2}\text{s}^{-1}$  (B) in control (white bars) or *Stratiotes* (green bars) medium. Bars represent average values ( $n = 5$ ) with error bars (1 SE). ns = not significant effects, \* = significant effects ( $p < 0.05$ ).



**Figure 5.3** Exponential growth rate,  $r$ , of the four phytoplankton cultures (NIVA-CYA 140 = toxic *Microcystis*; NIVA-CYA 43 = non-toxic *Microcystis*; *N. limnetica* = *Nannochloropsis limnetica*; *S. obliquus* = *Scenedesmus obliquus*) grown at light intensities of 105 (**A**) and 35  $\mu\text{mol m}^{-2}\text{s}^{-1}$  (**B**) in control (white bars) or *Stratiotes* (green bars) medium. Bars represent average values ( $n = 5$ ) with error bars (1 SE). ns = not significant effects, \* = significant effects ( $p < 0.05$ ).





**Figure 5.4** Initial biovolume doubling time,  $G^*$ , of the four phytoplankton cultures (NIVA-CYA 140 = toxic *Microcystis*; NIVA-CYA 43 = non-toxic *Microcystis*; *N. limnetica* = *Nannochloropsis limnetica*; *S. obliquus* = *Scenedesmus obliquus*) grown at light intensities of 105 (A) and 35  $\mu\text{mol m}^{-2}\text{s}^{-1}$  (B) in control (white bars) or *Stratiotes* (green bars) medium. Bars represent average values ( $n = 5$ ) with error bars (1 SE). ns = not significant effects, \* = significant effects ( $p < 0.05$ ).

## 5.5 Discussion

*Stratiotes* water significantly inhibited all phytoplankton species. This provides indications for our first hypothesis that *S. aloides* exudates can hamper the growth of phytoplankton. Allelopathic effects of exudates from *S. aloides* mainly prolonged the lag phase. For all phytoplankton strains we found an inhibitory effect of *Stratiotes* water on the initial biovolume doubling time,  $G^*$ , even though the exponential growth rate was sometimes slightly stimulated by *Stratiotes* water.

Growth inhibition of the cyanobacterium, *Microcystis*, caused by exudates from aquatic macrophytes was previously reported by Nakai et al. (2000) and Körner & Nicklisch (2002). Growth inhibition of *Nannochloropsis limnetica* has not been described before. Not all phytoplankton species in our experiment, however, were always inhibited by the presence of *S. aloides* exudates. *S. obliquus* was not inhibited at the high light intensity ( $105 \mu\text{mol m}^{-2}\text{s}^{-1}$ ). This is in accordance with observations of Jasser (1995) and Mulderij et al. (2003, Chapter 3): no inhibitory effect on *S. obliquus*. Additionally, Blindow & Hootsmans (1991) found differential allelopathic effects of several charophyte species. Some were inhibitory, while others had no effect on phytoplankton growth.

When looking at inhibitory effects of macrophytes on phytoplankton, one should also take the lag phase and exponential growth phase into account. A focus only on the lag phase would overestimate the inhibitory effect, while a focus only on the exponential growth rate would do the opposite.

In the presence of exudates from *S. aloides* we observed an effect of the light intensity on phytoplankton. Only for *S. obliquus*, the overall inhibitory effect of *Stratiotes* water was significantly stronger at  $35 \mu\text{mol m}^{-2}\text{s}^{-1}$  than at  $105 \mu\text{mol m}^{-2}\text{s}^{-1}$ . In accordance with our second hypothesis and the one of Reigosa et al. (1999), possible light limitation at  $35 \mu\text{mol m}^{-2}\text{s}^{-1}$  may have increased the susceptibility of *Scenedesmus* to allelopathic substances present in *Stratiotes* water.

Furthermore, we compared the responses ( $G^*$ ) of cyanobacteria and non-cyanobacterial phytoplankton species. As in the study of Wium-Andersen et al. (1982), we did not find the cyanobacteria to be more sensitive to exudates from *S. aloides* than the non-cyanobacterial species and therefore we reject the hypothesis that cyanobacteria are more sensitive to allelopathic substances than other phytoplankton species. In contrast to our hypothesis, however, Jasser (1994) and Körner & Nicklisch (2002) observed a higher sensitivity in cyanobacteria.

Only between the toxic and non-toxic cyanobacterial strain we observed differences in the response. The toxic cyanobacterium NIVA-CYA 140 was more sensitive to allelopathic substances from *S. aloides* than the non-toxic NIVA-CYA 43, supporting our hypothesis that toxic cyanobacteria are more sensitive than non-toxic cyanobacteria. This differential sensitivity to exudates from *S. aloides* may have effects on the succession of these strains and maybe also on the composition of phytoplankton in natural ecosystems, but more detailed studies are needed to justify this.

Several (submerged) macrophytes exhibit allelopathic activity on phytoplankton, but with varying strength. The allelopathic effect of extracts (Nakai et al. 1996) and exudates (Nakai et al. 1999) of several aquatic macrophytes was tested and *Myriophyllum* was shown to be the macrophyte with the strongest inhibitory effect on phytoplankton. Charophytes appear to have relatively weak inhibitory effects on phytoplankton growth (Blindow & Hootsmans 1991, Mulderij et al. 2003, Chapter 3). Our study showed that *Stratiotes* water can inhibit phytoplankton growth up to 50 % ( $G^*$ , and more if only the lag phase is considered).

Our experiment, however, may have lead to an underestimation of the allelopathic effect, because there was no continuous addition of *S. aloides* exudates like in e.g., coexistence experiments. Nevertheless we showed a strong inhibitory effect of *Stratiotes* on the growth of phytoplankton. So, *S. aloides* and other macrophytes with a relatively strong allelopathic activity may play a more important role (than e.g., charophytes) in increasing water transparency in aquatic ecosystems than they already do via other reinforcing mechanisms suggested for macrophytes (Scheffer et al. 1993a, Jeppesen et al. 1998a).

Another point of discussion is the movement of natural *S. aloides* plants through the water column over the season. Especially in spring and early summer, when *S. aloides* is still submerged, allelopathy may be a good strategy to compete with phytoplankton. Later in the season, when the plants start to become partly emergent,

allelopathy may be less important. In this period, alternative processes like the shading effect of *S. aloides* on phytoplankton may be more important. So, the extension of the lag phase during the submerged phase of the macrophyte seems the best alternative in the competition for light with phytoplankton, because there is (nearly) no growth of phytoplankton. On the contrary, if only the phytoplankton growth rate was affected, numbers of phytoplankton would still increase. It, however, does not mean that the light intensity is not important during the submerged growth phase of *S. aloides*.

Allelopathy alone may not be effective enough to decrease the density or change the composition of phytoplankton communities, but in natural aquatic ecosystems phytoplankton is influenced by a combination of competitive interactions (nutrient limitation, light limitation, and/or a grazing by zooplankton and macroinvertebrates). For some phytoplankton species, more than for others, this makes it more difficult to cope with an extra competitive interaction like allelopathy (Reigosa et al. 1999).

Additionally, experiments conducted in the laboratory under optimal conditions (e.g., light and nutrients) are likely to underestimate the potential of allelopathy in natural aquatic habitats. Several studies have already investigated the outcome of these combined interactions between macrophytes and phytoplankton under field conditions (Mjelde & Faafeng 1997, Gross 2000), but this subject deserves more detailed field studies in macrophyte stands to determine the ecological significance of allelopathy in shallow aquatic ecosystems. Also, the development of models that specifically simulate the relative contribution of allelopathy in shallow aquatic ecosystems in relation to other processes like light and nutrient limitation can help to understand the ecological significance of allelopathy in the field (Mulderij et al. *submitted*, Chapter 8).

In conclusion, our study demonstrates that *S. aloides* exudates extend the initial biovolume doubling time of phytoplankton. The allelopathic potential of *S. aloides* exudates was light-, and in the case of cyanobacteria strain-specific. In combination with other effects of macrophytes on shallow aquatic ecosystems (Scheffer et al. 1993a, Jeppesen et al. 1998a), allelopathic inhibition of phytoplankton may be a significant process in suppressing phytoplankton growth in *S. aloides* stands.

In particular, the extension of the phytoplankton lag phase can be a useful strategy for *S. aloides* for exploiting the light conditions in spring. In this period phytoplankton starts to grow and especially then it will be useful for *S. aloides* plants to be able to excrete allelopathic substances and delay the growth of phytoplankton and possibly also other phototrophic organisms until the plants reach the water surface and thus win the competition for light.

### **Acknowledgements**

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# Field experiments



Introduction of artificial *Stratiotes* plants for the field experiments in Lake Naardermeer.

Picture: G. Mulderij





Allelopathic effects of the aquatic macrophyte, *Stratiotes aloides*, on natural phytoplankton populations

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# Allelopathic effects on natural phytoplankton populations

## 6.1 Abstract

A survey of different locations in The Netherlands showed that the density of phytoplankton (except cyanobacteria) was always higher in samples collected outside *S. aloides* stands than in samples taken from between the rosettes of *S. aloides*. Analyses of water samples collected during the survey revealed that nutrient limitation was unlikely to have caused the lower phytoplankton biomass in the vicinity of *S. aloides*.

The *in situ* incubation experiment in lakes in the Danube Delta, Romania, gave strong indications for the presence of allelopathic activity against phytoplankton in *S. aloides* stands. The growth rate of natural phytoplankton communities exposed to water from *S. aloides* stands was significantly lower than that of communities that had not been in contact with *S. aloides* exudates.

A laboratory microcosm experiment showed a significantly lower phytoplankton biomass in treatments with *S. aloides* exudates. Nutrient concentrations and the light intensity were high enough to assume that the lower phytoplankton biomass could not be explained by nutrient or light limitation.

This study demonstrates the allelopathic effect of *S. aloides* on natural phytoplankton. Allelopathy may not be the steering factor in structuring shallow lake ecosystems or the functioning thereof, but it could be a factor in controlling the density and structure of natural phytoplankton communities.

## 6.2 Introduction

The term 'Allelopathy' is generally used to describe any direct or indirect effect (mainly negative) of one plant on another through the production of chemical compounds released into the environment (Molisch 1937, Rice 1979, Lampert & Sommer 1997). Possible allelopathic effects of submerged aquatic macrophytes have mainly been studied for charophytes (*Chara globularis* and *C. aspera*), *Ceratophyllum demersum* and *Myriophyllum spicatum*. Anthoni et al. (1980) and Wium-Andersen et al. (1982) suggested allelopathic effects of charophytes on the growth of phytoplankton, and more recently such effects have become well established (Mulderij et al. 2003, Chapter 3, Berger & Schagerl 2004). Forsberg et al. (1990), on the contrary, argued the allelopathic activity of *Chara* is unlikely to be of ecological importance under natural conditions. Compounds originating from *C. demersum* and *M. spicatum* also appear to inhibit phytoplankton (Gross et al. 1996, Körner & Nicklisch 2002).

The macrophytes *Stratiotes aloides* L. (Hydrocharitaceae), Water soldier, is native to Europe and Siberia (Cook & Urmi-König 1983). *S. aloides* propagates both vegetatively and generatively, but the vigorous vegetative reproduction by means of tillers and turions, plays a dominant role and often results in very dense stands (Cook & Urmi-König 1983). An important part of the life cycle of *S. aloides* is submerged (Bloemendaal & Roelofs 1988) as the plants overwinter as turions or entire (but rootless) plants on the sediment surface, where the decay of the macrophytes creates a thick layer of organic sediment (sapropel).

During the submerged phase in early spring, allelopathy would be a possible mechanism to gain competitive advantage in the struggle for light. However, convincing proof the allelopathic inhibition of phytoplankton by *S. aloides* is lacking. Brammer (1979) studied the 'exclusion' of phytoplankton in the proximity of *S. aloides*, but was not able to show unequivocally that allelopathic substances from *S. aloides* were causing the low phytoplankton densities in a Swedish and a Polish lake. The most likely explanation for the low phytoplankton densities was growth limitation of phytoplankton through a lower availability of nutrients (Brammer 1979, Brammer & Wetzel 1984). Wium-Andersen (1987) did observe an inhibitory effect of *S. aloides* extracts on photosynthesis by the diatom *Nitzschia palea* and a natural phytoplankton population. Jasser (1995) studied the allelopathic activity of *S. aloides* extracts in dialysis bags with phytoplankton and showed inhibitory effects that became stronger when extract concentrations increased. Usenko et al.

(2002) showed that several macrophytes, including *S. aloides*, may cause algicidal effects via the production of phenolic acids. The main shortcoming of the studies of Jasser (1995) and Wium-Andersen (1987) is that they are conducted with extracts, and therefore do not prove that allelopathic substances are actually excreted by macrophytes. Recently, Mulderij et al. (2005a, 2005b, Chapters 4 & 5) showed that exudates from *S. aloides*, may have allelopathically inhibited the growth of several phytoplankton species in laboratory experiments.

Laboratory experiments demonstrate the role of allelopathy under controlled conditions, but do not provide knowledge on the importance of allelopathy under natural conditions. To understand how important allelopathy can be under natural circumstances field experiments and surveys with/of natural populations of target and donor species are necessary. Observations of different Dutch *S. aloides* stands often showed clear water in the vicinity of *S. aloides*, whereas the water further away from the plants was more turbid or covered with filamentous algae (Mulderij & Smolders, *unpublished data*). We tested the hypothesis that allelopathy may be an important mechanism for *S. aloides* in the competition with algae. Here we describe the results from a survey of different Dutch *S. aloides* stands in The Netherlands, from an *in situ* incubation experiment with a natural phytoplankton population and *Stratiotes* water, and from a laboratory microcosm experiment.

## 6.3 Materials and methods

### 6.3.1 Field survey

On 12 and 15 July 2003 five Dutch *S. aloides* stands were surveyed (Zegveld 4°51'E, 52°07'N; De Bilt 5°11'E, 52°07'N; Naardermeer 5°06'E, 52°19'N; Meije 4°47'E, 52°08'N and Westbroek 5°07'E, 52°09'N). Water samples collected close to the rosettes of *S. aloides* and at ca. 5 m distance from the *Stratiotes* stands (control). These samples (500 mL in glass bottles) were then stored cold until arrival in the laboratory. Back in the laboratory, 20 mL of each (unfiltered) sample was fixed with a Paraformaldehyde (PF) and Glutaraldehyde (GA) solution (final concentration in sample: 0.01% PF and 0.1% GA). These samples were stored at 4°C prior to analysis by flow cytometry. Filtered (Whatman GF/C, Ø 47 mm) water samples (20 mL) for nutrient analyses were frozen until chemical analysis.

To determine the concentration of phytoplankton (cells mL<sup>-1</sup>), an EurOPA flow cytometer (TNO, Delft, The Netherlands), equipped with a 633 nm Helium Neon (red) and a 488 nm Argon ion laser (200 mW, Coherent, model Annova 90) was used. The flow rate was 20 µL min<sup>-1</sup> and a minimum of 1000 particles per sample was counted. Further, the EurOPA is equipped with a 1 mm diameter flow cell which allowed large particles to be analysed. This flow cytometer distinguished cell densities of cyanobacteria and those of other phytoplankton groups (further details in Dionisio Pires et al. *in press*).

### 6.3.2 Danube Delta field incubations

The aim of this experiment was to test if *S. aloides* exudates also cause allelopathic inhibition of a natural phytoplankton population *in situ*. Lake Cuibul cu Lebede is situated in the Gorgova-Isac complex of the Romanian Danube Delta (Oosterberg et al. 2000). A large part of this lake is covered with *S. aloides*. We collected two types of water from this lake: 'Stratiotes water' from *S. aloides* stands, and 'control water' from outside the *S. aloides* stands (distance *Stratiotes* vs control sites: ca. 30 m). Both types of water were filtered (0.2 µm cellulose acetate membrane filters, Ø 47 mm, Schleicher & Schuell) to remove zoo- and phytoplankton.

Lake Roşu (situated in the eastern part of the Danube Delta) is turbid and dominated by phytoplankton (cyanobacteria and diatoms). We collected water from Lake Roşu as a phytoplankton inoculum for the incubation experiment, because, in contrast to Lake Cuibul cu Lebede, this lake contained a sufficient phytoplankton concentration, mainly dominated by cyanobacteria. The water of this lake was filtered (nylon filter, mesh size 80 µm) to remove large zooplankton to avoid zooplankton grazing during the experiment. Thereafter the water was filtered over a nylon plankton net (15 µm) to concentrate the phytoplankton. This concentrated phytoplankton community was added (7.66 µg Chl-a L<sup>-1</sup>) to 750 mL polycarbonate bottles filled with 500 mL filtered water from Lake Cuibul cu Lebede (from outside or inside the *S. aloides* stands) spiked with nutrients (1 mM sodium nitrate, 0.14 mM potassium phosphate).

The bottles were incubated between 9 and 13 June 2002 in a randomized block design ( $n = 4$ ) on a pontoon, in the Danube Delta. To protect the bottles from direct sunlight, the bottles were placed under a roof (made of white fabric). The average temperature of the phytoplankton cultures (during daytime) was 25°C. To prevent sedimentation of phytoplankton, the cultures were mixed every 2 hours during daytime by rotating the bottles several times. The growth of phytoplankton in the closed bottles with control and *Stratiotes* water was determined by chlorophyll analysis ( $\mu\text{g L}^{-1}$ ) with a phyto-PAM Plankton analyzer (Walz Mess- und Regeltechnik GmbH, Effeltrich, Germany). Chlorophyll levels in both treatments at the start and end (5 days) of the experiment were then compared.

### 6.3.3 Laboratory microcosm experiment

The aim of this experiment was to determine the (allelopathic) effect of *S. aloides* exudates and the presence of *S. aloides* sediment on the growth of phytoplankton. Possible effects of substances released from the sediment are usually not tested, but this experimental set-up enabled exclusion of inhibitory or stimulatory effects of the substances possibly released from the sediment.

The *S. aloides* plants were derived from a laboratory culture which was established in July 2001 using plants originating from a ditch in Tienhoven (52°10'0N 5°4'60E), near Utrecht, The Netherlands. The macrophytes were grown in a 500-L aquarium with copper-free tap water and ca. 10 cm sediment originating from the same site in Tienhoven. Every second week the aquarium was refilled with fresh copper-free tap water (Mulderij et al. 2005a, 2005b, Chapter 4 & 5). The plants were exposed to a 16 : 8 hour light : dark cycle ( $30 \mu\text{mol m}^{-2}\text{s}^{-1}$ ) at 18°C.

In total, 24 microcosms (20 L) were established. Eight microcosms were filled only with 15 L synthetic macrophyte medium (Mulderij et al. 2005a, Chapter 4, Table 4.1) and served as controls. The remaining sixteen microcosms received synthetic macrophyte medium and a five cm layer of sediment obtained from the same site in Tienhoven as where the macrophytes were collected. Eight of the last sixteen microcosms with sediment also received one *S. aloides* plant (diameter 15 - 20 cm, 25 - 30 g DM) per microcosm. Hence the experiment comprised three treatments (each  $n = 8$ ): (1) medium only, (2) medium + sediment, and (3) medium + sediment + *Stratiotes*. The microcosms were kept at 18°C in a water bath and received continuous light of  $137 \mu\text{mol m}^{-2}\text{s}^{-1}$ .

At the start of the experiment, water from each of the 24 microcosms was filtered (Whatman GF/F, Ø 47 mm) and used to establish two replicate 8-L alga cultures (in 10 L aquaria). After the transfer of culture water from the 24 (macrophyte) microcosms to the 48 alga cultures, an excess of nutrients was added from concentrated stock solutions (Woods Hole medium with extra  $\text{NaHCO}_3$ , Simons et al. 1999) to avoid nutrient limited growth of the algae during the experiment. Thereafter, small amounts (0.13 g FM ~ 0.003 g DM) of a natural population of filamentous algae (predominantly *Spirogyra* sp.), carefully rinsed with Woods Hole medium, were incubated in the 10-L alga aquaria. These aquaria were kept at identical conditions as the microcosms (18°C and  $137 \mu\text{mol m}^{-2}\text{s}^{-1}$ ).

After 11 days the dry mass of the alga cultures was determined. During the experiment, however, phytoplankton most likely introduced with the filamentous algae inoculum, quickly started to dominate the alga aquaria until hardly any filamentous algae were left. The dry mass determinations of phytoplankton at the end of the experiment were performed by filtering 25 - 200 mL alga culture water on pre-dried Whatman GF/C filters (Ø 47mm) and drying at 105°C for 24 hours. Additionally, a Dentan FN5 turbidity meter was used to determine the turbidity of the water in the three treatments (medium, medium and sediment, and medium, sediment and *Stratiotes*). Particles other than algal cells and (most likely) bacteria were absent from the cultures, as the water from the microcosms was gently removed, filtered (Whatman GF/F, Ø 47mm) and transferred into the alga cultures, so that no sediment particles were transferred to the alga cultures. Therefore, we assume that the measured turbidity in the 10-L cultures was entirely caused by phytoplankton (and possibly some bacteria).

At the end of the experiment 500 mL (in glass bottles) of each alga culture was collected and fixed with Lugol's solution for phytoplankton analysis by light microscopy (Leitz, Fluovert inverted microscope, 400x magnification). With this analysis we could provide information on the nature of the particles causing turbidity of the water in the algae aquaria (phytoplankton or sediment particles). This analysis further focussed on the most dominant phytoplankton species, rather than on exact cells densities and, therefore, no cell counts were performed.



#### 6.3.4 Chemical analyses

The pH was determined using a combination glass electrode with an Ag/AgCl internal reference (Orion Research, Beverly, USA). Carbon dioxide (CO<sub>2</sub>) concentrations were measured using an infrared carbon analyser (model PIR-2000, Horiba Instruments, Irvine, USA). Chloride (Cl) concentrations were determined colorimetrically with a Technicon II Auto Analyzer, according to O'Brien (1962). Nitrate and ammonium (NO<sub>3</sub> and NH<sub>4</sub>) were measured colorimetrically with a Traacs 800+ auto-analyser, using hydrazine sulphate (Technicon 1969) and silicate (Grasshoff & Johannsen 1977), respectively. Orthophosphate (o-PO<sub>4</sub>) was determined colorimetrically with a Technicon AAI system, using ammonium molybdate (Henriksen 1965). Potassium (K) and sodium (Na) were measured by flame photometry (FLM3 Flame Photometer, Radiometer, Copenhagen, Denmark). Aluminium (Al), calcium (Ca), iron (Fe), magnesium (Mg), manganese (Mn), total phosphorus (TP), silicium (Si), sulphur (S), and zinc (Zn) were determined by inductively-coupled plasma emission spectrophotometry (Spectro Analytical Instruments, type Spectroflame, Kleve, Germany). Nutrient analyses (TN and TP) in the Danube Delta field experiment were carried out by the Institute for Inland Water Management and Waste Water Treatment (RIZA), Lelystad, The Netherlands.

#### 6.3.5 Statistical analyses

The nutrient concentrations determined in the field survey and the chlorophyll data from the field incubation in the Danube Delta were analysed by means of Paired-samples *t*-tests ( $\alpha = 0.05$ ). The flow cytometer analysis of cyanobacterial cell densities and densities of other phytoplankton species were analysed with a Wilcoxon's signed rank test ( $\alpha = 0.05$ ). All other data (nutrient concentrations in the field incubation and microcosm experiment and the turbidity and algal biomass (dry mass) in microcosm experiment) were analysed by means of ANOVA at a significance level of  $p < 0.05$  (Fowler et al. 1998, Sokal & Rohlf 1995). Prior to assessment by ANOVA, the estimated values for the parameters were tested for normality (Kolmogorov-Smirnov test,  $\alpha = 0.05$ ) and homoscedasticity (Levene's test,  $\alpha = 0.05$ ). All data followed a normal distribution and most variances were homogeneous, except for the turbidity and Al, Fe, Mn, S, and o-PO<sub>4</sub> concentrations in the microcosm experiment. Therefore, those data were log- or square root transformed. When these transformations did not remove heteroscedasticity (Al and Mn concentrations) an  $\alpha$ -value of 0.01 was used where normally an  $\alpha$ -value of 0.05 is used as the criterion for significance (Fowler-Walker & Connell 2002). If ANOVA revealed significant effects, a Tukey test ( $p < 0.05$ ) was performed to group homogeneous means (Fowler et al. 1998). Analysis of the data for the paired alga cultures filled

with water from a single microcosm showed no significant differences. Therefore, the data of those duplicates were pooled per treatment ( $n_{\text{final analysis}} = 8$ ). All analyses were performed in SPSS version 11.5 (Dytham 1999).

## 6.4 Results

### 6.4.1 Field survey

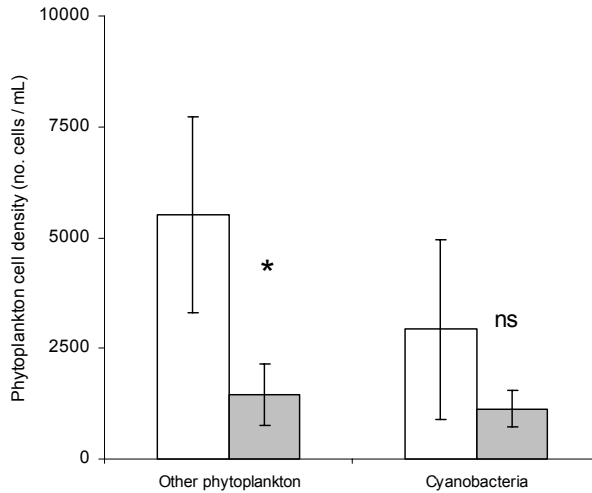
The cell densities for phytoplankton species (excluding cyanobacteria) were significantly ( $p = 0.04$ ) lower in close vicinity of *S. aloides* stands than further away (Fig. 6.1). No significant differences in cell concentration for cyanobacteria were found ( $p = 0.345$ ). The density of phytoplankton other than cyanobacteria outside *Stratiotes* was 3.7 times higher than close to the *S. aloides* plants.

Nutrient analyses showed that the concentration of Al, Cl, Fe,  $\text{NH}_4$ , Si, TP and o- $\text{PO}_4$  in both type of samples (within and outside *S. aloides*) were significantly ( $p < 0.03$ ) correlated with each other (Pearson's product-moment correlation between 0.92 - 0.99). However, the Paired-samples *t*-test did not reveal any significant differences between water samples taken in- or outside the *S. aloides* stand. Other element concentrations (see *Chemical analyses*) were not significantly different in- or outside *S. aloides* stands.

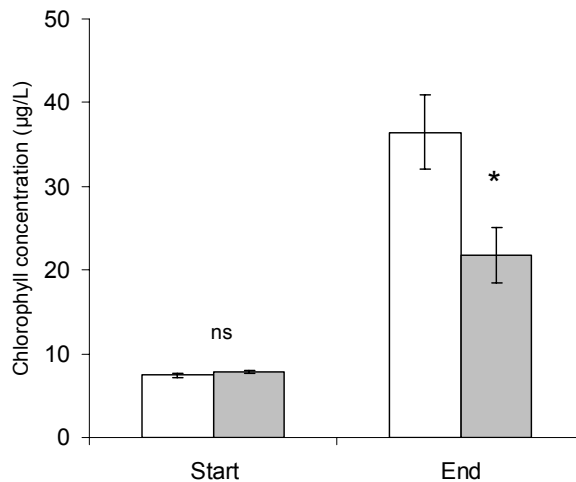
### 6.4.2 Danube Delta field incubations

At the start of the experiment no significant differences in average chlorophyll concentration were found between control or *Stratiotes* water (Fig. 6.2). At the end of the experiment significantly ( $p = 0.038$ ) higher chlorophyll concentrations were measured in the bottles with control water than in bottles with *Stratiotes* water (Fig. 6.2).

Nutrient analyses of water collected in *S. aloides* stands and control water collected outside *S. aloides* stands before addition of nutrients showed that the concentration of N in water collected in the *S. aloides* stand was  $0.18 \text{ mg L}^{-1}$ , while it was significantly lower ( $0.08 \text{ mg L}^{-1}$ ,  $p = 0.025$ ) outside the stands. Phosphorus concentrations inside and outside *S. aloides* stands in Lake Cuibul cu Lebede were not different with an average value of  $0.7 \text{ mg L}^{-1}$ . The concentrations of N ( $0.08 \text{ mg L}^{-1}$ ) and P ( $0.9 \text{ mg L}^{-1}$ ) and the pH values (8.05) at the end of the experiment were not significantly different between control and *Stratiotes* treatments.



**Figure 6.1** Phytoplankton cell density in water samples taken outside (white bars) and inside (green bars) *S. aloides* stands during the field survey. Bars represent average values ( $n = 5$ ) with error bars (1 SE). The significant difference ( $p = 0.04$ ) between the open water and plant stands is indicated with \*. ns = non-significant.



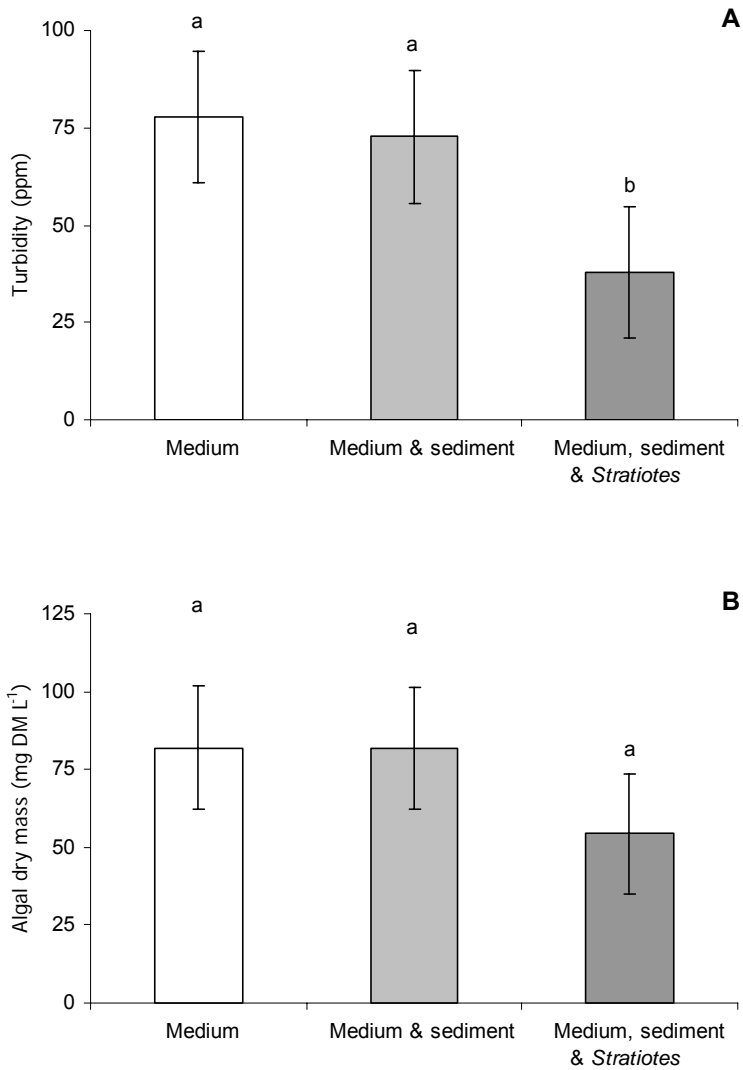
**Figure 6.2** Chlorophyll concentration in controls (white bars) and *Stratiotes* treatments (green bars) at the start and end of the Danube Delta experiment. Bars represent average values ( $n = 4$ ) with error bars (1 SE). The significant difference between the treatments (control and *Stratiotes*) is indicated with \*. The non-significant difference is indicated with 'ns'.

### 6.4.3 Laboratory microcosm experiment

The experiment started with an inoculum of filamentous algae (mainly *Spirogyra* sp.), but the system quickly became dominated by phytoplankton, mainly chlorophyte species such as *Scenedesmus*, *Monoraphidium*, *Gonium*, and sometimes *Pediastrum*. These phytoplankton species were most likely introduced with the inoculum of filamentous algae, even though we carefully rinsed them. At the end of the experiment a strong effect of *S. aloides* on the turbidity of the water in the alga cultures was evident. The turbidity in the cultures that received water in which *Stratiotes* had grown was significantly ( $p < 0.001$ ) lower (39 ppm) than the turbidity in the cultures that received water from the microcosms with only medium or with medium and sediment (79 and 73 ppm, Fig. 6.3a). A similar trend towards a lower dry mass of phytoplankton in the *Stratiotes* treatments could be seen (Fig. 6.3b), but this difference was not significant ( $p = 0.242$ ).

Microscopic analysis of the alga cultures showed no differences in phytoplankton species composition occurred. *Scenedesmus*, *Monoraphidium* and *Gonium* were most dominant in all cultures. Microscopic analyses further showed that the turbidity was caused by high numbers of phytoplankton cells and not by other particles, e.g., sediment particles.

At the start of the experiment no significant differences in the pH of the microcosms were found (average pH 7.48). At the end of the experiment the pH in the microcosms slightly, but differed significantly ( $p = 0.003$ ) between the treatments. The pH in the microcosms with *S. aloides* was 7.99, while the pH in the controls and the treatments with sediment was 8.43 and 8.34, respectively.



**Figure 6.3** Turbidity (A) and dry mass (B) in the alga cultures that received water from the macrophyte aquaria with medium only (white bars), medium + sediment (green bars) or medium + sediment + *Stratiotes* (dark green bars). Bars represent average values ( $n = 8$ ) with error bars (1 SE). Significant differences between the treatments are indicated with different letters (Tukey test,  $\alpha = 0.05$ ).

## 6.5 Discussion

All three experiments do provide indications of allelopathic activity of *S. aloides* against phytoplankton. The field survey showed that phytoplankton densities (excluding cyanobacteria) outside *S. aloides* stands were always higher than within these stands. The results from the *in situ* incubation experiment also suggest that something else than nutrients controls phytoplankton development in the presence of *S. aloides*. Lastly, the laboratory microcosm experiment showed a significantly lower turbidity and a slightly (but not significantly) lower phytoplankton dry mass in the cultures that received *S. aloides* exudates.

Although light limitation of phytoplankton under dense layers of *S. aloides* could have played a role in the observations from the field survey, similar observations came from the other two experiments when light limitation could not play a role. Hence, the experiments suggest that exudates from *S. aloides* may play a role in the occurrence of lower phytoplankton densities close to *S. aloides*.

The growth of phytoplankton in freshwater ecosystems is generally considered to be limited by the availability of phosphorus (Schindler 1977, Elser et al. 1995) or nitrogen (Jansson et al. 1996, 2001). Brammer (1979) and Brammer & Wetzel (1984) suggested that the uptake of potassium by *S. aloides* may limit the growth of phytoplankton under natural conditions. In our field survey, however, we never found significantly lower concentrations of potassium or other important nutrients in the presence of *S. aloides* plants. Jaworski et al. (2003) observed that the growth rate in cultures of the diatom *Asterionella formosa* was independent of the potassium concentration above  $0.7 \mu\text{mol L}^{-1}$ , while several planktonic chrysophytes suffer growth inhibition at potassium concentrations higher than  $130 \mu\text{mol L}^{-1}$  (Lehman 1976, Sandgren 1988). Jaworski et al. (2003) conclude that potassium limitation of phytoplankton species is rather unlikely in aquatic ecosystems as potassium concentrations are rarely extremely low. The average potassium concentration in *Stratiotes* stands in our field survey was  $127 \mu\text{mol L}^{-1}$ , which is between the previously described limiting concentrations.

Our observations of low phytoplankton densities close to *S. aloides* in natural ecosystems are most likely the result of several interacting processes (e.g., light limitation, grazing and allelopathy). As a consequence, laboratory experiments conducted under relatively optimal conditions are likely to underestimate the allelopathic effect, because phytoplankton may be less sensitive to allelopathic substances under less stressful conditions. Phytoplankton species growing under nutrient stress are thought to be more susceptible to allelopathic substances (Reigosa et al. 1999). However, to avoid nutrient limitation, we always added an excess of nutrients to the phytoplankton cultures.

Allelopathy experiments conducted with charophyte exudates and the green alga *C. minutissima* did not show that this alga was more sensitive to *Chara* exudates under P-limitation (Mulderij et al. 2003, Chapter 3). Fitzgerald (1969) showed similar results with the macrophyte, *Ceratophyllum*, and phytoplankton subjected to P-limited conditions, but did show allelopathic effects under nitrogen limitation. Also P-limited cultures of the cyanobacterium *Synechococcus elongatus* were not more sensitive to *S. aloides* extracts than P-sufficient cultures, while the opposite was observed for cultures of the green alga *S. obliquus* (Mulderij et al. *submitted*, Chapter 9). Recently, Mulderij et al. (*submitted*, Chapter 9) showed that K-limited cultures of the cyanobacterium *Synechococcus elongatus* were less sensitive to *S. aloides* extracts than non-limited cultures. The growth of non-limited cultures was inhibited at increasing extract concentrations, while the growth of K-limited cultures was stimulated.

We showed inhibitory effects of *S. aloides* on phytoplankton growth with exudates instead of extracts, indicating that allelopathic substances are actually excreted by *S. aloides* (see criteria of Willis 1985, page 17). Allelopathic inhibition of phytoplankton by exudates of *S. aloides* was previously shown in laboratory experiments (Mulderij et al. 2005a, 2005b, Chapters 4 & 5), but never under natural conditions. Moreover, we did not observe any effects of the sediment on the growth of phytoplankton in the laboratory experiment, suggesting that substances possibly released from the sediment do not affect phytoplankton abundance or composition.

Our results suggest that allelopathic effects of *S. aloides* on phytoplankton can occur under natural conditions, but further studies are required to evaluate the extent and magnitude of such allelopathic effects *in situ*.

## **Acknowledgements**

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Allelopathic inhibition of filamentous algae by  
the aquatic macrophyte *Stratiotes aloides*

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# Filamentous algae

## 7.1 Abstract

Observations of Dutch *Stratiotes aloides* L. (Hydrocharitaceae) stands showed that filamentous algae and *S. aloides* often co-occur. However, filamentous algae were absent in close vicinity of *S. aloides* visual as gaps in mats of filamentous algae. We hypothesize that these gaps are caused by allelopathic activity of *S. aloides*.

We determined the relative importance of resource competition versus a form of interference competition (allelopathy) and conducted a field survey of stands with *S. aloides*. Thereafter, we conducted field experiments where we introduced artificial (non-allelopathic) macrophytes in natural stands of *S. aloides* and compared the natural and artificial *S. aloides* plants in terms of colonization with filamentous algae.

The results from this study support the hypothesis that *S. aloides* has allelopathic effects on the growth and development of filamentous algae. The majority of the nutrients occurred in similar concentrations in water samples taken from the filamentous algae and *S. aloides* stands, and artificial *S. aloides* plants were rapidly colonized by filamentous algae, while the natural *S. aloides* plants remained nearly free of these algae.

With this study we emphasize that the debate of resource versus interference competition should no longer focus on the existence of allelopathy. It should rather stress the relative importance of these processes.

## 7.2 Introduction

Research on allelopathic effects of macrophytes on phytoplankton has shown that various aquatic plants possess the ability to excrete compounds that inhibit phytoplankton growth. Examples of allelopathic macrophytes are charophytes (*Chara*, *Nitella*, e.g., Berger & Schagerl 2004), rigid hornwort (*Ceratophyllum*, e.g., Gross et al. 2003b), and water milfoil (*Myriophyllum*, e.g., Gross & Sütfield 1994). The allelopathic activity of *S. aloides* has been studied less frequently. *S. aloides* is an important macrophyte as it is a rapid colonizer (Erixon 1979) that will tend to exclude many plant species when growing in small aquatic ecosystems.

Brammer (1979) studied the allelopathic effect of *S. aloides* on phytoplankton, but competition for essential nutrients together with changes in the ionic composition of the water were more likely explanations for the decline of phytoplankton in the proximity of *S. aloides* than allelopathy. Brammer & Wetzel (1984) showed that, especially during its submerged phase, *S. aloides* can markedly change concentrations of potassium, sodium and calcium in the water. Other studies provided evidence in favour of the allelopathy hypothesis. Jasser (1995) conducted laboratory experiments with *S. aloides* extracts and found inhibitory effects on the growth of cyanobacteria (*Anabaena*, *Lyngbya*, and *Oscillatoria*). In the same study, a field experiment with transparent plastic bags and extracts of *S. aloides* showed declining cyanobacterial densities at increasing concentrations of *S. aloides* extract were added. These studies illustrate the ongoing debate on whether lower phytoplankton densities in the vicinity of macrophytes are caused by real allelopathic interactions (a form of interference competition) or by nutrient competition.

In addition to allelopathic effects of *S. aloides* on phytoplankton, field observations suggested allelopathic interactions between *S. aloides* and filamentous algae. *S. aloides* is a dioecious, perennial aquatic macrophyte with an exceptional life-cycle. It overwinters at the lake sediment and in spring the macrophyte becomes buoyant and floats to the water surface where it flowers and reproduces during summer (Bloemendaal & Roelofs 1988, see also Fig. 1.6 Chapter 1). In autumn the macrophyte sinks to the sediment again. Development of filamentous algae starts at the lake bottom. In spring, the developing filamentous algae become buoyant and float to the water surface (Scheffer 1998), creating dense mats.

During a survey of several Dutch *S. aloides* stands studying the allelopathic interaction between *S. aloides* and phytoplankton (Mulderij et al. *submitted*, Chapter 6), gaps in the mats of filamentous algae at places where *S. aloides* plants occurred were observed. Based on these findings, we hypothesize that the aquatic macrophyte *S. aloides* negatively influences the growth/development of filamentous algae by excretion of allelopathic substances, resulting in gaps in the mats of filamentous algae.

Alternative explanations for the observation of these gaps may be (1) nutrient limitation, (2) movement of the macrophyte once occurring in the mats of filamentous algae, and (3) the occurrence of the macrophyte on the water surface before filamentous algae appeared there (timing). To assess the relative importance of allelopathy on the interaction between *S. aloides* and filamentous algae *in situ*, we conducted a field survey and performed two field experiments where we introduced artificial macrophytes in natural stands of *S. aloides* and compared colonization of filamentous algae on both types of plants (natural and artificial/non-allelopathic plants).

## 7.3 Materials and methods

### 7.3.1 Field survey

A total of 18 natural Dutch *S. aloides* stands, in ditches exposed to similar environmental conditions, were sampled: six in *De Krimpenerwaard* (4°35' E, 51°55' N), four in *Tienhoven* (5°05'E, 52°10'N), three in *Zegveld* (4°51'E, 52°07'N) and five in *Giethoorn* (6°05'E, 52°45'N). In all of these stands *S. aloides* plants co-occurred with filamentous algae (mostly *Spirogyra* sp. and *Cladophora* sp.). We collected a water sample (500 mL) in the middle of the *S. aloides* rosettes and in the filamentous algae and determined the nutrient concentrations (see *Chemical analyses*) after filtration (Whatman GF/C, Ø 47mm) of the water samples to see if nutrient limitation could be an alternative explanation for the absence of filamentous close to *S. aloides*. Sampling was carried out on 16 - 19 and 25 July 2002.

### 7.3.2 Field experiments

We conducted the field experiments in a ditch close to Lake Naardermeer, The Netherlands (5°06'E, 52°19'N). In the spring of 2003 and 2004 we introduced 20 artificial, non-allelopathic plants (Fig. 7.1), with a similar morphology as *S. aloides*, at two locations in the ditch (*Loc. 1* and *Loc. 2*, ca. 100 m distance from each other). This resulted in combined stands of artificial plants and natural *S. aloides*, later in the season. Unfortunately, the *S. aloides* plants at *Loc. 1* remained submerged in 2004. Therefore, we were not able to determine the development of filamentous algae in the vicinity of *S. aloides* at *Loc. 1* in that year.



**Figure 7.1** Artificial/non-allelopathic plants introduced in 2003 at Location 1 (*Loc. 1*). Note the onset of filamentous algae development between the artificial plants. Natural *S. aloides* plants did not emerge on the surface yet (see *Discussion*).

Picture: G. Mulderij



During the growing season (every second week) we took a pooled water sample at two sites per location: from 20 rosettes (if possible, see below) of natural *S. aloides* plants and from 20 rosettes of artificial (non-allelopathic) plants. These samples were filtered over 0.2  $\mu\text{m}$  cellulose acetate membrane filters ( $\varnothing$  47 mm, Schleicher & Schuell). Back in the laboratory the water samples were frozen until analysis (see *Chemical analyses*). On each sampling occasion we photographed the plants to determine the development of filamentous algae around natural and artificial plants (see *Statistical analyses*).

We were also interested in the moment of appearance of *S. aloides* on the water surface relative to the appearance of filamentous algae. Therefore, we started the second experiment (2004) before natural *S. aloides* plants and/or filamentous algae appeared on the water surface. Around May - June the natural plants started to appear on the water surface (Figs. 7.6), but not directly in high enough numbers ( $n = 20$ , Fig. 7.6). The sampling size of the natural plants was not constant during the study period, due to the seasonal succession of *S. aloides*. We thus had an unbalanced design with a fixed number of artificial plants versus a variable number of natural *Stratiotes* plants.

The main purpose of our field experiment was to test for differences in the colonization of filamentous algae on natural and artificial *S. aloides* plants. The colonization was categorized in three groups: (1) free of filamentous algae, (2) intermediately overgrown with filamentous algae, and (3) completely overgrown with filamentous algae.

### 7.3.3 *Chemical analyses*

In the laboratory, the pH of the samples from the field survey was determined with a combination glass electrode with an Ag/AgCl internal reference (Orion Research, Beverly, USA). The alkalinity of the survey samples was determined by titrating 50 mL down to pH 4.2 with 0.01 M HCl.

All following analyses were conducted for samples of the survey and the field experiments. Carbon dioxide ( $\text{CO}_2$ ) concentrations were measured using an infrared carbon analyser (model PIR-2000, Horiba Instruments, Irvine, USA). Nitrate and ammonium ( $\text{NO}_3$  and  $\text{NH}_4$ ) were measured colorimetrically with a Traacs 800+ auto-analyser, using hydrazine sulphate (Technicon 1969) and silicate (Grasshoff & Johannsen 1977), respectively. Orthophosphate ( $\text{o-PO}_4$ ) was determined colorimetrically with a Technicon II Auto Analyzer, using ammonium molybdate (Henrik-

sen 1965). Potassium (K) and sodium (Na) were measured by flame photometry (FLM3 Flame Photometer, Radiometer, Copenhagen, Denmark). Aluminium (Al), calcium (Ca), iron (Fe), magnesium (Mg), manganese (Mn), total phosphorus (TP), silicon (Si), sulphur (S), and zinc (Zn) were determined by inductively-coupled plasma emission spectrophotometry (Spectro Analytical Instruments, type Spectroflame, Kleve, Germany).

#### **7.3.4 Statistical analyses**

Prior to analysis of the data of the pH, alkalinity, and nutrient concentrations, the data were tested for normality (Kolmogorov-Smirnov test,  $\alpha = 0.05$ ) and homogeneity of variances (Levene's test,  $\alpha = 0.05$ ). The  $\text{NH}_4$  data of the field experiment in 2003 were not normally distributed and therefore log-transformed. All data were analysed by means of Paired-samples  $t$ -tests ( $\alpha = 0.05$ , Fowler et al. 1998, Sokal & Rohlf 1995).

We analysed the categorical data of the field experiments with artificial macrophytes and found highly significant results (see *Results, Field experiments*), and therefore decided not to increase the number of categories. The colonization of filamentous algae on each location was classified by plant type (artificial, natural), sampling date, and sampling year. The  $H_0$  was that colonization of filamentous algae on each location is independent of plant type, sampling date and sampling year. The  $\chi^2$  statistic ( $\alpha = 0.05$ ) was used to test the  $H_0$ . All statistical analyses were performed in SPSS version 11.5 (Dytham 1999).

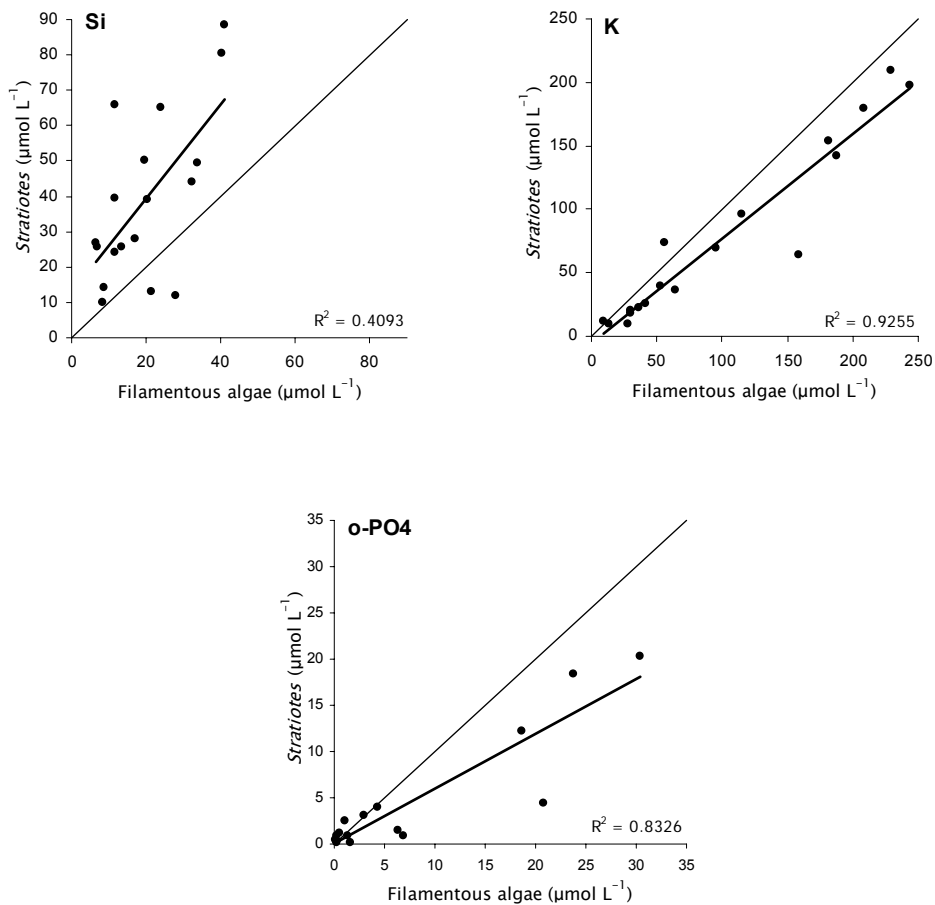
## 7.4 Results

### 7.4.1 Field survey

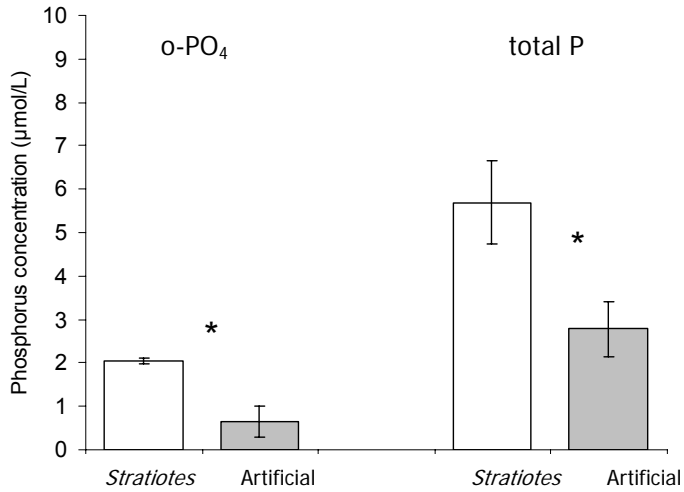
The pH and alkalinity of water samples taken in the vicinity of *S. aloides* and in the filamentous algae did not significantly differ ( $p = 0.120$  and  $p = 0.136$ ). The field survey showed that nutrient concentrations in the rosettes of *S. aloides* were often significantly correlated with the concentration found in locations with filamentous algae (Pearson's product-moment correlation: 0.51 - 1.00). The Paired-samples *t*-test, however, only showed significant differences in average concentrations of o-PO<sub>4</sub>, K and Si between water samples taken in the vicinity of *S. aloides* and water samples taken from locations with filamentous algae (Fig. 7.2). Si was significantly ( $p < 0.001$ ) higher in the vicinity of *S. aloides* rosettes than close to the filamentous algae, but the opposite was true for o-PO<sub>4</sub> and K: significantly lower concentrations ( $p = 0.026$  and  $p < 0.001$ , respectively) were found close to *S. aloides*.

### 7.4.2 Field experiments

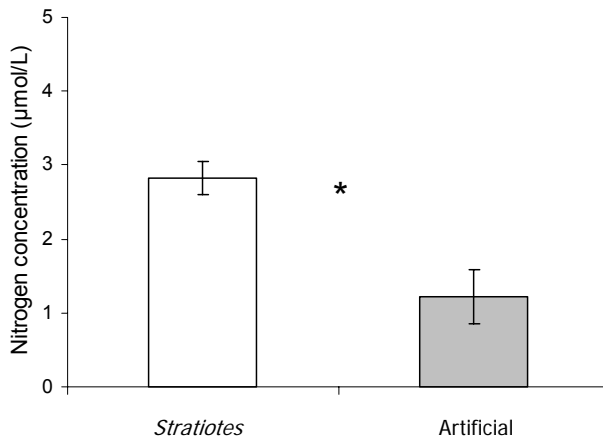
In 2003, we did not observe any significant differences between nutrient concentrations in water samples taken close to *S. aloides* or close to the artificial plants at *Loc. 1*. At *Loc. 2* we only observed a significant difference for o-PO<sub>4</sub> and total P (Fig. 7.3). Concentrations of o-PO<sub>4</sub> and total P found in the vicinity of *S. aloides* were significantly higher ( $p = 0.019$  and  $p = 0.039$ ) than the concentrations found in water samples taken close to the artificial macrophytes (Fig. 7.3). In 2004, we found no significant effects except for the higher NO<sub>3</sub> concentrations ( $p = 0.037$ ) close to *S. aloides* at *Loc. 1* (Fig. 7.4). For the other nutrients and all nutrient concentrations at *Loc. 2* no significant differences were found ( $p > 0.05$ ).



**Figure 7.2** Concentrations of silicon (Si), potassium (K) and orthophosphate (o-PO<sub>4</sub>) in water samples taken during the field survey of 18 Dutch *S. aloides* stands. The samples were collected in the vicinity of *S. aloides* and close to the filamentous algae.



**Figure 7.3.** Concentrations of orthophosphate (o-PO<sub>4</sub>) and total phosphorus (TP) in the vicinity of *S. aloides* and close to artificial plants at Loc. 2, 2003. Bars represent average values ( $n = 7$ ) with error bars (1 SE). \* indicates significant differences ( $p < 0.05$ ) between natural *S. aloides* and artificial plants.

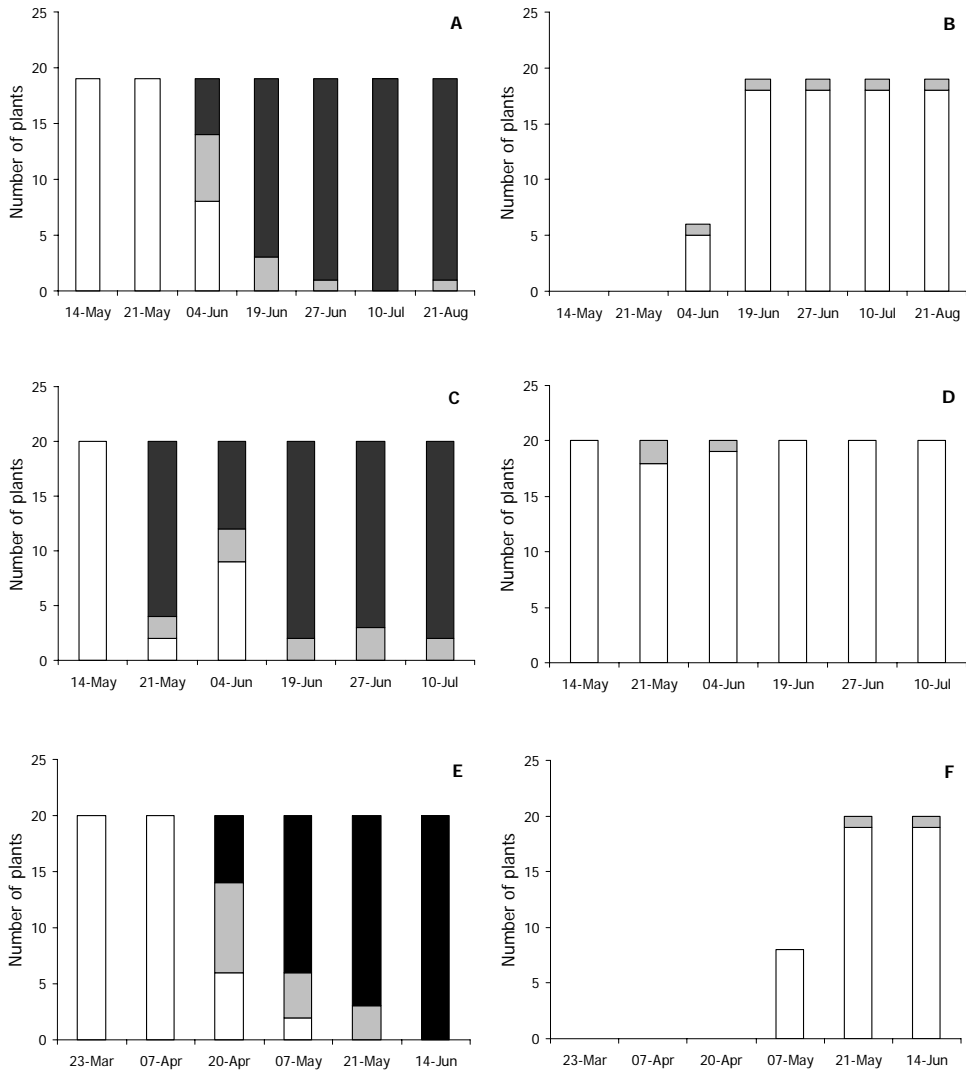


**Figure 7.4.** Concentrations of NO<sub>3</sub> in the vicinity of *S. aloides* and close to artificial plants at Loc. 1, 2004. Bars represent average values ( $n = 7$ ) with error bars (1 SE). \* indicates significant differences ( $p < 0.05$ ) between natural *S. aloides* and artificial plants.

Analysis of development of filamentous algae showed that most artificial plants became heavily overgrown with filamentous algae, while nearly all natural *Stratiotes* plants remained free of filamentous algae during the entire experiment in both years (Figs. 7.5 - 7.6). Chi-square tests showed that, on each location, natural *Stratiotes* plants were significantly less overgrown with filamentous algae than artificial plants (Pearson  $\chi^2$  Loc. 1: 78.4,  $p < 0.001$ ; Pearson  $\chi^2$  Loc. 2: 170.9,  $p < 0.001$ ). At Loc. 2 the observations did not differ between years (Pearson  $\chi^2$  Loc. 2: 2.533,  $p = 0.279$ ). Effects of subsequent years could not be tested for Loc. 1 because we could only conduct an experiment at this location in 2003. Furthermore, on each location the observations differed significantly over the season for both type of plants (Pearson  $\chi^2$  Loc. 1: 55.0,  $p < 0.001$ ; Pearson  $\chi^2$  Loc. 2: 110.9,  $p < 0.001$ ). Especially the artificial macrophytes became heavily overgrown with filamentous algae towards the end of the experiment.



**Figure 7.5** Natural *S. aloides* plant (on the left) surrounded by filamentous algae and three artificial (non-allopathic) plants (see white circles) covered with filamentous algae at Loc. 1 in 2003.



**Figure 7.6** Colonization of artificial (A, C, E) and natural (B, D, F) *S. aloides* plants (number of plants) with filamentous algae during the experiment in 2003 at *Loc. 1* (A, B) and *Loc. 2* (C, D) and the experiment in 2004 at *loc. 2* (E, F). Plants were classified in three categories: completely free of filamentous algae (white), completely overgrown (dark green), or slightly covered with filamentous algae (green).

## 7.5 Discussion

Our field experiments showed highly significant differences in the colonization of natural and artificial *S. aloides* plants by filamentous algae. The observations could not be explained in terms of nutrient limitation of filamentous algae. The nutrient concentrations in the vicinity of *S. aloides* were not lower than in the vicinity of filamentous algae. With respect to the ongoing debate on the importance of nutrient competition versus that of allelopathic interactions (a form of interference competition), our observations in the field experiments provided strong indications for the existence of allelopathic effects of *S. aloides* on filamentous algae.

For the majority of the nutrients measured in the field survey we did not find significant differences in the concentrations close to *S. aloides* and close to filamentous algae. The survey, however, did show significantly lower  $\text{o-PO}_4$  and K values in the vicinity of *S. aloides*, suggesting that nutrient ( $\text{o-PO}_4$  and K) limitation could play a role in the absence of high densities of filamentous algae close to *S. aloides* plants. These observations correspond with those of Brammer (1979) and Brammer & Wetzel (1984) who demonstrated that competition for essential nutrients together with changes in the ionic composition of the water, rather than allelopathy, were more likely explanations for lower phytoplankton densities close to *S. aloides* plants. Mulderij et al. (*submitted*, Chapter 6) and Jaworski et al. (2003) argued that potassium limitation under natural conditions was rather unlikely, as *in situ* concentrations are often not extremely low.

An alternative explanation for our observations could be that the gaps in filamentous algae are a result of the morphology and movement of *S. aloides*, another type of interference. The gaps could be created when *S. aloides* plants, once emerging on the water surface in the mats of filamentous algae slightly move around (by wind or wave action) and further open the gaps in the filamentous algae. This explanation would, however, only hold when *S. aloides* occurs at the water surface before filamentous algae start to appear there. The filamentous algae usually start to grow at the sediment, and thereafter become buoyant and float to the water surface (Scheffer 1998). When *S. aloides* plants are already present at the water surface, the developing filamentous algae can (most likely) only move along the side of the *S. aloides* rosettes to reach the water surface. Small movements of the plants may then create more space around themselves. *S. aloides*, however, appears at the water surface after the filamentous algae, indicating that it is unlikely that the gaps in filamentous algae are only created by the morphology of *S. aloides*. Artificial (non-alle-



lopathic) plants with a similar morphology (and way of moving by wind/waves) to natural *S. aloides* plants, on the contrary, became completely overgrown with filamentous algae. We further observed that young *S. aloides* plants were able to penetrate the dense mats of filamentous algae and appeared on the water surface after creating a gap in the layers of filamentous algae, which is in favour of the allelopathy hypothesis.

Light limitation of phytoplankton due to shading by macrophytes may also play a role in the interaction between these two primary producers (Sand-Jensen 1989, Ozimek et al. 1990). We, however, assume that shading plays a less important role in the interaction between *S. aloides* and filamentous algae as both species were present at the water surface, suggesting that they are subjected to similar light conditions. Moreover, filamentous algae appeared on the water surface before *S. aloides* did, indicating that during the early movement of filamentous algae from the sediment towards the water surface in spring, these algae could hardly have been light limited as a consequence of the presence of *S. aloides*.

The use of artificial *S. aloides* plants enabled us to mimic the presence of *S. aloides* as a structural component of the aquatic food web. Higher densities of zooplankton (Jeppesen et al. 1998, Irvine et al. 1990) and macrofauna (Higler 1977) seem to be related to the presence of macrophytes which function as refuges or habitat. High zooplankton densities can create a high grazing pressure on algae. However we doubt that zooplankton grazes upon filamentous algae too. Fulton (1988) conducted experiments with herbivorous zooplankton species, filamentous diatoms, filamentous green algae, and cyanobacteria, and showed that the zooplankton species *Daphnia pulex* and *Bosmina longirostris* avoided the green alga *Ulothrix* sp. (similar to *Spirogyra* sp. and *Cladophora* sp. present in our experiments). Higler (1977) performed experiments with artificial *S. aloides* plants and showed that, compared to living plants, artificial plants could accommodate comparable numbers and about 90% of the same species of macroinvertebrates. Based on the results from Fulton (1988) and Higler (1977) we assume that grazing by zooplankton or macrofauna on filamentous algae as determining factor for the absence of filamentous algae in the vicinity of *S. aloides* is rather unlikely.

This is the first study of allelopathic effects of *S. aloides* on filamentous algae. Our study supports the hypothesis that, in addition to allelopathic effects on phytoplankton, *S. aloides* has allelopathic effects on the density and development of filamentous algae. The results from this study emphasize that the ongoing debate of resource competition versus allelopathy should no longer focus on the existence of allelopathy, but rather on assessment of the relative importance of these two processes.

### **Acknowledgements**

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# Theory & chemistry





# 8

The effects of two macrophytes on phytoplankton growth: a model to estimate the contribution of allelopathy *in situ*

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# Allelopathy model

## 8.1 Abstract

Submerged aquatic macrophytes are important in shallow lakes because they stabilize the macrophyte-dominated state by increasing lake water transparency in various ways. One of these is the excretion of substances inhibitory to phytoplankton. This phenomenon is called *allelopathy*. We analysed the relative contribution of allelopathy to inhibitory effects of macrophytes on phytoplankton.

We developed a model describing phytoplankton growth including the effects of two different macrophytes (*Chara* sp. and *Stratiotes aloides*), who are both known to excrete allelopathic substances. With this model we analyzed single and combined effects of shading (1), resuspension (2), nutrient competition (3) and allelopathy (4) on the biomass development of phytoplankton.

Shading of phytoplankton by *Chara* sp. was estimated to be negligible as this macrophyte species has a growth form close to the sediment. Nutrient competition seems to cause somewhat lower phytoplankton densities. Allelopathic activity of *Chara* sp. caused a slight growth reduction of phytoplankton, while the most pronounced effect seems to be related to reduction of particle (sediment particles but also algal cells) resuspension.

*S. aloides* is a macrophyte that floats on the water surface during spring and summer and it may have a high potential shading effect in these seasons. Additionally, it has a strong allelopathic capacity, sometimes resulting in a growth reduction of phytoplankton (up to 80%). Nutrient competition with phytoplankton and reduction of resuspension seem negligible for this macrophyte.

The model analysis suggests that the relative contribution of allelopathy may be low or negligible for species like *Chara*. Only for species, like *S. aloides*, it may be a significant. Further, the highly different growth forms of the two tested macrophytes may substantially influence the allelopathic interaction (and possibly also other processes) between them and phytoplankton.

## 8.2 Introduction

Submerged aquatic macrophytes are important for food web interactions and environmental quality of shallow lakes (Carpenter & Lodge 1986, Jeppesen et al. 1998a). The presence of macrophytes in aquatic habitats can completely alter the ecosystem. Lake water transparency and species diversity of e.g., zooplankton and macroinvertebrates increases with the occurrence of macrophytes (Scheffer 1998). Many abiotic and biotic components of aquatic ecosystems are involved in the stimulatory effects of aquatic macrophytes on the water transparency of shallow lakes (Jeppesen 1998, Scheffer 1998). Among other processes, macrophytes reduce resuspension, provide shelter for zooplankton and young fish, reduce nutrient levels, provide a habitat for macroinvertebrates, and excrete allelopathic substances that inhibit the growth of other photoautotrophs, such as epiphyton and phytoplankton (Scheffer 1998).

Macrophytes affect their environment, but obviously the environment has effects on macrophytes too. As light is a major limiting factor in macrophyte growth, the positive effect of macrophytes on lake water transparency makes that their growth is self-stabilizing. This positive feedback is suggested to cause alternative stable states in shallow lakes (e.g., Scheffer et al. 1993a). According to this theory, a lake can have either clear water with macrophytes or turbid water with phytoplankton as main primary producers. This phenomenon is important for lake managers as the shift from turbid to clear water is expected to occur at much lower nutrient levels than the opposite shift, a process called 'hysteresis' (Scheffer et al. 1993a).

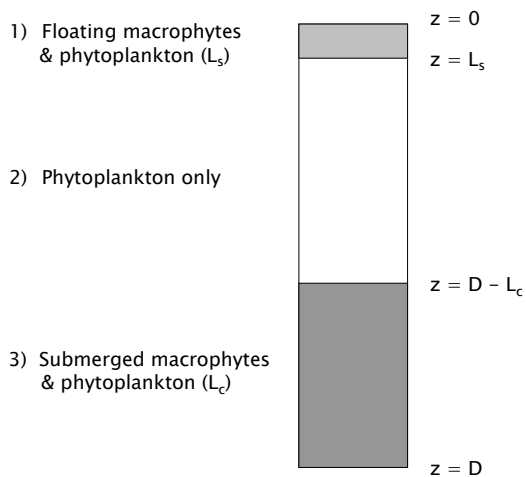
It has been shown in laboratory experiments that the inhibition of phytoplankton growth by allelopathic substances from some macrophytes can be up to 80% (e.g., Körner & Nicklisch 2002, Mulderij et al. 2005b, Chapters 5). The potential role of allelopathy *in situ* still remains unclear and controversial (Forsberg et al. 1990, Van Donk & Van de Bund 2002). The main problem is that allelopathic interactions in the field are less well studied. Allelopathy and several other processes acting in macrophyte beds (e.g., shading, reduced resuspension, nutrient competition) interfere with each other and the relative importance of all mechanisms differs strongly between lakes and species.

An alternative way to get more insight in the role of allelopathy *in situ* is the use of models. However, only a few models consider allelopathic interactions. Moreover, most of these models describe terrestrial ecosystems (e.g., An et al. 1993), which are structurally different from aquatic ecosystems. The only models that describe 'aquatic' allelopathic interactions are developed by Chattopadhyay (1996), Mukhopadhyay et al. (1998, 2003), Tapaswi et al. (1999) and Zhen & Ma (2002). They all use modified versions of the Lotka-Volterra competition model. All these models are, however, very theoretical and they only consider allelopathic interactions between phytoplankton species.

Though there are several models on phytoplankton growth (e.g., Huisman & Weissing 1994, 1999, Gragnani et al. 1999) and some on macrophyte growth (e.g., Scheffer et al. 1993b, Hootsmans 1994, Van Nes et al. 2002, 2003), to our knowledge there is no model that includes allelopathic effects of macrophytes on phytoplankton growth. In the present paper, we constructed a model to analyse the singular and combined effects of shading, nutrient uptake, resuspension of algal cells and the excretion of allelopathic substances by *Chara* sp. and *S. aloides*, on the development of phytoplankton. We have chosen these two species as their allelopathic capacity is well studied in the laboratory (Wium-Andersen et al. 1982, Blindow & Hootsmans 1991, Mulderij et al. 2003, 2005a, 2005b, Chapters 3 - 5) and the species have different growth forms. *Chara* sp. is a bottom-dwelling species, while *S. aloides* is a species that is submerged in autumn and winter, but becomes buoyant in spring and floats on the water surface during summer (Bloemendaal & Roelofs 1988). As the dynamics of allelopathic substances in aquatic ecosystems remain unclear, the model focuses on the inhibitory effects of allelopathy on phytoplankton growth only.

### 8.3 Model description

We modified the phytoplankton models of Huisman & Weissing (1994, 1999) to describe the effects of floating (*S. aloides*) and submerged (*Chara sp.*) macrophytes. We assume that phytoplankton is distributed equally over the whole water column, whereas submerged aquatic vegetation is distributed equally over a part of the water column (Fig. 8.1).



**Figure 8.1** Position of floating and submerged macrophytes and phytoplankton in the water column of a shallow lake.  $D$  is the water depth,  $L_s$  is the thickness of the layer with floating vegetation (*S. aloides*),  $L_c$  is the height of submerged vegetation (*Chara sp.*).

The gross phytoplankton production is integrated over the whole water column,  $D$ . We describe phytoplankton ( $A$ ) growth with the following differential equation (see Table 8.1 for parameter values of the model):

$$\frac{dA}{dt} = \frac{1}{D} \int_0^D p(I_z, N, P) dz - l_A A$$

in which  $p(I_z, N, P)$  is the gross phytoplankton production as a function of the available amount of light ( $I_z$ ), nitrogen ( $N$ ), and phosphorus ( $P$ ). Little is known about the effect of different concentrations of the active compounds on different biomasses of phytoplankton. As a first approach, allelopathy is assumed to cause growth limitation by a fixed factor  $(1 - f_{all})$ , as a percentage of the phytoplankton growth (Mulderij et al. 2003, 2005a, 2005b, Chapters 3 - 5). In the loss factor of phytoplankton ( $l_A$ ) we include the effect of reduced resuspension. Nutrient and light limitation of phytoplankton are described with the following Monod functions:

$$p(I_z, P, N) = r_A A \frac{I_z}{h_{I,A} + I_z} \frac{N}{h_n + N} \frac{P}{h_p + P} (1 - f_{all})$$

The amount of light in the water column ( $I_z$ ) available for phytoplankton growth is dependent on self-shading and shading by macrophytes. To calculate the light availability we divided the water column into three parts (Fig. 8.1): Layer 1 from 0 to  $L_s$ ; Layer 2 from  $L_s$  to  $D - L_c$ ; Layer 3 from  $D - L_c$  to  $D$ . Phytoplankton is assumed to be present in all three layers, while *S. aloides* (as floating macrophyte,  $S$ ) is only present in layer 1 ( $L_s$ ) and the submerged macrophyte *Chara* ( $C$ ) is only present in layer 3 ( $L_c$ ).

**Table 8.1** Parameter values of the model, and their units.

Parameter	Description	Unit	Default value	Source
$C$	<i>Chara</i> biomass	$\text{g m}^{-2}$	0 – 300	Field observations
$S$	<i>Stratiotes</i> biomass	$\text{g m}^{-2}$	0 – 300	Field observations
$r_A$	Phytoplankton growth rate	$\text{d}^{-1}$	0.5	Scheffer & Rinaldi 2000
$r_n$	Turnover rate N	$\text{d}^{-1}$	0.25	
$r_P$	Turnover rate P	$\text{d}^{-1}$	0.2	Hudson et al. 1999
$Y_{A,N}$	N:C ratio phytoplankton	–	0.25	Muhammetoğlu & Soyupak 2000
$Y_{A,P}$	P:C ratio phytoplankton	–	0.025	Muhammetoğlu & Soyupak 2000
$f_{all}$	Limitation factor allelopathy	–	0	Based on Mulderij et al. 2003, Chapter 3
$h_{l,A}$	Half saturation light limitation phytoplankton	$\mu\text{E m}^{-2}\text{s}^{-1}$	30	Bates 1976
$h_n$	Half saturation nitrogen limitation	$\text{g m}^{-3}$	0.025	Muhammetoğlu & Soyupak 2000
$h_P$	Half saturation phosphorus limitation	$\text{g m}^{-3}$	0.003	Riegman & Mur 1984
$l_A$	Phytoplankton loss rate	$\text{d}^{-1}$	0.1	Scheffer & Rinaldi 2000
$l_0$	Light intensity water surface	$\mu\text{E m}^{-2}\text{s}^{-1}$	1000	Kirk 1994
$k_b$	Background light attenuation	$\text{m}^{-1}$	0.2	Huisman et al. 2002
$k_c$	Light attenuation coefficient submerged vegetation	$\text{m}^2 \text{g}^{-1}$	0.02	Ikusima 1970*
$k_s$	Light attenuation coefficient floating vegetation	$\text{m}^2 \text{g}^{-1}$	0.02	Ikusima 1970*
$k_A$	Light attenuation phytoplankton	$\text{m}^2 \text{g}^{-1}$	0.2	Kirk 1994
$Y_{C,N}$	Effect of <i>Chara</i> on N	$\text{d}^{-1}$	0.01	Van Donk et al. 1993
$D$	Water depth	m	2	Field observation
$L_C$	Maximum height <i>Chara</i>	m	0.35	Van Nes et al. 2003
$L_S$	Thickness top layer <i>Stratiotes</i>	m	0.10	Field observation
$s_P$	Equilibrium P	$\text{g m}^{-3}$	0.3	Varied between 0–1
$s_N$	Equilibrium N	$\text{g m}^{-3}$	3	Arbitrary realistic value

\*assumed to be the same as for *Chara* sp.

Water depth  $D$  (m), Submerged vegetation (*Chara* sp.)  $C$  ( $\text{g m}^{-2}$ ), Floating vegetation (*Stratiotes aloides*)  $S$  ( $\text{g m}^{-2}$ ) parameter default values were arbitrarily chosen.

We assume that floating and submerged vegetation is distributed evenly within its layer. For each of the three layers we applied the law of Lambert-Beer, adjusted for shading by macrophytes (Ikusima 1970) and phytoplankton (Huisman & Weissing 1994):

$$I_z = I_{in} e^{-k_b z - k_A A - \sum C k_C \frac{z}{\Delta z_{layer}}} = I_{in} e^{\left(-k_b - k_A A - \sum \frac{C k_C}{\Delta z_{layer}}\right) z} = I_{in} e^{-k_{layer} z}$$

The average limitation of phytoplankton in each layer is obtained by integrating the following Monod equation:

$$p(I)_{layer} = \frac{1}{\Delta z_{layer}} \int_0^{\Delta z_{layer}} \frac{I_z}{I_z + h_{I,A}} dz$$

Huisman & Weissing (1994) showed that the integral of this formula for a water column with phytoplankton can be solved as follows:

$$p(I)_{layer} = \frac{1}{\Delta z_{layer}} \int_0^{\Delta z_{layer}} \frac{I_z}{I_z + h_{I,A}} dz = \frac{1}{\Delta z_{layer} K_{layer}} \ln \left( \frac{h_{I,A} + I_{in}}{h_{I,A} + I_{out}} \right)$$

Each layer in the water column has its own light attenuation coefficient, ( $K_{layer}$ ) and its own incoming and outgoing light intensity ( $I_{in}$  and  $I_{out}$ ). As we modelled light attenuation by macrophytes in an analogous way as phytoplankton (Huisman & Weissing 1994) we can apply this formula for each of the three layers (Table 8.2). The total light limitation experienced by phytoplankton is the weighted average over all three layers:

$$p(I)_{total} = \frac{z_S p(I)_{layer1} + (D - z_S - z_C) p(I)_{layer2} + z_C p(I)_{layer3}}{D}$$

**Table 8.2** Light attenuation coefficient in the three layers in a water column (see Fig. 8.1).

Layer	$\Delta z_{\text{layer}}$	$k_{\text{layer}}$	$I_{\text{in}}$	$I_{\text{out}}$
1	$L_S$	$k_b + k_A A + S \frac{k_S}{L_S}$	$I_0$	$I_0 e^{-(k_b + k_A A) L_S + S k_S}$
2	$D - L_C - L_S$	$k_b + k_A A$	$I_0 e^{-(k_b + k_A A) L_S + S k_S}$	$I_0 e^{-(k_b + k_A A)(D - L_C) + S k_S}$
3	$D - L_C$	$k_b + k_A A + C \frac{k_C}{L_C}$	$I_0 e^{-(k_b + k_A A)(D - L_C) + S k_S}$	$I_0 e^{-(k_b + k_A A) D + S k_S + C k_C}$

We described the dynamics of the nutrients ( $N$  and  $P$ ) in a simplified way, similar to models used for chemostat cultures (Edelstein-Keshet 1988). We assumed a fixed nutrient load ( $r * s$ ) and a first order decay (Huisman & Weissing 1999). Furthermore, nutrients required for phytoplankton production were taken into account. In contrast to influences of phytoplankton, those of the two macrophytes on the dynamics of phosphorus are not incorporated in the model because a steady state was assumed for macrophyte growth, and therefore they do not take up nutrients. The remaining effect of phytoplankton on the dynamics for phosphorus ( $P$ ) was described with the following equation:

$$\frac{dP}{dt} = r_P (s_P - P) - \gamma_{A,P} \frac{1}{D} \int_0^D P(I_z, N, P) dz$$

Submerged aquatic macrophytes in shallow eutrophic lakes can have a significant effect on the nitrogen availability (Van Donk et al. 1993, Scheffer 1998). Probably this effect is negligible for *S. aloides*, as this poorly rooted species will most likely not be able to stimulate nitrification-denitrification processes through the transport of oxygen to the sediment significantly, like charophytes can (Kufel & Kufel 2002). We assumed that the effect of *Chara* sp. results in a decrease of the nitrogen concentration proportionally with the biomass (parameter  $\gamma_{C,N}$ ). Furthermore, nitrogen dynamics were modelled analogously to the phosphorus dynamics:

$$\frac{dN}{dt} = r_N (s_N - \gamma_{C,N} C - N) - \gamma_{A,N} \frac{1}{D} \int_0^D P(I_z, N, P) dz$$

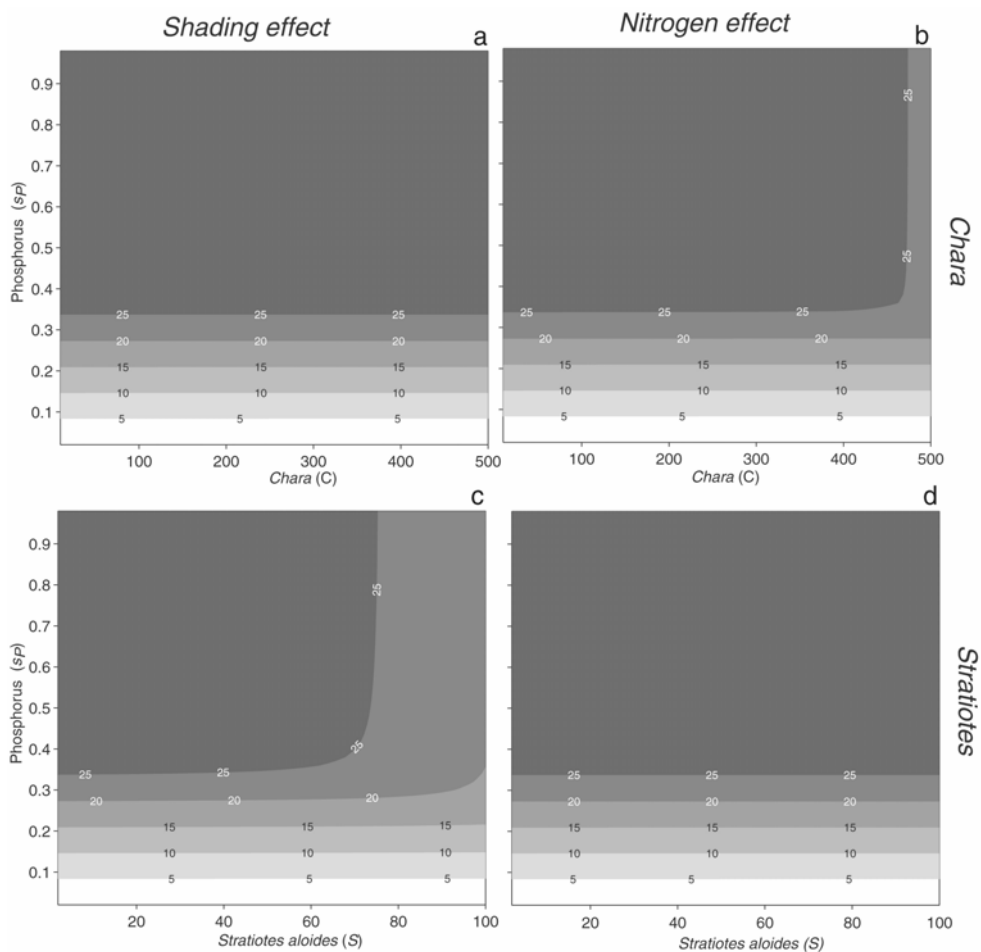


We implemented the model using the software package GRIND for MATLAB (free available at [www.dow.wau.nl/aew/grind](http://www.dow.wau.nl/aew/grind)), which uses a Runge-Kutta method to solve the differential equations. We used a numerical scanning procedure to evaluate the effect of pairs of parameters on the attractor of the model. Both parameters were changed in 50 small steps. After setting each new combination of parameters, the model was stabilized during 500 days by simulation and the equilibrium phytoplankton biomass was plotted using a standard interpolation function of MATLAB.

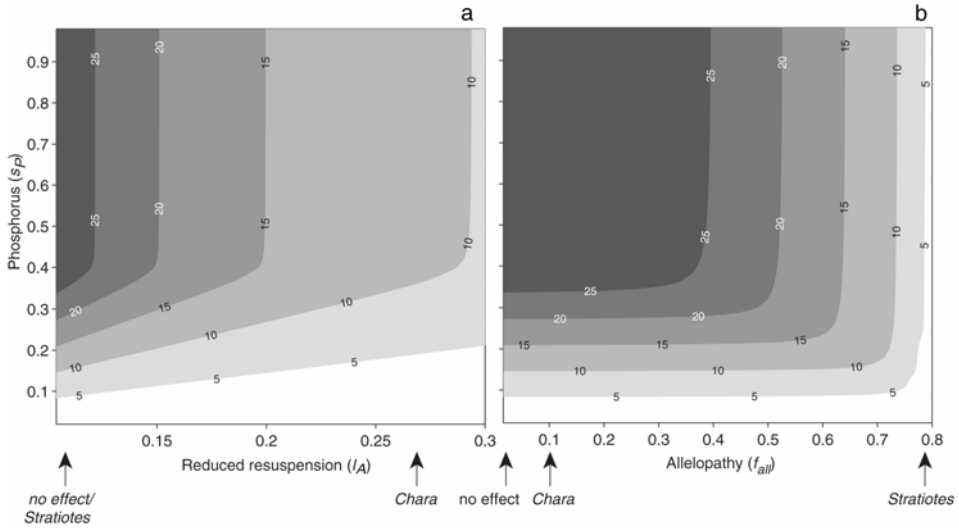
## 8.4 Results

Over a large part of its parameters the model has one stable equilibrium and one unstable trivial equilibrium. As nutrients are modelled in a similar way as in standard chemostat models (Edelstein-Keshet 1988, Huisman & Weissing 1994), the model can perform damped oscillations, but we found this behaviour only at unrealistic parameter values and in a small part of the parameter space. The simple model behaviour facilitates the analysis of this model, as most parameters only affect the equilibrium biomass.

We concentrated our analysis on the effect of the single clearing mechanisms (Figs. 8.2 & 8.3) and on the combined effects (Fig. 8.4) of macrophytes on the phytoplankton biomass. The effect of shading by both macrophyte types varied considerably. The low-growing *Chara* had a negligible shading effect, whereas the floating *Stratiotes* at high densities ( $> 75 \text{ g m}^{-2}$ ) had a strong effect on phytoplankton (Figs. 8.2a and 8.2c), even at a lower nutrient status ( $s_p < 0.1 \text{ g m}^{-3}$ ). The effect of the plants on phytoplankton via nitrogen limitation was negligible for both (Figs. 8.2b and 8.2d).



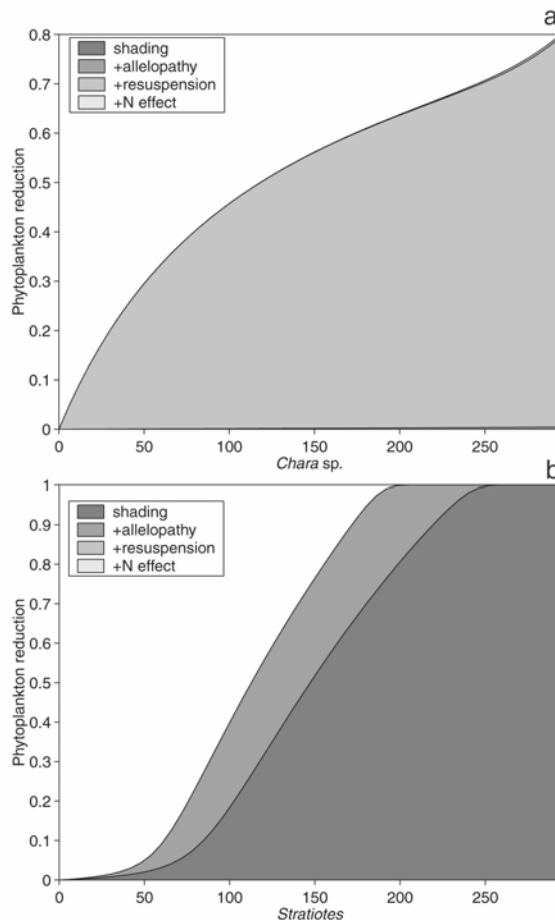
**Figure 8.2** **A** Effect of shading by *Chara* sp. ( $C$ , g m<sup>-2</sup>) and phosphorus ( $sP$ , g m<sup>-3</sup>) on the total phytoplankton biomass (g m<sup>-3</sup>, see numbers and different green scales in the graphs). **B** Effect of stimulated denitrification by *Chara* sp. ( $C$ , g m<sup>-2</sup>) and phosphorus ( $sP$ , g m<sup>-3</sup>) on the total biomass of phytoplankton (g m<sup>-3</sup>). **C** Effect of shading by *Stratiotes aloides* ( $S$ , g m<sup>-2</sup>) and phosphorus ( $sP$ , g m<sup>-3</sup>) on the total biomass of phytoplankton (g m<sup>-3</sup>). **D** Effect on denitrification by *S. aloides* ( $S$ , g m<sup>-2</sup>) and its influence on the total phytoplankton biomass (g m<sup>-3</sup>) at different phosphorus levels ( $sP$ , g m<sup>-3</sup>).



**Figure 8.3** **A** Effect of the phytoplankton loss rate via reduced resuspension ( $I_A$ ,  $d^{-1}$ ) and phosphorus ( $sP$ ,  $g\ m^{-3}$ ) on the total biomass of phytoplankton ( $g\ m^{-3}$ , see numbers and the different green scales in the graphs). **B** Effect of allelopathic inhibition ( $f_{AI}$ ) and phosphorus ( $sP$ ,  $g\ m^{-3}$ ) on the total biomass of phytoplankton ( $g\ m^{-3}$ ).

Other effects of these macrophytes may be their role in the sedimentation (reduced resuspension) of phytoplankton cells (Fig. 8.3a) and allelopathic interactions (Fig. 8.3b). Already at low phosphorus levels, reduction of resuspension by *Chara* has some effects (Fig. 8.3a). Our model shows that the effect of *Chara* via allelopathy is negligible, whereas *S. aloides* has a strong effect on phytoplankton biomass. The effect was stronger at higher P concentrations (Fig. 8.3b).

Finally, we simulated the combined effects of shading (Figs. 8.2a and 8.2c), denitrification (Figs. 8.2b and 8.2d), reduced resuspension of algal cells (Fig. 8.3a) and allelopathy (Fig. 8.3b) with fixed values for total P ( $s_P = 0.3\ mg\ L^{-1}$ ) and water depth (2 m). The effects of allelopathy and resuspension were assumed to depend linearly on the plant biomass to their maximum biomass ( $300\ g\ m^{-2}$ ). The model showed that, for *Chara*, the reduction of resuspension was by far the most important process (Fig. 8.4a), while for *S. aloides* shading and allelopathy were estimated to be the most important processes (Fig. 8.4b).



**Figure 8.4** **A** The predicted effect of *Chara* sp. on the biomass reduction of phytoplankton via a combination of the effects of shading, allelopathy, resuspension and denitrification (= N effect). The maximum allelopathy at 300 g m<sup>-2</sup> is set to 0.1 and the maximum resuspension effect to 0.25. For other parameters see Table 8.1. **B** The predicted effect of *Stratiotes aloides* on the biomass reduction of phytoplankton via a combination of shading, allelopathy, resuspension and nutrient (N) effect. The maximum allelopathy at 300 g m<sup>-2</sup> is set to 0.8, the resuspension effect is assumed to be negligible. Other parameters are depicted in Table 8.1.

## 8.5 Discussion

Our model showed that the relative contribution of allelopathy is strongly dependent on the macrophyte species. Though both species have strong positive effects on water transparency (Brammer 1979, Jasser 1995, Van den Berg et al. 1998a, Mulderij et al. 2005a, 2005b), the responsible mechanisms were very different. Inhibition of phytoplankton via allelochemicals was suggested to be only an important mechanism for *S. aloides*. Remarkably, shading by *Chara* seems to be insignificant, especially in relatively turbid water, when the light intensity is already low near the bottom. Of course the shading effect will be larger in shallower water when *Chara* is present in a larger part of the water column. For floating macrophytes like *S. aloides* and probably also for species like *Potamogeton pectinatus*, shading may be an important mechanism. Increased nitrification does not seem to be a strong effect, probably because phytoplankton can still grow at low nutrient concentrations.

We only studied two macrophyte species, of which the allelopathic capacities are well studied. On basis of growth form of the macrophyte and data about their allelopathic effects found in literature we can use this study to estimate the effects of other species too. Other macrophytes, like *Ceratophyllum*, *Elodea*, *Myriophyllum* or *Potamogeton* are intermediates between the bottom-dwelling charophytes and floating *S. aloides*. Shading of these species may thus be intermediate. These macrophytes were also tested for allelopathic activity, but with variable results. *Potamogeton pectinatus* inhibited the growth of the green alga *Scenedesmus* (Blindow & Hootsmans 1991), but Körner & Nicklisch (2002) showed no inhibitory effects of this macrophyte on green algae, cyanobacteria and diatoms. Nakai et al. (1999), however, tested two *Potamogeton* species (*P. oxyphyllus* and *P. crispus*) for their activity against cyanobacteria and found that *P. crispus* had no inhibitory effect at all, while one of the cyanobacteria was inhibited by *P. oxyphyllus*. *Myriophyllum* and *Ceratophyllum*, which have a growth form relatively similar to *Potamogeton*, are macrophytes of which allelopathic interactions have often been described (see review Gross 2003a). Furthermore, *Myriophyllum* seemed to possess a higher algicidal activity than *Ceratophyllum* (Nakai et al. 1999, Körner & Nicklisch 2002). So, the allelopathic activity of plants seems to be strongly dependent on the species and highly variable.

Our model is a minimal model and it is easy to analyse. Although we incorporated the most important parameters and settings, this approach has its limitations. We neglected some relevant mechanisms and oversimplified others. The main uncertainty is about the fate (production, diffusion and decay) of allelopathic substances in the water column. However, all this relevant information is unknown as it is yet even unclear what the chemical composition of the allelopathic substances excreted by *S. aloides* is. Early studies on charophytes (Anthoni et al. 1980; Wium-Anderson et al. 1982) showed that dithiane and trithiane had inhibitory effect on a diatom and on natural phytoplankton. *Myriophyllum* is known to produce hydrolysable polyphenols. For *S. aloides*, there are indications that moderately lipophilic compounds (Mulderij et al. *submitted*, Chapter 9) or phenolic compounds (Usenko et al. 2002) may play a role in this macrophyte.

Reigosa et al. (1999) stated that the effect of allelochemicals can be affected by many environmental factors as these influence both the target and donor species. The role of allelopathy has been shown to enhance under stress conditions, like nutrient or light limitation (Reigosa et al. 1999). Gross (2003b), described that total bioactive hydrolysable polyphenol levels in the submerged freshwater angiosperm *Myriophyllum spicatum* (= donor) were highly influenced by light, while the nitrogen availability had an effect on tellimagrandin II levels. In the field, phytoplankton (= target) is probably exposed to more stressful conditions than in the laboratory, and therefore more sensitive to allelopathic substances. We neglected the role of stress in our model and used allelopathy data that were determined in the laboratory (Mulderij et al. 2003, 2005a, 2005b, Chapters 3 - 5). This could have lead to an underestimation of the role of allelopathy in *Chara*, especially under low phosphorus levels.

We have not included seasonal effects in the model. The effect of vegetation will be different in different parts of the year. In spring the macrophyte biomass will probably be too low to have a significant effect. Moreover, *S. aloides* has a special seasonal cycle: it is submerged during autumn and winter and it floats on the water surface during spring and summer (see Fig. 1.6, Chapter 1). This migration through the water column may have its effects on the importance of the different processes discussed earlier.

Another simplification is that we did not include the effects of direct uptake of nutrients by both species, as we assume that they have reached their maximum biomass. However, in spring, when the population growth is strong, especially floa-

ting *S. aloides* will take up considerable amounts of nutrients from the water column (Brammer 1979; Brammer & Wetzel 1984), which may cause nutrient limitation in phytoplankton. Reduced resuspension seems to be the most important effect of charophytes. As reduced resuspension is a local effect, it also explains why *Chara* beds in Lake Veluwemeer have caused local clear water areas with steep transparency gradients (Scheffer et al. 1994). Interestingly all these effects are predicted to be mainly important in eutrophic and hypertrophic situations. If there is strong nutrient limitation, most effects are unimportant.

Furthermore, we did not include the uptake of other nutrients than N and P. Especially *S. aloides* can take up considerable amounts of  $K^+$ ,  $Na^+$  and  $Ca^{2+}$  (Brammer 1979). This uptake could especially cause potassium limitation in phytoplankton. However, the growth rate of the freshwater diatoms *Asterionella formosa*, *Diatoma elongatum* and phytoflagellate *Plagioselmis nannoplanctica* was independent of the potassium concentration above  $0.8 \mu\text{mol/L}$  (Jaworski et al. 2003). Also chrysophytes (*sensu* Sandgren 1988) seemed to occur over a potassium range of  $3.8\text{--}1611 \mu\text{mol L}^{-1}$ . Comparison of these values with the average potassium concentration found in natural Dutch freshwater ecosystems with *S. aloides* (average  $106 \mu\text{mol L}^{-1}$ , De Lyon & Roelofs 1986), regarding a depletion induced by *S. aloides*, suggests that potassium is unlikely to limit the growth of phytoplankton, and was therefore not included in the model.

We have not included the refuge effect of macrophytes and zooplankton grazing on phytoplankton in the model. Including the refugium effect of aquatic macrophytes is beyond the scope of this study, as this is hard to implement in a simple way. It would require dynamic modelling of zooplankton in more than one compartment.

Our model is a first attempt to understand the ecological role of allelopathy under natural conditions. It showed the differential allelopathic effects of two macrophytes on phytoplankton. More research under natural conditions, experiments revealing the different modes of action of aquatic allelochemicals and identification of their chemical structure would help to get a better understanding of the ecological relevance of allelopathy between macrophytes and phytoplankton.





Allelopathic activity of *Stratiotes aloides* on phytoplankton:  
towards identification of allelopathic substances.

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## Chemical identification

### 9.1 Abstract

The allelopathic activity of the aquatic macrophyte, *Stratiotes aloides*, was determined with laboratory experiments. Active compounds excreted into the medium or present in plant tissue were extracted using standard procedures and solid phase extraction (SPE). The activity towards various cyanobacteria and chlorophytes was tested in two different bioassay systems using agar plates and liquid cultures of phytoplankton.

Extracts and exudates of *S. aloides* affected phytoplankton growth. Similarly, SPE-enriched exudates and enriched water from a natural *S. aloides* stand caused inhibition of target species, however, also some controls were active. Phytoplankton species exhibited differential sensitivity to extracts of *S. aloides*. We observed both inhibitory and stimulatory effects on phytoplankton growth. In general, more cyanobacteria than other phytoplankton species were inhibited and the inhibition of cyanobacteria was stronger. Nutrient (P or K) limitation of *Synechococcus elongatus* decreased the sensitivity of this species towards allelochemicals from *S. aloides*, but not that of *Scenedesmus obliquus*.

The allelopathically active compound(s) from *S. aloides* are moderately lipophilic and most likely no phenolic compounds. Our results indicate that allelopathy, besides nutrient interference and shading, might also account for the low phytoplankton densities in the vicinity of *S. aloides* plants, at least during certain phases of the life-cycle of *S. aloides*.

## 9.2 Introduction

Aquatic macrophytes play a crucial role in stabilizing the clear water state in shallow mesotrophic and eutrophic lakes (Scheffer 1998, Burks et al. *in press*). They can stimulate changes to clear water situations by a range of mechanisms (see review Van Donk & Van de Bund 2002). One of those mechanisms is the excretion of inhibitory substances that reduce phytoplankton growth (= *Allelopathy*, e.g., Gross 2003a). The release of these compounds may be an effective trait of submerged macrophytes to gain competitive advances against other photoautotrophs.

Only a few allelochemicals from submerged aquatic macrophytes are structurally elucidated (Wium-Andersen et al., 1982; Gross et al., 1996). Previous experiments (Mulderij et al. 2005a, 2005b) provided evidence for allelopathic interference of *S. aloides* with phytoplankton species, but the nature of those active compound(s) was not investigated.

*S. aloides* is a dioecious, perennial aquatic macrophyte, native to Europe and Siberia (Cook & Urmi-König, 1983). An important part of its life cycle is submerged (Bloemendaal & Roelofs 1988) and the plants overwinter as turions or entire (but rootless) plants on the lake bottom. The macrophyte becomes buoyant in spring as a result of increased photosynthesis and remains floating on the water surface until autumn (De Geus-Kruyt & Segal 1973). Especially during its submerged phase, *S. aloides* may be favoured by the ability to produce allelopathic compounds that may inhibit phytoplankton growth.

Other mechanisms for the exclusion of phytoplankton in the proximity of *S. aloides* have been proposed. The availability of potassium or sodium might be limiting for phytoplankton growth in *S. aloides* stands and further, co-precipitation of phosphates with calcium might be an important mechanism (Brammer 1979; Brammer & Wetzel 1984).

Other authors, however, indicated allelopathic activity of *S. aloides*. Extracts of the macrophyte showed inhibitory effects even at the lowest concentration tested (2 g FM/L, Jasser 1995). Usenko et al. (2002) showed that phenolic acids might also be responsible for algicidal effects of *S. aloides*.

Both studies, however, did not prove that *S. aloides* excreted the allelopathic compounds, an important issue to assess the ecological importance of allelopathic interactions (see criteria Willis, Chapter 1, page 17). Recent laboratory experiments showed that exudates of *S. aloides* inhibited the green alga *Scenedesmus obliquus* and induced colony formation in this alga (Mulderij et al. 2005a, Chapter 4). Further, allelopathic effects of *S. aloides* exudates on the cyanobacterium *Microcystis aeruginosa* and the eustigmatophyte *Nannochloropsis limnetica* were observed (Mulderij et al. 2005b, Chapter 5).

We performed bioassay-directed extractions and fractionations to elucidate the chemical characteristics of allelopathic substances in extracts and exudates of *Stratiotes*, and we were especially interested whether the active compounds are hydro- or lipophilic. We also tested if the sensitivity of target organisms to *S. aloides* would increase when they were cultured under nutrient limiting conditions, as Reigosa et al. (1999) stated that target organisms under stress (e.g., nutrient limitation) become more susceptible to additional stressors (e.g., allelopathy) than equivalent target organisms not further stressed.

## 9.3 Materials and Methods

### 9.3.1 Macrophyte culture

The *S. aloides* plants originated from a ditch in Tienhoven (52°10'0"N, 5°4'60"E), The Netherlands, and were collected in July 2003. The plants were carefully rinsed with tap water and transferred into 500-L aquaria filled with 10 cm sediment from the same ditch and with copper-free tap water. The macrophytes were grown at a density comparable to moderate densities in the field (ca. 10 g FM/L), and water was renewed fortnightly. The aquaria were illuminated ( $30 \mu\text{mol m}^{-2}\text{s}^{-1}$ ) 16 h per day and the water temperature was 18°C. At the start of an experiment, plants were transferred to sediment-free aquaria filled with macrophyte medium (Mulderij et al. 2005a, Chapter 4, Table 4.1).

### 9.3.2 Target organisms

We used axenic monocultures of twelve phytoplankton species, consisting of cyanobacteria, green algae and an eustigmatophyte (Table 9.1). The cyanobacteria, *Anabaena* sp. and *A. variabilis* P9, were used in agar diffusion assays (ADAs, see Bioassays), while the other cyanobacteria, the green algae and the eustigmatophyte were used in liquid culture assays (LCAs, see Bioassays). All cyanobacteria were cultured in cyanobacteria medium (Jüttner et al. 1983 with modifications as in Gross et al. 1991), while all green algae and *Nannochloropsis* were cultured in modified WC medium (Guillard & Lorentzen 1972, modified by Lürling 1999, Table 9.1).

**Table 9.1** Origin of the phytoplankton species (target organisms) used in the bioassays.

Species	Culture code	Origin	Medium
<b>Cyanobacteria</b>			
<i>Anabaena</i> sp. <sup>A</sup>	PCC 7120	D. Erhard*	Cyanobacteria
<i>Anabaena variabilis</i> P9 <sup>A</sup>	ATCC 29413	D. Erhard*	Cyanobacteria
<i>Microcystis aeruginosa</i> <sup>L</sup>	CYA 43	NIVA	Cyanobacteria
<i>Microcystis aeruginosa</i> <sup>L</sup>	CYA 140 (toxic)	NIVA	Cyanobacteria
<i>Microcystis aeruginosa</i> <sup>L</sup>	V131	E. Kardinaal**	Cyanobacteria
<i>Microcystis aeruginosa</i> <sup>L</sup>	PCC 7820	Pasteur Collection	Cyanobacteria
<i>Synechococcus elongatus</i> <sup>L</sup>	SAG 89.79	D. Erhard*	Cyanobacteria
<b>Green algae</b>			
<i>Chlorella minutissima</i> <sup>L</sup>	211/52	CCAP	Modified WC
<i>Chlamydomonas reinhardtii</i> <sup>L</sup>	CHL 13	NIVA	Modified WC
<i>Scenedesmus obliquus</i> <sup>L</sup>	276/3A	CCAP	Modified WC
<i>Selenastrum capricornutum</i> <sup>L</sup>	CHL 1	NIVA	Modified WC
<b>Eustigmatophyte</b>			
<i>Nannochloropsis limnetica</i> <sup>L</sup>		Krienitz 1998/3	Modified WC

\* From long-term culture Limnological Institute, University of Konstanz.

\*\* Isolate Lake Volkerak, University of Amsterdam

<sup>A</sup> used in a agar diffusion assays

<sup>L</sup> used in a liquid assays

Phosphate- (P) limited cultures of *S. obliquus* and *S. elongatus* and potassium- (K) cultures of *S. elongatus* were established by first centrifuging (6 min., 2300 rpm) aliquots of non-limited cultures twice. After each centrifugation step the supernatant was removed and cells were resuspended in P- or K-limited medium. P-limited medium was created by substituting 50  $\mu\text{M}$   $\text{K}_2\text{PO}_4$  by 1  $\mu\text{M}$   $\text{K}_2\text{PO}_4$  and 40  $\mu\text{M}$   $\text{KCl}$ . K-limited medium was created by substitution of 50  $\mu\text{M}$   $\text{K}_2\text{HPO}_4$  with 1  $\mu\text{M}$   $\text{K}_2\text{HPO}_4$  and 40  $\mu\text{M}$   $\text{Na}_2\text{HPO}_4$  (Alahari & Apte 2004). The P- or K-limited batch cultures (200 mL) were grown for at least 9 days on an orbital shaker (110 rpm) at 22°C, 50  $\mu\text{mol PAR m}^{-2}\text{s}^{-1}$  with a photoperiod of 16 : 8 (L : D).

### 9.3.3 Extraction

*S. aloides* plants were carefully rinsed with tap water, shock-frozen with liquid nitrogen, and immediately lyophilized for 24 hours. This material was homogenized and stored in the dark at room temperature until further use. Later, this plant material was extracted (1 mL solvent per 10 mg plant dry mass, DM) for 2 hours at room temperature under continuous stirring. As solvents we used water, methanol and acetone in different mixtures (50% or 70% [v/v] in water, and 100%). Extracts were filtered (Whatman GF/F,  $\varnothing$  47mm) to remove plant particles, evaporated under vacuum to dryness and resuspended in 50% [v/v] aqueous methanol at a final concentration equivalent to 100 mg extracted dry mass per mL. Extracts were stored at -20°C. Solvent controls were performed using the same procedure without adding plant material.

### 9.3.4 Fractionation of crude extracts

Crude extracts were fractionated using solid phase extraction (SPE). An aliquot of crude extract (20 mg extracted DM) dissolved in 2 mL 50% [v/v] aqueous methanol, was diluted 1 : 25 with water and passed over a preconditioned SPE-C18 cartridge (Varian Bond Elute, 3cc, 2 g sorbens) and the eluate was collected. Then the cartridge was stepwise eluted with each two reservoir volumes (10 mL) of 40, 60, 70, 80, 90 and 100% [v/v] methanol (in water). Those seven fractions were collected separately, evaporated to dryness, and redissolved in 50% [v/v] aqueous methanol for further use in bioassays (ADA, LCA, see Bioassays).

### 9.3.5 Heat stability and polyvinylpyrrolidone (PVPP) test

Crude extracts of *S. aloides* were heated for 5 min. at 95°C. Immediately thereafter the extracts were cooled down in ice, evaporated to dryness, and resuspended in 50% [v/v] aqueous methanol. To test for the presence of phenolic compounds, we conducted a polyvinylpyrrolidone (PVPP, Sigma P6755) test modified after Loomis & Battaile (1966). A suspension of PVPP in water (2 g PVPP/20 mL water)

was added to an aliquot of the crude extract (v/v 1:1) and stored overnight at 4°C. Thereafter, the suspension was centrifuged (5 min., 4000 g) and the supernatant was used in the bioassays.

### 9.3.6 Exudation

Possible allelopathic compounds, exuded into the water by *S. aloides*, were investigated with two approaches: **Laboratory approaches** (*set-ups* 1 & 2): Incubating single or multiple plants in microcosms, and a **field approach** (*set-up* 3): Collecting water from a natural *S. aloides* stand in Lake Naardermeer (nature reserve).

In *set-up* 1 (Single plant cultures) we filled 1.5-L glass vials (Ø 10 cm, 20 cm height) with 1 L macrophyte medium (Mulderij et al. 2005a, Chapter 4, Table 4.1) and one *S. aloides* plant (25 - 30 g FM) from the laboratory culture. Control vials received only medium. All vials were kept at 16°C and approximately 60 µmol PAR m<sup>-2</sup>s<sup>-1</sup> (14 hour photoperiod). After 1, 2 and 5 days, the culture water of three vials (one control and two with *S. aloides*) was used for analyses.

In *set-up* 2 (microcosms), two 50-L aquaria were filled with 30 L synthetic macrophyte medium. One aquarium served as control and was filled with medium, while the other aquarium was filled with medium and eight *S. aloides* plants (ca. 200 g FM) originating from Giethoorn (52°45'0N, 6°5'0E, The Netherlands), collected the week before the start of the experiment. After 1, 2 and 3 days, one litre water of each aquarium was collected for further analysis.

For *set-up* 3, water samples (1 L) were collected in the vicinity of *S. aloides* plants. As a control, two sites without *S. aloides* were sampled: one in the same ditch (*Ditch* 1) just outside the *S. aloides* stand (5 m) and the other in an other ditch (300 m further) in the nature reserve (*Ditch* 2). In ditch 2, *S. aloides* plants were absent during the whole experiment. Samples were collected between 7 April and 14 June 2004.

All samples (*set-up* 1, 2 & 3) were filtered over Whatman GF/F (Ø 47 mm) and then over 0.2 µm cellulose acetate membrane filters (Ø 47 mm, Schleicher & Schuell). Potentially exuded allelopathic compounds were trapped on preconditioned C18-filters (Ø 47 mm, 3M Empore, Phenomenex) and eluted with 100% methanol (*set-up* 1) or with 50%, 90%, and 100% methanol (*set-ups* 2 & 3). All fractions were evaporated to dryness and resuspended in 50% [v/v] aqueous methanol.



### 9.3.7 Bioassays

We conducted two bioassay types under sterile conditions: Agar diffusion assays (ADAs, Flores & Wolk 1986, Gross et al. 1991) and liquid culture assays (LCAs, Schrader et al. 1997, slightly modified by Erhard & Gross *submitted*).

#### 9.3.7.1 Agar diffusion assay (ADA)

Extracts or SPE fractions (0.5 to 3 mg extracted plant DM) were spotted in multiple intervals onto 1% agar plates, so that the diameter never exceeded 5 mm. Subsequently, a second layer containing cyanobacteria medium, target cells and heated 4% agar (final agar strength: 1% ) was added. Target organisms were inoculated at an optical density (OD) of 40 mAU at 530 nm. For one week the agar plates were incubated at 28°C and 80  $\mu\text{mol PAR m}^{-2}\text{s}^{-1}$  (L : D 16 : 8). Exudates were spotted in concentrations equivalent to 40, 120, and 240 mL (*set-up 1*: equal to 1.2, 3.6, and 7.2 g FM) or 600 mL culture water (*set-up 2 & 3*: equal to 3.96 g FM for *set-up 2*). Extracts, exudates and SPE fractions with algicidal activity caused clearing areas in the algal lawn. All ADAs were carried out at least in duplicate.

#### 9.3.7.2 Liquid assay (LCA)

Each of the four rows on a 24-well plate was assigned six different concentrations of A) extract + target cells, B) extract, C) solvent control + target cells, or D) solvent control. Extracts were pipetted into the wells and the solvent was evaporated. Per well, 2 mL culture with target cells (OD<sub>530nm</sub> 40 mAU) in the respective medium (Table 9.1) were added. Plates were incubated for one week at either 21°C and 60  $\text{PAR m}^{-2}\text{s}^{-1}$  L : D 16 : 8 (NIOO-KNAW, Centre of Limnology, The Netherlands) or at 28°C and 60  $\text{PAR m}^{-2}\text{s}^{-1}$  under continuous illumination (University of Konstanz, Germany), depending on the phytoplankton species. All LCAs were conducted in triplicate and the growth of target cells, as percentage relative to the control, was calculated for each extract concentration as follows:

$$\text{Growth [as \% of control]} = \frac{\text{Row A} - \text{Row B}}{\text{Row C} - \text{Row D}} \times 100\%$$

The first LCA (LCA 1) was conducted with four different concentrations of *Striatotes* extract (0, 0.75, 1.5 and 3.75 mg DM/mL) and all target organism. In the second LCA (LCA 2) lower extract concentrations were used (0, 0.5, 1.0 and 1.5 mg DM/mL extract). In this way we determined which extract concentration and which test organisms should be used in following assays. The two most sensitive species of cyanobacteria and green algae were chosen as test organism for the next LCAs.

We conducted two short term LCAs with 3 mg DM/mL *S. aloides* extract, to test if allelopathic effects of *S. aloides* extract become apparent and how long they persist without extra addition of fresh extract. The first short term LCA (LCA 3) with *S. elongatus* and *S. obliquus*, was sampled after 0, 2, 4, 6, 8, 24, and 48 h incubation. In the second short term LCA (LCA 4) with *S. elongatus* only, we sampled after 24, 48, 72 and 216 h incubation.

The next LCA (LCA 5) was conducted with extract concentrations ranging from 0 to 5.5 mg DM/mL at increments of 0.5 mg DM/mL. This enabled the determination of threshold levels of inhibition/stimulation of the growth of *S. elongatus* and *S. obliquus* in the presence of *S. aloides* extract. LCAs 3 & 5 were also performed with nutrient (K or P) limited *S. obliquus* and *S. elongatus* cells.

Two other LCAs were conducted with SPE fractions of the crude extract (LCA SPE) and PVPP treated extract (LCA PVPP) added in four concentrations (0, 1, 3, and 5 mg DM/mL).

### 9.3.8 Statistical analyses

The data of the ADAs (diameter of clearing zones) were analysed with Student's *t*-tests ( $\alpha = 0.05$ , Fowler et al. 1998). The influence of *S. aloides* extracts on the growth of target cells in LCAs ( $OD_{530nm}$  values) was assessed by means of ANOVA ( $\alpha = 0.05$ ). Prior to the ANOVA, all data were tested for normality (Kolmogorov-Smirnov test,  $\alpha = 0.05$ ) and homoscedasticity (Levene's test,  $\alpha = 0.05$ ). The data followed a normal distribution, but sometimes heteroscedasticity was observed. Therefore, log-transformations were carried out. If these transformations did not remove heteroscedasticity, an  $\alpha$ -value of 0.01 was used (Fowler-Walker & Connell 2002). When the ANOVA revealed significant effects, a Tukey test ( $\alpha = 0.05$ ) was applied to group homogeneous means.

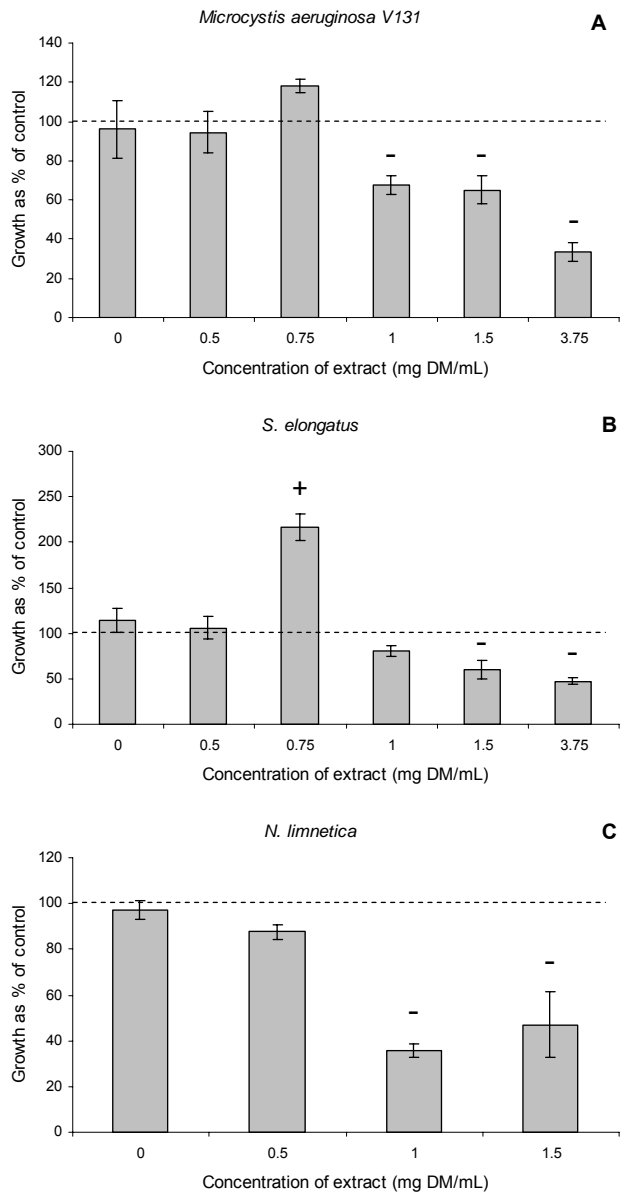
## 9.4 Results

### 9.4.1 Allelopathic activity of *Stratiotes* extracts

ADAs with *Anabaena* sp. and *A. variabilis* showed that *Anabaena* sp. was not significantly inhibited by any of the extracts. *A. variabilis* was much more sensitive and was significantly inhibited by 50% and 70% methanol or acetone extracts. Increasing extract concentrations caused increased larger clearing zones. The strongest inhibition of *A. variabilis* was observed when lyophilized *S. aloides* tissue was extracted with the 50% [v/v] aqueous acetone extract. This solvent was subsequently used for further extractions.

*M. aeruginosa* V131 was the most sensitive strain, exhibiting a significant ( $p < 0.001$ ) growth inhibition in *LCA 1* of 32% already at 1 mg DM/mL (Fig. 9.1a). *S. elongatus* was inhibited (56%) by extract concentrations above 1.5 mg DM/mL (Fig. 9.1b). Other cyanobacteria showed no significant response (*LCA 1*) or even a significant growth stimulation (*LCA 2*), depending on the experiment (Table 9.2). While all tested green algae and the eustigmatophyte *N. limnetica* exhibited no significant inhibition or stimulation in *LCA 1*, we observed an inhibition above 1 mg DM/mL with *N. limnetica* (Fig. 9.1c) and a stimulation of all chlorophytes at 0.5 or 1 mg DM/mL in *LCA 2* (Table 9.2).

Short term *LCA 3* showed a significant ( $p < 0.001$ ) growth stimulation of *S. elongatus* (130%) and *S. obliquus* (282%) after 48 hours incubation in *S. aloides* extract, while short term *LCA 4* exhibited a significant ( $p = 0.002$ ) inhibition of *S. elongatus* only after 216 h ( $45 \pm 2\%$ ).

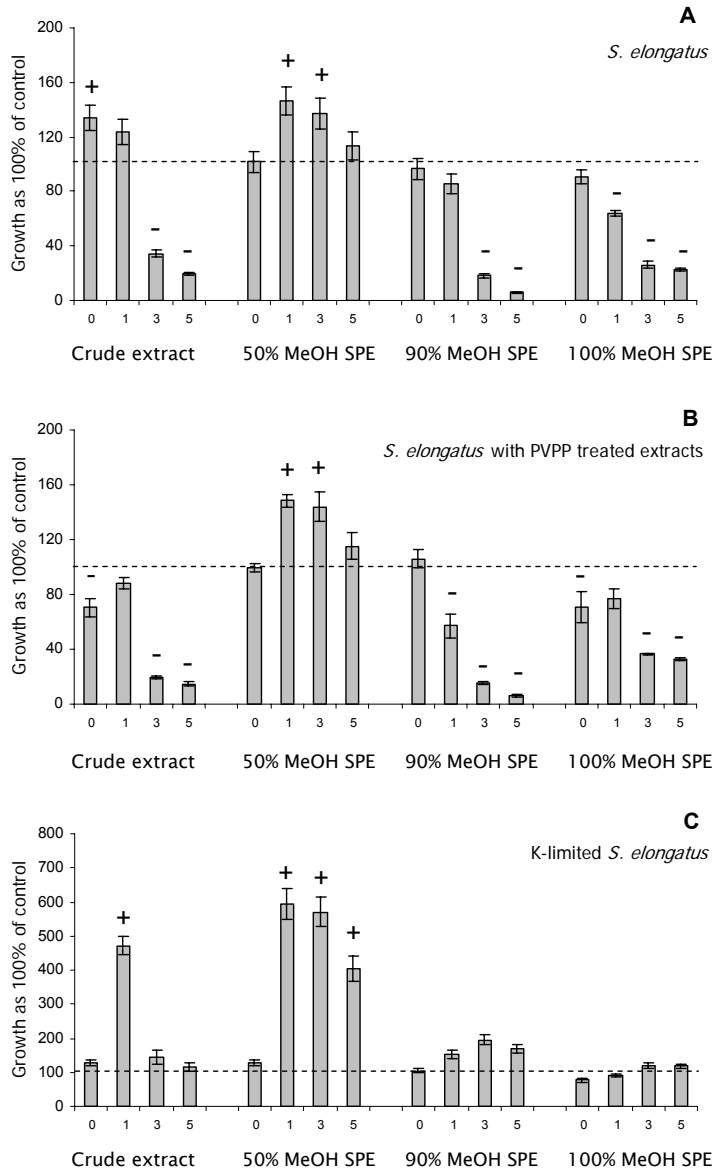


**Figure 9.1** Growth relative to the control of *Microcystis aeruginosa* (A), *Synechococcus elongatus* (B), and *Nannochloropsis limnetica* (C) at different concentrations of *S. aloides* extract in the first two liquid assays. Bars indicate average values ( $n = 3$ ) with error bars (1 SE). Significant inhibitory (-) and stimulatory (+) effects on the growth of the phytoplankton species are indicated.

**Table 9.2** Results statistical analyses of *LCA 1* & *LCA 2*. Effects of extract addition were either stimulatory (+), inhibitory (–) or not clear (0) with threshold values indicated in parenthesis. The ANOVA, was carried out on series of 24 values per phytoplankton species (3 replicates, 2 treatments: control and *S. aloides*, and 4 extract concentrations: 0, 0.75, 1.5 and 3.75 mg DM/mL (*LCA 1*), or 0, 0.5, 1.0 and 1.5 mg DM/mL (*LCA 2*).

	LCA 1		LCA 2	
	0 – 3.75 mg DM/mL		0 – 1.5 mg DM/mL	
	p-value	Effect	p-value	Effect
<b>Green algae</b>				
<i>C. minutissima</i>	0.603	0	< 0.001	+ (0.5)
<i>C. reinhardtii</i>	0.402	0	< 0.001	+ (0.5)
<i>S. obliquus</i>	0.057	0	< 0.001	+ (0.5)
<i>S. capricornutum</i>	0.635	0	< 0.001	+ (1.0)
<b>Eustigmatophyte</b>				
<i>N. limnetica</i>	0.222	–	< 0.001	– (1.0)
<b>Cyanobacteria</b>				
<i>M. aeruginosa</i> CYA 43	0.044*	–	< 0.001	+ (0.5)
<i>M. aeruginosa</i> CYA 140	0.317	0	< 0.001	+ (0.5)
<i>M. aeruginosa</i> V131	0.056	–	< 0.001	– (1.0)
<i>M. aeruginosa</i> PCC 7820	0.230	–	< 0.001	+ (0.5)
<i>S. elongatus</i>	0.033	– (1.5)	0.100	– (1.5)

\*criterion for significance was 0.01, see *Statistical analysis*.



**Figure 9.2** Growth relative to the control in non-limited (**A**, **B**) and K-limited cultures (**C**) of *Synechococcus elongatus* when exposed to different concentrations (0, 1, 3 and 5 mg DM/mL) of *S. aloides* extract (crude extract) or SPE fractions (50%-, 90%-, and 100% methanol) either treated (**B**) or not treated (**A**, **C**) with poly-vinylpyrrolidone (PVPP). Bars indicate average values ( $n = 3$ ) with error bars (1 SE). Significant inhibitory (-) and stimulatory (+) effects on the growth of the phytoplankton species are indicated.

### 9.4.2 Fractionation of crude extracts

The ADA with *A. variabilis* and SPE fractions of crude extract, showed significant inhibitory effects of the 80% and 90% methanol SPE fractions (Table 9.3). The LCA with crude extract and its SPE fractions showed significant ( $p < 0.001$ ) effects on the growth of *S. elongatus* (Fig. 9.2a). Crude extract and the 90% and 100% methanol SPE fractions showed significant inhibitory effects at concentrations of 3 and 5 mg DM/mL, and the 100% fraction also already at 1 mg DM/mL, between 36 and 94% (Fig. 9.2a). The 50% SPE fraction, on the contrary, showed significant stimulatory effects at concentrations of 1 and 3 mg DM/mL (Fig. 9.2a).

**Table 9.3** Algicidal activity of SPE-fractions of crude *Striatotestes* extract (50% acetone) on *A. variabilis*. The aliquots were applied in two concentrations in the agar diffusion assay.

Fraction	Clearing area diameter (cm)	
	2 mg DM	3 mg DM
Flow through	–	–
40% Methanol	–	–
60% Methanol	(+) <sup>ns</sup>	(+) <sup>ns</sup>
70% Methanol	–	–
80% Methanol	(0.7)*	(0.8)*
90% Methanol	0.8*	1.1*
100% Methanol	(+) <sup>ns</sup>	(+) <sup>ns</sup>

( ) inhibition less strong. Algal cells not completely absent in the clearing areas, only lower densities observed.

<sup>ns</sup> not significantly different from control.

\* significantly different from control.

### 9.4.3 Heat stability and polyvinylpolypyrrolidone (PVPP) test

The ADAs showed that *S. aloides* extracts remained allelopathically active after heating. The clearing zone of untreated or heat-treated extracts did not differ significantly. Further, three ADAs showed that PVPP treatment of *S. aloides* crude extract did not or only slightly remove inhibitory effects. PVPP treatment neither removed the inhibitory activity of the extract in an LCA (LCA PVPP) with *S. elongatus* and of *S. aloides* extract (Fig. 9.2a vs 9.2b).

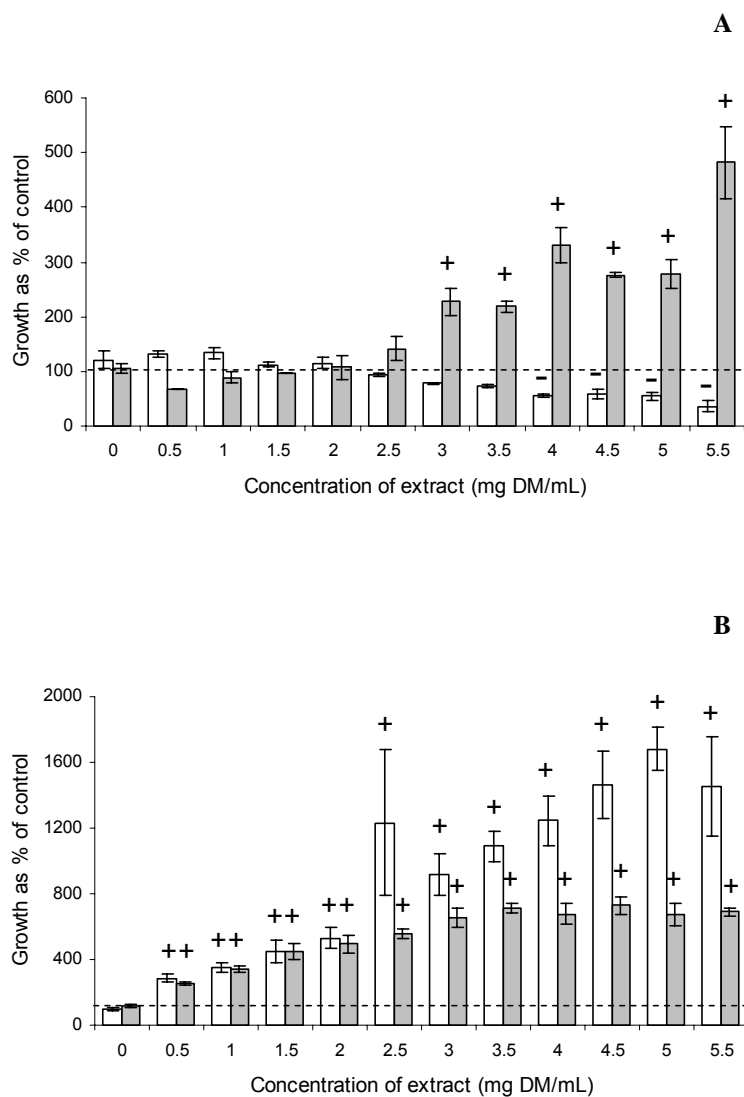
#### 9.4.4 *Phosphorus and potassium limitation of S. elongatus and S. obliquus*

The short term LCA 4 showed significant stimulatory effects of *S. aloides* extract on P-limited cultures of *S. elongatus* and *S. obliquus*. The stimulatory effects on P-limited cultures were stronger (130 vs 414% for *S. elongatus*; 282 vs 844% for *S. obliquus*) and occurred earlier (after 24 instead of 48 h) than on non-limited cultures.

LCA 5 with *S. elongatus* showed significant ( $p < 0.001$ ) stimulatory effects on P-limited cultures above extract concentrations of 2.5 mg DM/mL (Fig. 9.3a), while non-limited cultures were significantly ( $p < 0.001$ ) inhibited ( $> 45\%$ ) by concentrations above 4.0 mg DM/mL (Fig. 9.3a). P-limited and non-limited *S. obliquus* cultures, were significantly ( $p < 0.001$ ) stimulated (up to 1600%) by the presence of *S. aloides* extract at concentrations above 0.5 mg DM/mL (Fig. 9.3b), with the most pronounced effects for non-limited cultures (Fig. 9.3b).

K-limited *S. elongatus* cultures were less strongly inhibited than non-limited ones. Whereas the non-limited *S. elongatus* cultures were significantly ( $p < 0.001$ ) inhibited by crude extract and the 90% and 100% SPE fraction (Fig. 9.2a), the K-limited cultures showed no significant inhibitory effects at all (Fig. 9.2c). The 50% SPE fraction exhibited a significant growth stimulation on both non-limited and K-limited cultures of *S. elongatus*.





**Figure 9.3** Growth relative to the control in non-limited (white bars) and P-limited (green bars) cultures of *Synechococcus elongatus* (A) and *Scenedesmus obliquus* (B) over a range of *S. aloides* extract concentrations. Bars indicate average values ( $n = 3$ ), with error bars (1 SE). + and - indicate significant inhibitory and stimulatory effects on the growth of the phytoplankton species.

### 9.4.5 Exudation

The ADA with *A. variabilis* showed significant clearing zones (> 6 mm) for SPE-enriched medium equivalent to 6 - 7 g FM plants (240 mL medium) after 5 days incubation (*set-up* 1). Another ADA (*set-up* 2) showed inhibitory effects of SPE-enriched medium equivalent to 4 - 5 g FM plants (600 mL medium) after one day incubation, but some controls also showed slight clearing areas.

ADAs with exudates from Lake Naardermeer (*set-up* 3) also showed a strong inhibition of *A. variabilis* (Table 9.4), with the strongest inhibitory effect found at 20 April (when *S. aloides* plants were still submerged). Controls, however, also showed inhibitory effects, but generally less strong than the exudates.

**Table 9.4** Results agar diffusion assay with *A. variabilis* and *S. aloides* exudates from three sites in ditches near Lake Naardermeer (C18 fractions). SPE-enriched aliquot equivalent to 600 mL water from the ditch.

Site description	Methanol fraction	Clearing area diameter (cm)				
		7 April*	20 April*	7 May	21 May	14 June
Inside <i>S. aloides</i> (Ditch 1)	50%		> 3.0	–	0.5	(1)
	90%		> 2.5	1.0	(0.7)	0.9
	100%		0.8	–	1.5	–
Outside <i>S. aloides</i> (Control, Ditch 1)	50%		1.5	–	1.0	(0.5)
	90%		2.2	(+)	0.8	(0.5)
	100%		1	(+)	–	1
No <i>S. aloides</i> (Control, Ditch 2)	50%		2.4	(+)	–	–
	90%	> 2.0	2.1	(+)	1.4	0.9
	100%		0.8	–	0.5	–

\* plants were still submerged

## 9.5 Discussion

In general, our results showed both stimulatory and inhibitory effects of *S. aloides* extract and exudates on phytoplankton growth, with more cyanobacteria than green algae being sensitive to allelopathic substances of *S. aloides*. Jasser (1994) and Körner & Nicklisch (2002) also showed similar results, but Mulderij et al. (2005b, Chapter 5) observed no significant differences in the sensitivity of cyanobacteria and green algae to allelopathic exudates from *S. aloides*.

Similar to Fitzgerald (1969), P-limitation of *S. elongatus* did not increase the sensitivity of this cyanobacterium to *S. aloides* extracts, while P-limited *S. obliquus* cultures showed opposite effects, corresponding with the hypothesis that nutrient stressed organisms are more susceptible to allelopathy (Reigosa et al. 1999). K-limitation of *S. elongatus* did not intensify its sensitivity to *S. aloides* extract. The stimulatory effects on K-limited target cells may have occurred because the extract acted as K-source. The extract might also have served as a P-source, because we observed both stimulatory and inhibitory effects of P-limited target cells upon extract addition. Organic phosphorus compounds might bind to C18 and may have been present in the 50% fraction, causing a stimulation of phytoplankton growth, while inhibitory compounds present in the 90 - 100% fractions resulted in adverse effects on phytoplankton growth. Brammer (1979) and Brammer & Wetzel (1984) stated that the absence of phytoplankton in the presence of *S. aloides* is caused by nutrient limitation rather than allelopathy. Recent measurements *in situ*, did not reveal differences in the nutrient content between *S. aloides* stands and *S. aloides*-free ditch water (Mulderij et al. *submitted*, Chapter 6). Both extract and exudates exhibited allelopathic activity in our assays. Since we observed both stimulatory and inhibitory effects in P- or K-limited cultures, we argue against the hypothesis of Reigosa et al. (1999) that allelopathy acts more severely on already otherwise stressed target cells.

ADAs with *S. aloides* exudates from laboratory cultures showed inhibitory effects after an incubation period of at least 3 days. Furthermore, exudates from a natural *S. aloides* stand in Lake Naardermeer showed inhibitory effects, but some controls also exhibited a slight activity which was, however, in general less active than the exudates. Inhibitory effects were strongest when *S. aloides* was still submerged, indicating that the plants might start excreting allelopathic compounds at an early life-stage. This would be an effective strategy, because early in spring the competition with other photoautotrophs is probably strongest. There are more indica-

tions for higher allelopathic activity in younger *S. aloides* plants than in older ones (Mulderij et al. 2005a, Chapter 4).

Compared to other studies (e.g., Gross et al. 1996, Gross et al. 2003b) and based on the biomass of *S. aloides* needed in both extract and exudate to achieve inhibition, the inhibitory effects of *S. aloides* shown in our study, are relatively weak. Mulderij et al. (2005a, 2005b, Chapters 4 & 5), on the contrary, observed inhibitory effects of *S. aloides* exudates on phytoplankton growth ranging between 8 and 51%.

Moreover, a comparison of the observations of Mulderij et al. (2005b) and this study showed that two strains of the cyanobacterium, *Microcystis aeruginosa* (NIVA-CYA 43 and NIVA-CYA 140), the eustigmatophyte, *Nannochloropsis limnetica*, and the green alga, *Scenedesmus obliquus*, were significantly inhibited by exudates of *S. aloides* (Mulderij et al. 2005b), while only *Nannochloropsis* was inhibited by *S. aloides* extracts (this study). The other phytoplankton species showed no effect or their growth was stimulated. The *S. aloides* extract may perhaps contain substances that stimulated the growth of phytoplankton, thereby masking the possible inhibitory effects of allelopathic compounds present in the extract. This indicates that the allelopathic activity of plant extracts does not always reflect the potential activity of the same substances once they are excreted.

The allelopathic activity of macrophytes further depends on the chemical nature of allelopathic substance(s) and on the rate at which they are produced and excreted. As a consequence, experiments with exudates are ecologically more relevant. Our first experiment with *S. aloides* exudates showed significant inhibitory effects of SPE-enriched *S. aloides* medium on the cyanobacterium *A. variabilis*, indicating that *S. aloides* may excrete the allelopathic substances. However, exudation experiments overall did not yet show a clear effect and differed from previous experiments using macrophyte culture filtrates (Mulderij et al. 2005a, 2005b, Chapter 4 & 5). This might be due to an incomplete binding on the C18 filters used, or because the active compounds are otherwise lost during the SPE process. The inhibitory effects found in field exudates of macrophyte-free control stations might be caused by humic compounds, which are also known to have allelopathic properties (Serrano, 1992; Mulderij et al., *in prep.*).

Knowing the chemical nature of allelopathically active compounds, facilitates studies of plant content, exudation and mode of action. Bioassays with PVPP treated extracts showed that the active substance(s) in *S. aloides* are most likely not of phenolic nature. Smolders et al. (2000) showed that the phenolic content of *S. aloides* leaves is relatively low compared to other macrophytes. Our results contradict those of Usenko et al. (2002) who proposed that the algicidal activity in *S. aloides* is based on phenolic acids. Further characterisation of the allelopathic compounds in *S. aloides* indicated that the allelopathic substance is heat-stable and moderately lipophilic.

## 9.6 Conclusion

Our assays showed stimulatory and inhibitory effects of *S. aloides* extract and exudates on phytoplankton, where cyanobacteria were more sensitive than other phytoplankton species. This differential sensitivity of phytoplankton species may affect the biomass and composition of phytoplankton populations under natural conditions. Nutrient limitation of phytoplankton cells did not increase their sensitivity to allelopathic extracts of *S. aloides*. The substance(s) responsible for allelopathic inhibition of phytoplankton are moderately lipophilic and most likely not phenolic compounds.

## Acknowledgements

We are grateful to Claudia Feldbaum for her assistance in the laboratory. Parts of this study were supported by the Schure-Beijerink-Popping fund (SBP-2001-29).



# Discussion



Street in the village Kortenhoef, The Netherlands, named after the submerged aquatic macrophyte *Stratiotes aloides* L. (Krabbescheer in Dutch).

Picture: G. Mulderij





# 10

Summarizing discussion

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## Summarizing discussion

### 10.1 Introduction

One of the referees of our papers started his/her comments with the following: *“Working with allelopathy is a real challenge. While studies of competition for resources generally are reviewed much more uncritically, any study concerning allelopathy is confronted with the postulation to exclude alternative explanations such as competition for resources or apparent competition.”* Harper (1977), for instance, criticized that most experiments that ‘show’ allelopathy actually do not show it, because alternative explanations were often not excluded. After four years of research, I agree with the statement of our referee that allelopathy remains a challenging field of research....

It is still hard to convince scientists that allelopathic interactions do occur. The experiments described in this thesis may help to change this indecisive viewpoint on the existence of allelopathic interactions between freshwater macrophytes and their competitors for light and nutrients: phytoplankton. Aquatic macrophytes in shallow lakes have a positive effect on the water transparency via several processes (Chapters 1 & 2). This thesis focussed on allelopathic effects of aquatic macrophytes on several phytoplankton species occurring in freshwater habitats. The experiments demonstrated allelopathic inhibitory effects of two macrophytes (*Chara* sp. and *Stratiotes aloides*) on phytoplankton. Further, differential sensitivity was observed between functional phytoplankton groups, but also within these groups. Nutrient limitation of target organisms did not often enhance the sensitivity of target cells to allelopathic substances. Other factors, important for the allelopathic inhibition of phytoplankton were the toxicity of the target species, the light intensity at which target species were cultured, and the age of the donors (macrophytes).

### 10.2 Allelopathic activity of charophytes

With respect to allelopathy, charophytes are among the most studied aquatic macrophytes. Steeman-Nielsen (1973) considered allelopathy to be the cause of an extremely low phytoplankton abundance in brackish water with extensive *Chara* beds. Further, relatively low periphyton densities on *Chara globularis* may indicate allelopathic activity of charophytes on periphyton communities (Howard-Williams 1978), although Blindow (1987), on the contrary, observed relatively high periphy-

ton densities on charophytes. Moreover, Crawford (1977, 1979) observed low phytoplankton densities in ponds with a dense *Chara vulgaris* vegetation. Chemical substances originating from *Chara* were shown to inhibit the photosynthesis of natural phytoplankton populations and of an epiphytic diatom in the laboratory (Anthoni et al. 1980, Wium-Andersen et al. 1982, Wium-Andersen et al. 1987). More recent studies on the allelopathic activity of charophytes are from Berger & Schagerl (2003, 2004), who worked with extracts of different charophyte species.

There is, however, still much debate about the relevance of allelopathic interactions between charophytes and phytoplankton under natural conditions. Forsberg et al. (1990), for instance, observed results that do not support the hypothesis of allelopathic interactions between *Chara* and phytoplankton, and therefore concluded that *in situ* allelopathic effects of *Chara* were absent. Blindow & Hootsmans (1991) found about 10% phytoplankton growth inhibition if exudates of *Chara globularis* were present. Chapter 3 of this thesis describes a recent study with charophyte exudates and demonstrates the allelopathic effect of a mixed culture of *Chara globularis* and *C. contraria* on the growth of the green algae, *Scenedesmus*, *Selenastrum* and *Chlorella*. Filtered water from this charophyte culture inhibited the growth of *Selenastrum* and *Chlorella*, but not that of *Scenedesmus*. This inhibitory effect was in the same order of magnitude as shown for other charophytes (Blindow & Hootsmans 1991). Similarly, this differential sensitivity of phytoplankton may influence the composition and biomass of phytoplankton communities *in situ*.

### 10.3 Allelopathic activity of *Stratiotes aloides*

Studies on the allelopathic growth inhibition of phytoplankton by the macrophyte *S. aloides* are scarce. Brammer (1979) reported the exclusion of phytoplankton in the proximity of *S. aloides*, but nutrient competition, rather than allelopathy, explained the lower phytoplankton densities in the presence of *S. aloides* (Brammer 1979, Brammer & Wetzel 1984). *S. aloides* extracts in dialysis bags were also shown to inhibit the growth of phytoplankton (Jasser 1995). This inhibitory effect increased with increasing extract concentrations. Usenko et al. (2002) demonstrated that *Stratiotes* tissue contained phenolic acids with algicidal effects.

However, the presence of allelopathic substances in the tissue of macrophytes does not prove the excretion of these substances into the surrounding water, where they can subsequently have their allelopathic effects on phytoplankton growth and development. Therefore, the laboratory experiments described in this thesis were conducted with macrophyte exudates. The growth of the green alga *Scenedesmus obliquus* was inhibited by the presence of *S. aloides* exudates (Chapter 4), whereas similar experiments with charophyte exudates did not show growth inhibition of *Scenedesmus* (Chapter 3). Moreover, *Scenedesmus* started to form colonies of 2 to 8 cells when the alga was exposed to *S. aloides* exudates (Chapter 4). Colonies of *Scenedesmus* may have higher sinking rates than single cells and, therefore, disappear faster from the upper water layers, reducing the competition for light and nutrients between phytoplankton and aquatic macrophytes.

The sensitivity of cyanobacteria to exudates from *S. aloides* was not higher than the sensitivity of other phytoplankton species (Chapter 5), but the study described in Chapter 9 showed that more cyanobacteria than green algae were sensitive to *Stratiotes* extract. Other studies (e.g., Jasser 1995, Körner & Nicklisch 2002) also demonstrated a higher sensitivity of cyanobacteria than other phytoplankton species.

#### 10.4 Factors influencing the extent of the allelopathic

This thesis also describes studies investigating if allelopathy was more effective under stress conditions (Reigosa et al. 1999). I conducted experiments with phytoplankton species that were cultured under sub-optimal nutrient or light conditions (Chapters 3, 5 & 9).

I conducted laboratory experiments with P-limited *Chlorella*, but observed that P-limitation did not cause a higher sensitivity of the green alga to allelopathic substances from *Chara* (Chapter 3). These observations correspond with the findings of Fitzgerald (1969), but are in contradiction with the hypothesis of Reigosa et al. (1999) that nutrient limited target species may be more sensitive to allelopathic substances than non-limited ones.

Nutrient (P and K) limited phytoplankton species were also exposed to extracts of *S. aloides* (Chapter 9). These experiments indicated that P-limitation of phytoplankton had differential effects on the green alga *Scenedesmus obliquus* and the cyanobacterium *Synechococcus elongatus*. Both P-sufficient and P-limited cultures of *S. obliquus* were stimulated in their growth in the presence of *S. aloides* extract, espe-

cially in non-limited cultures. The growth of P-limited *S. elongatus* cultures was stimulated in the presence of *S. aloides* extract, but inhibited in non-limited cultures. Similar to P-limited *S. elongatus*, K-limited cultures of *S. elongatus* were stimulated in the presence of *S. aloides* extract and also these observations were in contradiction with the hypothesis of Reigosa et al. (1999).

Also light influenced the response of *S. obliquus* to allelopathic exudates from *S. aloides* (Chapter 5). The inhibitory effect was stronger when *Scenedesmus* was cultured at a lower light intensity, what may indicate that light limited *Scenedesmus* cells become more sensitive to exudates from *S. aloides*. The eustimatophyte *Nannochloropsis limnetica*, and the cyanobacterium, *Microcystis aeruginosa*, were also inhibited by *S. aloides* exudates, but this effect was not further influenced by the light intensity.

Furthermore, these experiments showed that the allelopathic inhibition was stronger for the toxic than for the non-toxic *Microcystis* strain (Chapter 5). The toxic cyanobacterium may have needed its energy to produce toxins and could therefore cope less well with the adverse effects of allelopathic substances from *S. aloides*.

This thesis further described that the age of the macrophytes may also play a role. Charophytes (Chapter 3) and *S. aloides* (Chapter 4) seem to be more active when they are younger. Chapter 4, also describes that younger plants influenced the lag phase of phytoplankton, whereas older plants affected the phytoplankton growth rate.

## 10.5 Field observations and experiments

The allelopathic effects of *S. aloides* exudates on phytoplankton described in Chapters 4 & 5 were observations from laboratory experiments. To gain more insight into the role of *S. aloides* in natural ecosystems, and to understand the ecological importance of allelopathic effects I performed field experiments that focussed on the effect of macrophyte exudates on whole phytoplankton communities in natural ecosystems. The results from these experiments are described in Chapters 6 & 7.

Chapter 6 presents the results from an experiment with natural phytoplankton exposed to exudates of *S. aloides*, a field survey of five Dutch *S. aloides* stands and a field incubation of natural phytoplankton with *S. aloides* exudates from a lake in

the Romanian Danube Delta. A significantly lower water turbidity in treatments with *Stratiotes* exudates than in the controls was observed in the first experiment. The field survey showed that nutrient limitation is unlikely to be the explanation for low phytoplankton densities close to *S. aloides* as the nutrient concentrations were higher in the vicinity of *S. aloides* than further away from the plants. The field incubation also indicated allelopathic activity of *S. aloides* against phytoplankton, because significantly lower chlorophyll concentrations were observed in the incubations with *S. aloides* exudates than in the controls.

Field observations (Mulderij & Smolders, *unpublished data*) moreover suggested that not only phytoplankton, but also filamentous algae are influenced by the presence of *Stratiotes* exudates. Therefore, we conducted a second field survey and two field experiments with filamentous algae, natural, and artificial *S. aloides* plants in Lake Naardermeer (Chapter 7). The field survey showed that concentrations of K and o-PO<sub>4</sub> were significantly lower in the presence of *S. aloides*, and this probably explained the lower *in situ* abundances of filamentous algae close to *S. aloides* (Brammer 1979, Brammer & Wetzel 1984). The concentrations of K and o-PO<sub>4</sub>, however, were still too high to assume growth limitation of filamentous algae. The artificial plants in the field experiment became completely overgrown with filamentous algae toward the end of the entire experiment, whereas the natural *S. aloides* plants remained free of filamentous algae. Alternative explanations for the observation of gaps in covers of filamentous algae close to *S. aloides*, like light limitation, zooplankton and macrofauna grazing or physical factors appeared to be less likely than allelopathy. *S. aloides* and filamentous algae were exposed to similar light intensities as they both occurred on the water surface, for most of the experiment. Nutrient concentrations were not limiting for filamentous algae and grazing of zooplankton on filamentous algae was assumed to be negligible (Fulton 1988). The number of macrofauna species associated with natural and artificial *S. aloides* plants was equal (Higler 1977). The physical presence of artificial *S. aloides* plants was shown to have no significant effect on the growth and development of filamentous algae. Allelopathy seemed the most likely explanation for the observations in these experiments.

## 10.6 Allelopathy model

In Chapters 3 - 5 I have noted that *Chara* sp. and *S. aloides* had allelopathic effects on phytoplankton growth under laboratory conditions. Chapters 6 & 7 deal with such allelopathic effects under natural conditions. It is, however, difficult to perform good field experiments, because of the difficulty of having good controls. A theoretical model of our system would help to understand processes occurring in nature. Most models on aquatic allelopathy, however, are either too theoretical or only consider allelopathic interactions among phytoplankton species (Mukhopadhyay et al. 1998, 2003, Tapaswi et al. 1999). To study the importance of allelopathic effects of macrophytes on phytoplankton under natural conditions we developed a model (Chapter 8) that describes phytoplankton growth under the influence of the single and combined effects of shading, sediment resuspension, competition for nutrients, and allelopathy of macrophytes on the development of phytoplankton. The model showed that the most important process for *Chara* in promoting clear water seems to be the reduction of resuspension, whereas shading and allelopathy seem to be the most important processes for *S. aloides*. Our model, therefore, suggests that the relative contribution of allelopathy *in situ* is low or negligible for aquatic macrophytes like charophytes, but for species, such as *S. aloides*, it may be a significant process.

## 10.7 Chemical identification of *Stratiotes* extract

Our knowledge about the chemical characteristics of allelopathic substances is limited. For charophytes there are several indications that the allelopathic substances are sulphur compounds (e.g. di- and trithiane, Anthoni et al. 1980, Wium-Andersen et al. 1982). For *S. aloides*, Usenko et al. (2002) identified allelopathic compounds that were probably phenolic acids. Smolders et al. (2000) studied the phenolic content in leaves of aquatic macrophytes, but with a focus on such compounds as defences against pathogens and herbivores. These compounds could also have allelopathic properties. Smolders et al. (2000), however, showed that the phenolic content in leaves of *S. aloides* was relatively low compared with that in leaves of other macrophyte species. We determined the nature of allelopathic substances from *S. aloides* in a series of bioassays using extracts and exudates of the macrophytes (Chapter 9). The allelopathic compounds in *S. aloides* appeared to be moderately lipophilic and they are probably not phenolic.



Compared with similar studies on extracts of other macrophytes (e.g. Gross et al. 2003b) the allelopathic effect of *S. aloides* extracts, found in our experiments, was relatively weak. Mulderij et al. (2005b, Chapter 5), on the contrary, showed strong effects (up to 80%) of *S. aloides* exudates on the growth of green algae and cyanobacteria. This discrepancy between the effects of extract and exudates indicates the importance of the use of macrophyte exudates rather than extracts. Substances present in plant extracts can be allelopathically active, but they should also be excreted to have their effects on target organisms (e.g. Wium-Anderson 1987). Indications of allelopathic effects with exudates give stronger evidence for the presence of allelopathy than experiments with extracts (see criteria Willis, Chapter 1, page 17).

## 10.8 Concluding remarks

The main question in this thesis is if allelopathic effects of *Chara* spp. and *S. aloides* on phytoplankton growth exist. Following the six criteria of Willis (1985, Chapter 1, page 17) several patterns of inhibition of phytoplankton species by aquatic macrophytes were demonstrated using cell-free filtrates (Chapters 3 - 5). This suggests that allelopathic substances were released into the environment and (possibly) taken up by the target organisms (Criteria 3 - 5, Willis 1985). The observations could not exclusively be explained by processes other than allelopathy (Willis 1985, criterium 6). We should, however, focus more on the importance of allelopathy *in situ* and show that the criteria of Willis can also be fulfilled in field experiments. The field experiment described in Chapters 6 & 7 indicated the presence of allelopathy under natural conditions.

Although allelopathy has been subject of debate and scientific research for many years, some scientists still doubt the existence of allelopathy in nature. This thesis takes away some of this skepticism by providing information on the existence of allelopathy and its importance in natural ecosystems. There is an ongoing debate on resource competition versus allelopathy (e.g., Inderjit & Del Moral 1997). With help of works described in this thesis I would like to shift the emphasis of the discussion from the *existence* of allelopathy to its *importance* and to its comparison with other processes occurring in shallow lakes. Allelopathy may not be the steering factor in structuring or the functioning of shallow lake ecosystems, but it certainly is a factor in controlling the density and structure of phytoplankton communities. Allelopathy may have direct consequences for phytoplankton, but possibly

also indirect effects on the higher trophic levels of the aquatic food web (e.g. zooplankton), but more studies on this topic are needed.

## 10.9 Recommendations for future research

The experiments presented in this thesis add to our knowledge about allelopathic effects of macrophytes on phytoplankton in aquatic freshwater ecosystems, but proving allelopathy in an ecological meaningful way is not easy (Gross et al. *submitted*). More experiments, however, are needed to prove the general existence and importance of allelopathy in these ecosystems under different environmental and geographical conditions.

Follow-up studies should be designed to have a main focus on allelopathic effects under natural conditions. They could, for instance, be conducted with target and donor species that co-occur naturally. Moreover, the relative abundances of those species should be realistic and the use of crude extracts should be avoided or carried out simultaneously with use of exudates/cell-free filtrates.

Another aspect that needs more attention is the effect of macrophyte age and seasonality on the allelopathic activity of macrophytes (Chapters 3 & 4) and on the chemical identification of allelopathic compounds. Once the chemical nature of allelopathic substance(s) is identified, experiments elucidating in which life-stage and what part of the plant and in what season macrophytes produce allelopathic substances could be conducted.

Further, the relationship between the density of target organisms and the production of allelopathic substances by the donor could be investigated (Is this production inducible?). Identification of the chemical nature of allelopathic substances will possibly also lead to more knowledge about the concentration, dilution/degradation and mode of action of allelopathic compounds.

Simultaneously, new experiments should focus on the (indirect) effects of allelopathy on higher trophic levels in the aquatic food web, such as zooplankton and their predators. Therefore, upscaling of the present experimental set-ups (used under controlled conditions) would help to study the multitrophic impact of allelopathy in aquatic ecosystems.





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Samenvatting

# Inhoud

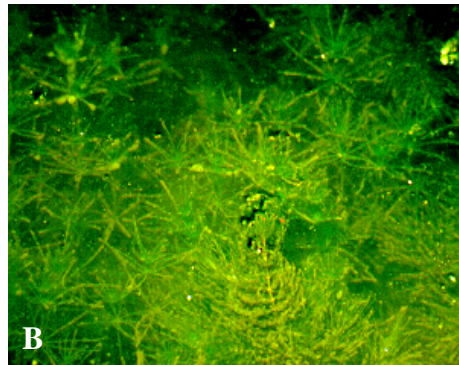
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# Samenvatting

## 1. Inleiding

Dit proefschrift beschrijft mijn promotieonderzoek van de afgelopen vier jaar (sept. 2000 – nov. 2004). Ik deed onderzoek aan chemische interacties tussen waterplanten en algen. Dit proces heet *Allelopathie*. Tijdens mijn onderzoek stonden twee waterplanten centraal: *krabbescheer* en *kranswier* (Fig. 1). In deze samenvatting beschrijf ik waarom en hoe ik dit onderzoek heb uitgevoerd, en wat de resultaten en conclusies zijn.

Tot 1950 was het water in Nederlandse meren vaak helder en groeiden er veel waterplanten (*macrofyten*). Later werden deze meren troebeler door explosieve groei van algen (*fytoplankton*) als gevolg van een toename van de hoeveelheid voedingsstoffen in het water. Dit proces noemen we *Eutrofiëring* en is ontstaan door de intensiever geworden landbouw en industrie en door de bevolkingsgroei. Het gevolg van eutrofiëring is een achteruitgang van de waterkwaliteit. De laatste jaren is er veel onderzoek gedaan naar de oorzaken, gevolgen en oplossingen van/voor eutrofiëring. Hieruit bleek onder andere dat de aanwezigheid van waterplanten van groot belang is voor een goede waterkwaliteit.



**Figuur 1** Krabbescheer (*Stratiotes aloides*, links) en kranswier (*Chara*, rechts).

Foto A: G. Mulderij

Foto B: [www.okstate.edu/artsci/biol3253/boomer/](http://www.okstate.edu/artsci/biol3253/boomer/)

### 1.1 *Waterplanten en waterkwaliteit*

Uit onderzoek is gebleken dat vooral de *ondergedoken waterplanten* (die onder de waterspiegel groeien), van groot belang zijn voor de biologische structuur en de waterkwaliteit van ondiepe meren. Ze hebben vaak een positief effect op de helderheid van het water wat veroorzaakt wordt door verschillende processen (zie Hoofdstuk 2). De meest belangrijke processen zal ik hieronder toelichten, omdat deze van belang zijn voor het begrip van mijn experimenten.

### 1.2 *Voedingsstoffen en licht*

Net als algen, maken waterplanten, voor hun groei, gebruik van zonlicht en voedingsstoffen. Algen kunnen over het algemeen alleen voedingsstoffen opnemen uit het water<sup>1</sup>, terwijl veel waterplanten ook nog voedingsstoffen uit de waterbodem kunnen opnemen (via hun wortels). Dit geeft ze een voordeel ten opzichte van de algen en blauwalgen/cyanobacteriën. De voedingsstoffen opgenomen door waterplanten zijn immers niet meer beschikbaar voor algen. In meren met veel waterplanten kunnen algen dus moeilijker overleven dan in vergelijkbare meren zonder waterplanten omdat er minder voedingsstoffen beschikbaar zijn. Deze competitie om voedsel is belangrijk bij het ontwerpen van experimenten. Om allelopathie aan te tonen moeten we er namelijk voor zorgen dat we competitie om voedsel tussen waterplant en alg uit kunnen sluiten, omdat we anders niet alleen naar het effect van allelopathie kijken.

Waterplanten kunnen ook zorgen voor beschaduwing van algen die zo minder licht krijgen en dus minder goed kunnen groeien. Vooral waterplanten die met hun bladeren op het wateroppervlak drijven (bijv. waterlelies en gele plomp) zijn erg goed in het beschaduwen van de hele waterkolom. Andersom kunnen algen (mits ze in grote getale aanwezig zijn) ook ondergedoken waterplanten beschaduwen, en er zo dus voor zorgen dat ondergedoken waterplanten slechter groeien.

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<sup>1</sup> Sommige blauwalgen/cyanobacteriën kunnen stikstof uit de lucht opnemen. Dit gebeurt via speciale cellen die heterocysten worden genoemd. Deze blauwalgen hebben dus een extra voordeel als er weinig stikstof in het water aanwezig is.



### 1.3 *Stabilisatie van de waterkolom*

Waterplanten zorgen verder voor een stabielere waterkolom, want het water binnen velden met waterplanten is minder in beweging dan het open water. Hierdoor zakken deeltjes die in het water zweven (bodemdeeltjes opgewerveld van de waterbodem, maar ook algencellen, afgestorven plantendelen, enz.) makkelijker en sneller uit naar de waterbodem en worden daar ook minder snel weer opgewerveld. Door dit proces van versneld uitzinken (*sedimenteren*) en de verminderde opwerveling (*resuspensie*) wordt het water helderder omdat er minder deeltjes in rond zweven.

### 1.4 *Allelopathie*

Een ander proces waarmee waterplanten kunnen zorgen voor helder water is *Allelopathie*. Waterplanten kunnen chemische stoffen in het water uitscheiden die de groei van fytoplankton kunnen remmen. Dit proces bestudeerde ik tijdens mijn promotieonderzoek. Eén van de definities van *allelopathie* luidt: *het ene organisme heeft een effect op het andere doordat het een stof of een mengsel van stoffen in zijn omgeving uitscheidt* (Hoofdstuk 1). Volgens deze definitie van de Duitse plantenfysioloog Prof. Hans Molisch gaat het hier alleen om chemische interacties tussen planten en dus niet om interacties met dieren die stoffen uitscheiden. Het doel van mijn promotieonderzoek was het aantonen van allelopathische activiteit van de twee eerder genoemde waterplanten (kranswier en krabbescheer) op de groei van fytoplankton.

## 2. **Opzet en uitvoering van het onderzoek**

Om allelopathische interacties tussen waterplanten en algen aan te tonen heb ik eerst laboratoriumexperimenten uitgevoerd. Met de hieruit voortgekomen kennis ontwierp ik veldexperimenten waarmee ik het belang van allelopathie onder natuurlijke omstandigheden onderzocht. Om deze bevindingen te ondersteunen is er ook een wiskundig model voor algengroei in aanwezigheid van waterplanten opgesteld. Tot slot ben ik met behulp van chemische analyses begonnen aan de identificatie van de allelopathische stof(fen) afkomstig van krabbescheer.

## 2.1 *Kranswier en krabbescheer*

*Kranswieren* (Fig. 1) zijn in Nederlandse meren belangrijke waterplanten. Dit komt vooral doordat ze hierin vaak als eerste opkomen en daarna snel kunnen groeien. Studies in het Veluwemeer in Nederland laten het belang van kranswieren zien. Boven kranswiervelden is het water vaak helderder dan in het open water. Wanneer de hoeveelheid kranswieren toeneemt, verdwijnt een groot deel van het fytoplankton. Onderzoekers concludeerden dat het ecologisch herstel van ondiepe meren samenhangt met de aanwezigheid van kranswieren. Voor deze waterplant zijn er reeds aanwijzingen voor *allelopathische activiteit* (de mogelijkheid om chemische stoffen aan te maken en uit te scheiden om de groei van andere organismen mee te remmen) gevonden. Net als kranswieren kan ook krabbescheer (Fig. 1) in korte tijd snel groeien en het water rond deze plant is vaak ook verbazend helder in vergelijking met water verder van de plant vandaan. Een allelopathische interactie tussen krabbescheer en fytoplankton zou hier een mogelijke verklaring voor kunnen zijn.

## 2.2 *Experimenten en verwachtingen*

Om aan te tonen dat er inderdaad *allelopathische interacties* tussen waterplanten en fytoplankton bestaan, deed ik verschillende laboratoriumexperimenten onder gecontroleerde omstandigheden (Hoofdstukken 3 t/m 5). Eerdere experimenten werden vaak alleen uitgevoerd met extracten<sup>2</sup> van waterplanten, maar het gebruik van extracten laat eigenlijk alleen zien dat er bepaalde stoffen in een plant zitten die een effect kunnen hebben op fytoplankton. Dit is echter geen bewijs dat de plant deze stoffen ook daadwerkelijk uitscheidt. Als waterplanten inderdaad chemische stoffen in het water uitscheiden en daarmee de groei van algen remmen, moeten we die (allelopathische) stoffen in het water terug kunnen vinden. Pas als deze stoffen (*exudaten*) in het water zitten en nog steeds een effect hebben op fytoplankton weten we dat ze een ecologisch belang hebben. Om allelopathie aan te tonen heb ik de groei van algen in water waar waterplanten in hebben gestaan, vergeleken met de groei van algen in water dat niet in contact is geweest met de waterplanten.

Verder wilde ik weten of er een relatie was tussen de leeftijd van de waterplanten en de mate van het *allelopatisch effect* (= groeiremming) op fytoplankton. Uit eerder onderzoek is namelijk gebleken dat jonge waterplanten vaak actiever zijn dan oudere waterplanten, en dit verwachtte ik terug te vinden in mijn proeven.

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<sup>2</sup> Extracten worden gemaakt door delen van een waterplant fijn te maken en met oplosmiddel te behandelen. Zo worden stoffen die normaal in de plant zitten opgenomen in het oplosmiddel. Daarna kan de allelopathische activiteit van deze extracten op fytoplankton (algen) worden getest.

Ook keek ik naar het allelopatische effect van waterplanten op algen en blauwalgen/cyanobacteriën die te weinig voedsel of licht kregen. In de literatuur werd namelijk beschreven dat algen en blauwalgen gevoeliger kunnen worden voor allelopathische stoffen wanneer ze stress ondervinden door niet-optimale groeiomstandigheden (bijv. licht- of voedselgebrek).

Resultaten uit laboratoriumexperimenten zeggen iets, maar niet alles. Om een idee te krijgen van het belang van allelopathie in de natuur, heb ik ook veldexperimenten uitgevoerd (Hoofdstukken 6 & 7). Ik vergeleek de hoeveelheid algen binnen en buiten krabbescheerplanten op verschillende locaties in Nederland, om te zien of er inderdaad minder fytoplankton in de nabijheid van krabbescheer voorkomt. Verder deed ik een experiment in een meer in de Roemeense Donau Delta waarbij de groei van natuurlijk fytoplankton in water met en zonder allelopathische stoffen van krabbescheer werd vergeleken (Hoofdstuk 6).

Uit observaties van verschillende natuurlijke vegetaties met krabbescheer bleek verder dat niet alleen de groei van fytoplankton werd beïnvloed door krabbescheer. Krabbescheer leek ook een effect te hebben op de groei van draadalgen (filamenteuze algen). Ik zag namelijk dat draadalgen bijna niet voorkwamen in de directe omgeving van krabbescheer. Een gat van zo'n 5 centimeter rondom de planten bleef vrij van deze algen. Om uit te zoeken of allelopathie hier ook de oorzaak van was, deed ik twee veldexperimenten in het Naardermeer (Hoofdstuk 7) en vergeleek de groei van draadalgen rondom krabbescheer en rondom plastic planten die veel leken op krabbescheer. Met deze plastic planten simuleerde ik de fysieke aanwezigheid van krabbescheer terwijl er geen allelopathische stoffen werden uitgescheiden.

Om de resultaten uit laboratorium- en veldexperimenten te ondersteunen heb ik ook gebruik gemaakt van een theoretisch model van 'mijn systeem': de waterplant en het fytoplankton (Hoofdstuk 8). Hiermee werd het effect van allelopathie in ondiepe meren gesimuleerd voor kranswier en krabbescheer. Deze studie werd in samenwerking met Dr. E.H. van Nes (Universiteit van Wageningen) uitgevoerd.

Tot nu toe heb ik het over allelopathie en allelopathische stoffen gehad, maar eigenlijk is nog niet precies bekend om welke chemische stof(fen) het gaat. In samenwerking met B. Mau en Dr. E.M. Gross (Universiteit van Konstanz, Duitsland) ben ik op zoek gegaan naar de chemische eigenschappen van de allelopathische stoffen afkomstig van krabbescheer (Hoofdstuk 9). Daarvoor deden we een reeks van chemische analyses en biotesten.

### **3. De resultaten**

#### **3.1 *Allelopathie***

De resultaten beschreven in hoofdstuk 3 laten zien dat stoffen uitgescheiden door een mix van verschillende kranswierensoorten de groei van groenalgen remmen. Hiermee bewees ik dat mijn experimentele opzet geschikt was om allelopathische interacties tussen een waterplant en fytoplankton aan te tonen. Vervolgens testte ik de reactie van verschillende algen op chemische stoffen uit krabbescheer met dezelfde proefopzet als voor kranswier (Hoofdstuk 3). Ook voor krabbescheer vond ik remmende effecten op de groei van verschillende algen (Hoofdstukken 4 & 5).

#### **3.2 *Voedingsstoffen en licht***

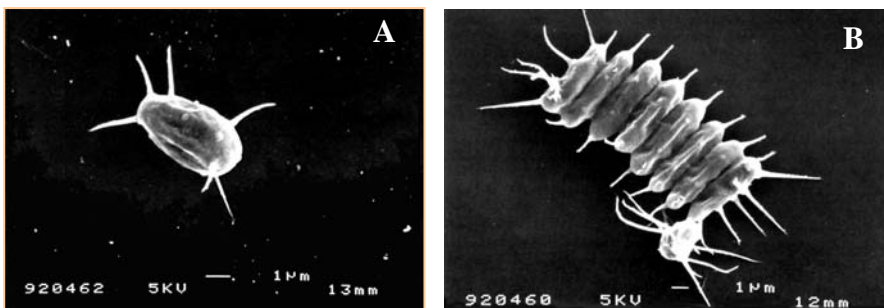
Fosfaatlimitatie (= gebrek aan fosfaat/voedselgebrek) resulteerde bij de groenalg *Chlorella* niet in een verhoogde gevoeligheid van deze alg voor allelopathische stoffen uit kranswieren (Hoofdstuk 3). Verder zag ik dat de groenalg *Scenedesmus* en de blauwalg *Synechococcus* in aanwezigheid van allelopathische stoffen uit krabbescheer zelfs beter groeiden dan de controle (zonder allelopathische stoffen) wanneer ze kaliumgebrek (= voedselgebrek) hadden (Hoofdstuk 9). Beide bevindingen zijn tegenstrijdig met de algemene gedachtengang en met onze voorspelling dat fytoplankton onder stress (bijv. fosfaat- of kaliumgebrek) gevoeliger wordt voor een extra stressfactor (zoals allelopathie). Voor de groenalg *Scenedesmus* met fosfaatgebrek vond ik wel de verwachte verhoging in gevoeligheid voor allelopathische stoffen en de groei van de alg werd dus geremd (Hoofdstuk 9). Verder werd deze groenalg ook sterker geremd wanneer die opgekweekt werd met te weinig licht (Hoofdstuk 5). De stress door gebrek aan fosfaat of licht zorgde waarschijnlijk voor een verhoogde gevoeligheid van de alg voor allelopathische stoffen uit krabbescheer. Dezelfde experimenten lieten echter zien dat de hoeveelheid licht die werd toegediend geen effect had op de respons van andere fytoplanktonsoorten.

### 3.3 *Leeftijd van waterplanten versus allelopathische activiteit*

In de hoofdstukken 3 & 4 laat ik voor kranswier en krabbescheer zien dat jongere planten allelopathisch actiever zijn dan oudere planten. Dit is waarschijnlijk logisch want vooral in het voorjaar, als de planten nog jong zijn en algen en waterplanten nog moeten gaan groeien, is de competitie tussen algen en waterplanten het sterkst. Het is juist in deze periode dus nuttig voor waterplanten om allelopathische stoffen te kunnen produceren en (belangrijker nog) ze uit te scheiden om zo algengroei te tegen te gaan.

### 3.4 *De groenalg *Scenedesmus*: kolonievorming*

De groei van de groenalg *Scenedesmus* werd geremd door allelopathische stoffen afkomstig van krabbescheer (Hoofdstuk 4). Verder zag ik dat de alg niet alleen als ééncellig organisme (Fig. 2a) bleef bestaan, maar dat de algencellen na celdeling ook aan elkaar bleven plakken: er werden kolonies van 2 tot 8 cellen gevormd (Fig. 2b). Al eerder werd er bij *Scenedesmus* kolonievorming waargenomen, maar dan in aanwezigheid van stoffen afkomstig van watervlooien. In dit geval bleek kolonievorming een afweermechanisme van de alg te zijn om niet gegeten te worden door watervlooien, want (grotere) kolonies zijn minder makkelijk eetbaar. Kolonievorming heeft echter ook nadelen. Kolonies zijn namelijk zwaarder en zinken daarvoor sneller uit naar de bodem van een meer. Dit is van belang bij de interactie tussen krabbescheer en *Scenedesmus*. In aanwezigheid van allelopathische stoffen groeide de alg slechter en werd zwaarder door kolonievorming. Krabbescheer zorgde dus, via groeiremming en het veroorzaken van kolonievorming, voor een verminderde hoeveelheid *Scenedesmus* in de directe omgeving en dus voor helderder water.



**Figuur 2** Ééncellige groenalg *Scenedesmus* (A) en *Scenedesmus* als kolonie van 8 cellen (B).

Foto's: Hessen & Van Donk, 1993

### 3.5 Verschillende algen, verschillende gevoeligheid

Naast groenalgen bevatten natuurlijke ecosystemen andere algen die ook gevoelig kunnen zijn voor allelopathische stoffen. Zo zag ik niet alleen effecten op de groei van *Scenedesmus* maar ook op de groei van de alg *Nannochloropsis* en twee blauwalgen/cyanobacteriën van de soort *Microcystis* (Hoofdstuk 5). De ene *Microcystis*-blauwalg was giftig, maar de andere niet. Na langere perioden van warm weer kunnen deze giftige blauwalgen/cyanobacteriën drijflagen vormen (Fig. 3). Hierdoor gelden in de zomer vaak zwemverboden (Fig. 4).

In mijn experimenten zag ik verder geen verschil in gevoeligheid voor allelopathische stoffen tussen blauwalgen en andere fytoplanktonsoorten (Hoofdstuk 5), terwijl andere onderzoekers hebben gevonden dat blauwalgen juist gevoeliger waren. Ik zag wel verschillen in de respons van groenalgen (Hoofdstuk 3). De groei van twee van de drie geteste groenalgen werden geremd door allelopathische stoffen afkomstig van kranswier.



**Figuur 3** Bloei van cyanobacteriën zichtbaar aan de oever van het IJsselmeer.

Foto: B.W. Ibelings



**Figuur 4** Alarmerende krantenkoppen (links) over het gevaar van giftige blauwalgen/ cyanobacteriën. Op het waarschuwingsbord bij het Engelse meer Belham tarn (rechts) wordt geadviseerd niet in het meer te zwemmen, het water niet te drinken, niet te vissen en uw hond niet te laten zwemmen.

Foto: B.W. Ibelings

Ook werd er binnen één blauwalg/cyanobacteriesoort (*Microcystis*) een verschil in respons op allelopathische stoffen van krabbescheer gevonden (Hoofdstuk 5). De groei van de giftige *Microcystis* variant werd sterker geremd dan de groei van de niet-giftige variant. Een mogelijke verklaring hiervoor is dat giftige blauwalgen al energie kwijt zijn aan het produceren van gifstoffen en hierdoor energie tekort komen om met de nadelige effecten van allelopathie om te gaan.

In verschillende experimenten werd de groei van sommige fytoplanktonsoorten sterker door allelopathische stoffen geremd dan de groei van ander soorten. Hierdoor kunnen verschuivingen optreden in de dichtheid en samenstelling van (natuurlijke) fytoplankton populaties in ondiepe meren en dat zou kunnen resulteren in veranderingen op hogere trofische niveau's<sup>3</sup> (bijv. zoöplankton populaties).

<sup>3</sup> **Trofisch niveau**, onderdeel van het voedselweb gedefinieerd door de manier van voedsel opnemen (producenten en consumenten). In een zoetwater voedselweb hebben we fytoplankton als producenten en zoöplankton (zoals watervlooien e.d.) als primaire consumenten. Watervlooien eten fytoplankton en worden zelf gegeten door ander zoöplankton en zelfs door vissen die daardoor secundaire (of tertiaire etc.) consumenten zijn.



### 3.6 *Veldexperimenten*

Tijdens een survey deed ik metingen aan natuurlijke krabbescheerpopulaties in Nederland en ook voerde ik een veldexperiment uit in de Donau Delta (Roemenië). Uit de survey bleek dat de hoeveelheid algen in de buurt van krabbescheer vaak lager was dan verder van de plant af (Hoofdstuk 6). Dit kon niet alleen worden verklaard door een tekort aan voedingsstoffen of lichtgebrek bij de algen, en wederom leek allelopathie een mogelijke verklaring te zijn. Ook het veldexperiment in de Donau Delta liet zien dat algen minder snel groeiden in water met, dan in water zonder allelopathische stoffen van krabbescheer.

Ook werd het voorkomen van draadalgen (filamenteuze algen) beïnvloed door de aanwezigheid van krabbescheer (Hoofdstuk 7). In de directe omgeving van krabbescheerplanten waren draadalgen afwezig (Fig. 5). Gedurende de lente en zomer zag ik dat plastic planten steeds verder overgroeid raakten, terwijl echte planten helemaal vrij van draadalgen bleven (Fig. 5). Wederom was dit effect niet te verklaren door de fysieke aanwezigheid van krabbescheer of door voedselgebrek van draadalgen en leek allelopathie de meest voor de hand liggende verklaring.



**Figuur 5** Een echte krabbescheerplant (links) en drie plastic planten die lijken op krabbescheer (drie witte cirkels). De echte plant bleef nagenoeg vrij van draadalgen, terwijl de plastic planten totaal overgroeid raakten.

Foto: G. Mulderij



### 3.7 *Wiskundig model voor algengroei*

Ons model beschrijft algengroei onder invloed van kranswieren en krabbescheer. Hiermee analyseerde ik de aparte en gecombineerde effecten van waterplanten op algen: 1) beschaduwning, 2) stabilisatie van de waterkolom, 3) competitie om voedingsstoffen, en 4) allelopathie. Uit de simulatie bleek dat allelopathie voor kranswieren minder belangrijk was dan voor krabbescheer. Daarnaast bleek bij krabbescheer dat de beschaduwning van algen, en het op die manier weg concurreren van algen, een belangrijke factor is. In de zomer drijft krabbescheer aan de oppervlakte en kan zo de hele waterkolom eronder beschaduwen. In het voorjaar echter, moet de plant nog van de waterbodem naar de oppervlakte komen en dan is beschaduwning waarschijnlijk een minder belangrijke factor. Hierdoor zou allelopathie dus vooral in het voorjaar een grote rol kunnen spelen. Kranswieren daarentegen, groeien het hele jaar dicht bij de bodem en hebben daarom een minder groot beschaduwend effect op fytoplankton. Bij kranswieren was vooral de stabilisatie van de waterkolom van belang. Er worden zo minder deeltjes van de bodem opgewerveld.

### 3.8 *Identificatie van allelopathische stoffen uit krabbescheer*

Ik heb met behulp van verschillende standaard biotesten de allelopathische activiteit van extracten van krabbescheer bepaald. Diverse fytoplanktonsoorten zijn getest op hun gevoeligheid voor extract van krabbescheer. De groei van sommige soorten werd geremd, terwijl andere soorten juist gestimuleerd werden. Verder zag ik dat de allelopathische activiteit van krabbescheerextract in vergelijking met extracten van andere waterplanten niet zo sterk was. Dit was echter tegenstrijdig met de activiteit van exudaten van krabbescheer (stoffen die reeds uitgescheiden zijn door de plant). In hoofdstuk 5 laat ik namelijk een groeiremming van 50% door exudaten van krabbescheer zien.

Het *extract* werd gemaakt door gevriesdroogd<sup>4</sup> plantenmateriaal te extraheren met oplosmiddel. De stoffen in het extract komen dus uit het plantenmateriaal, maar het is onduidelijk of deze normaal ook echt door de plant worden uitgescheiden, en of ze dan nog hetzelfde effect op fytoplankton hebben. Daarom heb ik ook geprobeerd om allelopathische stoffen te isoleren uit het water waar krabbescheer in heeft gestaan (*exudaten*, Hoofdstukken 4 & 5).

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<sup>4</sup> **Vriesdrogen** is een industriële methode die vanouds wordt toegepast voor het conserveren van levensmiddelen. Het principe van vriesdrogen is dat door de lage temperatuur de watermoleculen worden opgenomen in ijskristallen, waardoor de lucht extreem droog wordt en water aan het te vriesdrogen product of object wordt onttrokken.

De allelopathische stof bleek lipofiele<sup>5</sup> eigenschappen te bezitten en hoogst waarschijnlijk is het geen fenolachtige<sup>6</sup> verbinding. Om volledig uitsluitel te geven over de chemische samenstelling van de allelopathische stof(fen) zijn aanvullende bio-testen en chemische analyses nodig.

## 4. Conclusies

Met laboratoriumexperimenten is aangetoond dat de waterplanten kranswier en krabbescheer allelopathische effecten op fytoplankton hebben (Hoofdstukken 3 t/m 5). De groei van algen wordt geremd, sommige groenalgen gaan kolonies vormen, en algen die minder licht krijgen worden gevoeliger voor allelopathische stoffen. Verder waren er verschillen in gevoeligheid van fytoplanktonsoorten voor allelopathische stoffen. Dit kan invloed hebben op de dichtheid en samenstelling van (natuurlijke) fytoplanktonpopulaties. Veranderingen in de dominantie van fytoplanktonsoorten kunnen vervolgens verschuivingen in hogere trofische niveau's<sup>7</sup> van het voedselweb veroorzaken. In tegenstelling tot laboratoriumexperimenten uitgevoerd door andere wetenschappers beschrijft dit proefschrift onderzoek waarbij krabbescheer en kranswier de allelopathische stoffen ook daadwerkelijk uitscheiden en zo de groei van fytoplankton beïnvloeden.

In vergelijking met de vaak optimale groeicondities voor algen gedurende experimenten in het laboratorium zijn algen in natuurlijke systemen onderhevig aan een complex van stressfactoren. Daarom kunnen we niet om veldexperimenten heen als we iets over de rol van allelopathie in de natuur willen zeggen. Ook de beschreven veldexperimenten gaven sterke indicaties voor de aanwezigheid van allelopathie onder natuurlijke omstandigheden (Hoofdstukken 6 & 7). Het mysterie is echter nog niet volledig opgelost. Nieuwe (veld-)experimenten zullen de bevindingen uit voorgaande experimenten moeten ondersteunen en meer bewijsmateriaal voor het belang van allelopathie in andere natuurlijke systemen moeten geven.

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<sup>5</sup> **Lipofiel** betekent 'vetminnend' en is een eigenschap van sommige chemische stoffen. Lipofiele stoffen lossen goed op in apolair oplosmiddelen zoals oliën en vetten.

<sup>6</sup> **Fenol** (soms geschreven als phenol) of **fenyl alcohol**, is een verbinding bestaande uit een benzeenring waarvan 1 waterstof vervangen is door een hydroxylgroep(OH). De brutoformule is  $C_6H_5OH$ . Fenol behoort tot een groep aromatische organische stoffen die één of meerdere OH groepen heeft zoals fenol zelf en cresolen en xylenolen. Fenol is giftig bij opnamen via mond, inhalatie en huidabsorptie.

Ik zeg hier '*belang*' en niet '*bestaan*' van allelopathie omdat ik van mening ben dat we te lang stil blijven staan bij de discussie of allelopathie überhaupt kan bestaan. Volgens sommige wetenschappers voldoen de meeste experimenten niet aan alle voorwaarden om allelopathie echt aan te tonen. Met mijn proefschrift wil ik laten zien dat we van deze discussie af moeten stappen en moeten aantonen wat het belang van allelopathie is in vergelijking met andere relevante processen (zoals bijv. competitie om voedsel).

Nieuwe veldexperimenten met waterplanten en algen die van nature al in dezelfde ecosystemen voorkomen en met realistische concentraties aan allelopathische stoffen zullen een natuurgetrouwer beeld van de situatie geven en het belang van allelopathie kunnen bevestigen. Zo kunnen we een vollediger beeld vormen over de effecten van allelopathie op de soortsamenvestelling en dichtheid van fytoplankton en eventuele effecten op hogere niveaus in het voedselweb van ondiepe meren.





**Dankwoord**



## Dankwoord/Acknowledgements

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Tijdens mijn studie in Wageningen leerde ik, *Sandra* en *Ilya* en wederzijdse ouders, kennen. Door jullie weet ik dat het pas echt gezellig wordt als er 'Brabo's in de zaal zijn! Dank voor alle gezellige avonden in Wageningen en Roosendaal! Ik heb ervan genoten en dacht door jullie gezelschap zelfs niet eens aan mijn proefschrift, totaal ontspannen, knap he?

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Before I started my PhD project, I spend 7 months in Silkeborg at the National Environmental Research Institute, Denmark. Due to the nice cooperation with *Torben*, *Erik*, *Martin* and all the other colleagues in the Lake Group, I changed my mind and started to look for a PhD position. I will never regret this decision. Thanks for changing my mind!

En toen werd ze AIO in Nijmegen/Nieuwersluis, bij Prof. dr. E. van Donk. *Ellen*, je was een 'superchef'. Ik heb het erg gezellig met je gehad (en hoop dat dat nog niet voorbij is). Je bent altijd een grote vechtersbaas, vooral voor je AIO's. Daar waren wij natuurlijk mooi bij gebaat. Ik kon lekker 'meeliften' op jou netwerk. Ik herinner me nog de keren dat je me op congressen trots aan één van de 'geleerde dames en heren' voorstelde: "Hallo, dit is Gabi. Ze is mijn nieuwe AIO en werkt aan allelopathie. Ze heeft hele leuke resultaten en houdt overmorgen een verhaal..." Je verlaagde drempels, zeg maar.

Binnen FS leerde ik ook veel nieuwe collega's/vrienden kennen. Allereerst maar eens een bedankje naar mijn paranimfjes, *Miguel* (Dr. *Dreissena*) en *Arnout* (Dr. *Diatom*). Jongens, jullie waren er vanaf het begin bij. Dat jullie het al die tijd met me uit hebben gehouden?! Ik kon bij jullie altijd even komen spuien als me wat dwars zat. Hopelijk konden jullie ook zo bij mij terecht. *Arnout*, we gingen niet samen in de python, maar gelukkig wel samen naar de aula. En zelfs twee keer! *Miguel*, ook hartelijk dank voor alle hulp tijdens mijn experimenten en voor het lezen van mijn manuscripten. Je was een super kamergenoot en gelukkig is het daar niet bij gebleven. *Machteld*, jij hebt me ook van het begin meegemaakt. Samen konden we het wel winnen van de mannen op onze kamer, he? Ik vond het erg gezellig met je! En *Boris*, mijn andere kamergenoot, ik zal je nog één keer pesten met wat Nederlands gebrabbel: Je bent en blijft een mafkees! Ik hoop dat het goed met je gaat in Berlijn.

Wat later tijdens mijn promotie kwamen *Marjolijn* en *Irene* als AIO op het CL werken. Meiden, jullie kwamen op het goede moment. Ik was het wel zat: alleen tussen de heren AIO's (geintje, jongens). We hebben erg veel lol gehad, hier op het CL, in de klimhal, in Utrecht, op Bonaire, in Leiden, in Egypty, etc. *Wolf* en *Luis*, dank voor jullie hulp en het doorstaan van mijn eigenwijsheid m.b.t. statistiek. *Wolf*, *Luis*, en *Egbert van Nes*, hartelijk dank voor jullie hulp met het opzetten en runnen van modelletjes. *Lisette*, jij hielp me later enthousiast met statistiek en enge transformaties (wat zal het een opluchting geweest zijn voor *Wolf*). Ik vond onze samenwerking erg gezellig. *Ramesh*, hartelijk dank voor het corrigeren van een aantal van mijn manuscripten. *Nico*, eigenlijk ken ik jou van alle Cl-ers het langst.



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Mijn promotieonderzoek is eigenlijk ooit in Nijmegen gestart, maar al snel bleek dat Nieuwersluis een betere locatie was voor het uitvoeren van mijn experimenten. Daarom vertrok in na een half jaar naar Nieuwersluis. Toch bleven jullie, *Fons* en *Leon*, altijd erg betrokken bij mijn onderzoek. *Fons*, ik had altijd onzettend veel lol tijdens onze 'krabbescheerjachten'. Bedankt voor het enthousiasme waarmee je mijn manuscripten altijd zo snel van commentaar verzag. *Leon*, ik zag jou niet vaak, maar toch bedankt voor het meedenken en medeleven tijdens mijn promotieonderzoek. *Jan*, we hebben tijdens mijn promotie waarschijnlijk minder contact gehad dan we van tevoren hadden verwacht. Toch ben je deels verantwoordelijk geweest voor de realisatie van dit project. Dank daarvoor. *Germa*, *Fons* en *Roy*, dank voor jullie assistentie bij de chemische analyse van mijn watermonsters. De laatste Nijmegenaren die een apart plekje verdienen zijn *Bart*, *Mariëlle* en *Raju*. Dank voor het eeuwige enthousiasme waarmee jullie me altijd weer ontvingen!

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In de laatste fase van mijn promotie werd het privé allemaal erg zwaar. 18 november 2004 stierf mijn vader aan de gevolgen van longkanker. Papa, je kan fysiek niet meer bij mijn verdediging aanwezig zijn, maar je kleine meid gaat het toch echt flikken, ze wordt doctor! En ik weet dat jij op mijn schouder zit bij mijn verdediging! Tijdens het ziekbed en overlijden van mijn vader ben ik heel goed opgevangen door heel veel lieve mensen, zowel hier op het CL als daar buiten. Ik wil jullie allen hartelijk danken. Promoveren doe je niet alleen, maar rouwen zeker ook niet. *Syl*, jij en je moeder weten als geen ander wat het is om je vader/ echtgenoot veel te vroeg te verliezen. Jullie zijn schatten, en ik hoop dat we nog lang steun bij elkaar kunnen vinden. We blijven knokken!

Veel liefs, Gabi







Curriculum vitae



## Curriculum vitae

Gabi Mulderij was born in Nijmegen (The Netherlands) on 8 May 1977. After she had graduated at secondary school (Over-Betuwe college, Bemmelen, The Netherlands) in 1994 she went to Larenstein International Agricultural College (Velp, The Netherlands) and was trained as a laboratory technician. She finished this study in 1998 with a traineeship at the Radboud University Nijmegen under supervision of Prof. L.A.C.J. Voesenek, where she studied the hyponastic response of *Rumex palustris* upon flooding.

Between 1998-2000 she studied at the University of Wageningen (The Netherlands) and specialized in Aquatic Ecology and Water Quality Management. As final part of this study, she did a traineeship at the National Environmental Research Institute (NERI, Silkeborg, Denmark). During seven months she studied the effect of planktivorous fish on the autumn and winter dynamics of phytoplankton in two Danish lakes with a contrasting nutrient status, under supervision of Prof. E. Jepsen and Dr. M. Søndergaard.

In September 2000 she started her PhD project at the Centre of Limnology of The Netherlands Institute of Ecology (NIOO-KNAW). This project was conducted in cooperation with the Radboud University Nijmegen. She performed experiments under supervision of Prof. E. van Donk and A.J.P. Smolders, and studied the chemical effects of macrophytes on phytoplankton. The results from this study are described in this thesis and were presented at several (inter-)national conferences. Some of the experiments were conducted at the Limnological Institute of the University of Konstanz in cooperation with E.M. Gross and B. Mau. Gabi also collaborated several times with Dr. B.W. Ibelings (NIOO-KNAW, Centre for Limnology) and L. Török and M. Tudor (two PhD students of the Danube Delta National Institute, Tulcea, Romania) to study the effects of humic substances on the growth and composition of phytoplankton.

Since 1 December 2005 Gabi is employed at the Freshwater Biological Laboratory, University of Copenhagen, Denmark, where she is involved in an EU project (entitled PEPCY) to investigate the toxic properties and occurrence of cyanobacteria.





## Curriculum vitae

Gabi Mulderij werd op 8 mei 1977 geboren in Nijmegen. Na het behalen van haar HAVO diploma aan de scholengemeenschap Oost-Betuwe te Bemmelen (1994) ging ze naar de Internationale Agrarische Hogeschool Larenstein, te Velp, en behaalde daar in 1998 een diploma voor de studierichting Milieu-analyse. Hierbij liep ze o.a. stage op de toenmalige Katholieke Universiteit Nijmegen (Radboud Universiteit Nijmegen) en bestudeerde daar onder begeleiding van Prof. dr. L.A.C.J. Voesenek de respons van *Rumex palustris* (moeraszuring) op overstroming.

Van 1998 tot 2000 volgde ze een studie (Milieu-hygiëne) aan de Universiteit van Wageningen, waar ze zich specialiseerde in de richting Aquatische Ecologie en Waterkwaliteitsbeheer. Als afsluitend onderdeel van deze studie ging ze voor zeven maanden naar het National Environmental Research Institute (NERI) in Silkeborg (Denemarken), waar ze onder begeleiding van Prof. dr. E. Jeppesen en Dr. M. Søndergaard gedurende de herfst en winter van 1999/2000 onderzoek deed naar het effect van planktivore vis op de dynamiek van fytoplankton in twee meren met een contrasterend nutriëntgehalte.

In september 2000 startte ze haar promotieonderzoek op het Centrum voor Limnologie van het Nederlands Instituut voor Ecologie. Dit onderzoek werd uitgevoerd in samenwerking met de Radboud Universiteit Nijmegen. Ze deed dit onderzoek onder begeleiding van Prof. dr. E. van Donk en Dr. A.J.P. Smolders, en bestudeerde daarbij de chemische interactie tussen waterplanten en fytoplankton. De resultaten van het onderzoek staan in dit proefschrift beschreven en werden gepresenteerd op diverse (inter-)nationale congressen. Een aantal experimenten werd uitgevoerd op het limnologisch instituut van de Universiteit van Konstanz (Duitsland), in samenwerking met Dr. E.M. Gross en B. Mau. Ook werkte ze diverse malen samen met Dr. B.W. Ibelings en L. Török en M. Tudor (twee promovendi van het Danube Delta National Institute, Tulcea, Roemenië) om het effect van humuszuren op de groei en samenstelling van fytoplankton te bestuderen.

Sinds 1 December 2005 is Gabi werkzaam bij het zoetwaterlaboratorium van de Universiteit van Kopenhagen te Denemarken. Ze is daar betrokken bij een EU project (PEPCY) dat het voorkomen en de toxische effecten van cyanobacteriën/blauwalgen bestudeert.





List of publications



## List of publications

### Papers published

Mulderij G, Van Donk E & Roelofs JGM (2003)  
Differential sensitivity of green algae to allelopathic substances from *Chara*.  
*Hydrobiologia* 491: 261 - 271, Chapter 3 in this thesis.

Mulderij G, Mooij WM & Van Donk E (2005)  
Allelopathic growth inhibition and colony formation of the green algae  
*Scendesmus obliquus* by the aquatic macrophyte *Stratiotes aloides*.  
*Aquatic Ecology* 39(1): 11 - 21, Chapter 4 in this thesis.

Mulderij G, Mooij WM, Smolders AJP & Van Donk E (2005)  
Inhibition of phytoplankton by allelopathic substances from the aquatic  
macrophyte *Stratiotes aloides*.  
*Aquatic Botany* 82: 284 - 296, Chapter 5 in this thesis.

### Papers in press

Burks RL, Mulderij G, Gross EM, Jones JI, Jacobsen L, Jeppesen E & Van Donk E.  
Center stage: The crucial role of macrophytes in regulating trophic interactions in  
shallow lake wetlands.  
*In: Bobbink R, Beltman B, Verhoeven JTA & Whigham DF (Eds.) Wetlands:  
functioning, biodiversity conservation and restoration.*  
*Ecological Studies*, Chapter 2 in this thesis.

### Papers submitted

Mulderij G, Smolders AJP & Van Donk E.  
Allelopathic effects of the aquatic macrophyte *Stratiotes aloides* on natural phyto-  
plankton populations.  
Chapter 6 in this thesis.

**Papers submitted (continued)**

Mulderij G, Mau B, Senerpont Domis LN, Smolders AJP & Van Donk E.

Allelopathic inhibition of filamentous algae by the aquatic macrophyte *Stratiotes aloides*.

Chapter 7 in this thesis.

Mulderij G, Van Nes EH & Van Donk E

The effects of two macrophytes on phytoplankton growth: a model to estimate the contribution of allelopathy *in situ*.

Chapter 8 in this thesis.

Mulderij G, Mau B, Van Donk E & Gross EM

Allelopathic activity of *Stratiotes aloides* on phytoplankton: towards identification of allelopathic substances.

Chapter 9 in this thesis.

Gross EM, Hilt S, Lombardo P & Mulderij G

Searching for evidence of allelopathy in action - state of the art and open questions.

**Papers in preparation**

Mulderij G, Ibelings BW, Tudor M, & Török L.

Inhibition or stimulation? Effects of humic substances on phytoplankton growth in Danube Delta lakes.

Sørensen T, Mulderij G, Jeppesen E & Søndergaard M. Cascading effect of planktivorous fish during autumn and winter: enclosure experiments in two lakes with contrasting nutrient levels.







# Stellingen

behorende bij het proefschrift:

## **Chemical warfare in freshwater**

### **allelopathic effects of macrophytes on phytoplankton**

Gabi Mulderij

1. Allelopathie-experimenten met exudaten van waterplanten zijn ecologisch relevanter dan experimenten met extracten. *(Dit proefschrift)*
2. Verschillen in de gevoeligheid van fytoplanktonsoorten voor allelopathische stoffen kunnen gevolgen hebben voor de biomassa en samenstelling van fytoplanktonpopulaties. Dit kan vervolgens weer invloed hebben op de hogere trofische niveau's van het voedselweb. *(Dit proefschrift)*
3. De discussie of allelopathie bestaat zou moeten worden gesloten. We moeten nu onderzoeken wat de ecologische relevantie van allelopathie kan zijn.  
*(Dit proefschrift)*
4. Kolonievorming bij *Scenedesmus* is niet altijd een voordeel voor de alg. Inductie van kolonievorming door krabbescheer zorgt voor versnelde sedimentatie van de alg *Scenedesmus*. *(Dit proefschrift)*
5. Onderzoek doen aan allelopathische interacties is en blijft een uitdaging.
6. Aquatisch ecologen zonder duikbrevet hebben er niets van begrepen.
7. A scientist in his/her laboratory is not only a technician. He/She is also a child placed before natural phenomena which impress him like a fairy tale.  
*(Marie Curie, 1867 - 1934)*
8. The important thing in science is not so much to obtain new facts as to discover new ways of thinking about them. *(Sir William Bragg, 1862 - 1942)*
9. Roken maakt meer kapot dan je lief is.
10. Om vrijwillig te kunnen sterven moet je eerst geleefd hebben. *(Dave Mulderij)*
11. Euthanasie is de oplossing als deze door het leven zelf niet geboden wordt.