First publ. in: Hydrobiologia 584 (2007), pp. 89-100

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

Gabi Mulderij · Birgit Mau · Ellen van Donk · Elisabeth M. Gross

Abstract The allelopathic activity of the aquatic macrophyte, *Stratiotes aloides*, was determined with laboratory experiments. Active compounds exuded in 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. SPE-enriched exudates and enriched water from a natural *Stratiotes* stand caused inhibition of target species, however, also some controls were active. Phytoplankton species exhibited differential sensitivity to extracts of *S. aloides*. We observed inhibitory and stimulatory effects on phytoplankton. In general, more cyanobacteria than other phytoplankton species were inhibited, and the inhibition of cyanobacteria was stronger. In most cases, nutrient (P or K) limitation of *Synechococcus elongatus* and *Scenedesmus obliquus* decreased the sensitivity of these species towards allelochemicals from *Stratiotes aloides*, except for P-limited cultures of *Scenedesmus*. The allelopathically active compound(s) from *Stratiotes* 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 and filamentous algae densities in the vicinity of *Stratiotes* plants, at least during certain phases of the life-cycle of *Stratiotes*.

Keywords Allelopathy · Aquatic macrophyte · Exudate · Growth inhibition · Nutrient limitation

Guest editors: R. D. Gulati, E. Lammens, N. De Pauw & E. Van Donk
Shallow lakes in a changing world

G. Mulderij (✉) · E. van Donk
Department of Food Web Studies, NIOO-KNAW, Centre for Limnology, Nieuwersluis 3631 AC, The Netherlands
e-mail: g.mulderij@nioo.knaw.nl

E. van Donk
e-mail: e.vandonk@nioo.knaw.nl

G. Mulderij · E. van Donk
Department of Ecology, Aquatic Ecology and Environmental Biology Section, University of Nijmegen, PO Box 9010, Nijmegen 6500 GL, The Netherlands

B. Mau · E. M. Gross
Limnological Institute, University of Konstanz, PO Box M659, Konstanz 78457, Germany

B. Mau
e-mail: Mau.Birgit@web.de

E. M. Gross
e-mail: Elisabeth.Gross@uni-konstanz.de

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, 2003). 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, b) provided evidence for allelopathic interference of *Stratiotes aloides* with phytoplankton species, but the nature of the active compound(s) was not investigated. *S. aloides* is a dioecious, perennial aquatic macrophyte, native to Europe and Siberia (Cook & Urmi-König, 1983). A part of its life cycle is submerged (Bloemendaal & Roelofs, 1988) and the plants overwinter as turions or entire (but rootless) plants on the bottom of the lake. 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, *Stratiotes* might be favoured by the ability to produce allelopathic compounds that inhibit phytoplankton growth.

Other mechanisms for the exclusion of phytoplankton in the proximity of water soldier have been proposed. The availability of potassium or sodium might be limiting 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 *Stratiotes*. 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 be responsible for algicidal effects of *Stratiotes*. Both studies, however, did not prove that *Stratiotes* really excreted allelopathic compounds, an important issue to asses the ecological importance of allelopathic interactions. Recent laboratory experiments showed that exudates of *Stratiotes* inhibited the green alga *Scenedesmus obliquus* and induced colony formation in this alga (Mulderij et al., 2005a). Further, allelopathic effects of *Stratiotes* exudates on the cyanobacterium *Microcystis aeruginosa* and the eustigmatophyte *Nannochloropsis limnetica* were observed (Mulderij et al., 2005b).

We performed bioassay-directed extractions and fractionations to elucidate the chemical characteristics of allelopathic substances in extracts and exudates of *Stratiotes*, and were especially interested whether the active compounds are hydro- or lipophilic. We also tested if the sensitivity of target organisms to *Stratiotes* 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.

Materials and methods

Macrophyte culture

The *Stratiotes* 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 originating 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 μmol m^{-2} s^{-1}) 16 h per day and the water temperature was 18 ± 1°C. At the start of an experiment, plants were transferred to sediment-free aquaria with macrophyte medium (Mulderij et al., 2005a).

Target organisms

We used axenic monocultures of twelve phytoplankton species, consisting of cyanobacteria, green algae and an eustigmatophyte. *Anabaena* sp. PCC 7120 and *A. variabilis* P9 ATCC 29413 were used in agar diffusion assays (*ADAs*, see *Bioassays*), while the four *Microcystis aeruginosa* strains (NIVA-CYA 140 (toxic), NIVA-CYA 43, V131 (from E. Kardinaal), and PCC 7820, the latter two non-toxic), the cyanobacterium *Synechococcus elongatus* (SAG 89.79), the green algae *Chlorella minutissima* (CCAP 211/52), *Chlamydomonas reinhardtii* (NIVA-CHL 13), *Scenedesmus obliquus* (CCAP 276/3A), *Selenastrum capricornutum* (NIVA-CHL 1) and the eustigmatophyte *Nannochloropsis limnetica* (Krienitz 1998/3) 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 Lurling, 1999).

Phosphate- (P) or potassium- (K) limited cultures of *S. obliquus* and *S. elongatus* were established by centrifuging (6 min., 2,300 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 μM K_2PO_4 by 1 μM K_2PO_4 and 40 μM KCl. K-limited medium was created by substituting 50 μM K_2HPO_4 with 1 μM K_2HPO_4 and 40 μM Na_2HPO_4 (Alahari & Apte, 2004). P- or K-limited batch cultures (300 ml) were grown for at least 9 days on an orbital shaker (110 rpm) at 22°C, 50 μmol PAR m^{-2} s^{-1} with a photoperiod of 16:8 (L:D).

Extraction

Stratiotes plants were carefully rinsed with tap water, shock-frozen with liquid nitrogen, and immediately lyophilized for 24 h. 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) for 2 h 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) 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 (DM) per ml. Extracts were stored at –20°C. Solvent controls were performed using the same procedure without adding plant material.

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.

Heat stability and polyvinylpolypyrrolidone (PVPP) test

Crude extracts of *Stratiotes* 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 polyvinylpolypyrrolidone (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., 4,000 g) and the supernatant used in the bioassays.

Exudation

Possible allelopathic compounds, exuded into the water by *Stratiotes*, were investigated with three

approaches. Laboratory approaches (set-ups 1 & 2): Incubating single or multiple plants in microcosms. Field approach (set-up 3): Collecting water from a natural *Stratiotes* stand in Lake Naardermeer (nature reserve). In set-up 1 (single plant culture), we filled 1.5-l glass vials (∅ 10 cm, 20 cm height) with 1 l macrophyte medium (Mulderij et al., 2005a) and one *Stratiotes* plant (25–30 g FM) from the laboratory culture. Control vials only received medium. All vials were kept at 16°C and approx. 60 µmol PAR m^{-2} s^{-1} (L:D 14:10). After 1, 2 and 5 days, the culture water of three vials (one control and two with *Stratiotes*) was used for analyses. In set-up 2 (microcosms), two 50 l-aquaria were filled with 30 l macrophyte medium. One aquarium served as control and was filled with medium, while the other aquarium was filled with medium and eight *Stratiotes* plants (ca. 200 g FM) originating from Giethoorn (52°45′0″ N 6°5′0″ E, The Netherlands), collected the week before the start of the experiment. After 1, 2 and 3 days, 1l water of each aquarium was collected for further analysis. For set-up 3, water samples (1 l) were collected in the vicinity of *Stratiotes* plants. As a control, two sites without *Stratiotes* were sampled: one in the same ditch (Ditch 1) just outside the *Stratiotes* stand and the other in an other ditch (Ditch 2) in the nature reserve. In ditch 2, *Stratiotes* plants were absent during the whole experiment. Samples were collected between 7 April and 14 June 2004.

All samples (set-ups 1, 2 & 3) were filtered over Whatman GF/F and then over 0.2 µm celluloseacetate membrane filters (Schleicher & Schuell). Potentially exuded allelopathic compounds were trapped on preconditioned C18–filters (∅ 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.

Bioassays

We conducted two types of bioassays 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).

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. The agar plates were incubated at 28°C and 80 µmol PAR m^{-2} s^{-1} (L:D 16:8) for one week. Exudates were spotted in concentrations equivalent to 40, 120, and 240 ml culture water (set-up 1, equal to 1.2, 3.6 and 7.2 g FM) or 600 ml (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.

Liquid culture 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. Then, per well, 2 ml culture with target cells ($OD_{530\ nm}$: 40 mAU) in the respective medium were added. Plates were incubated for one week at either 21°C and 60 PAR m^{-2} s^{-1} L:D 16:8 (NIOO) or at 28°C and 60 PAR m^{-2} s^{-1} under continuous illumination (University of Konstanz), 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:

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

The first LCA was conducted with four different concentrations of *Stratiotes* extract (0, 0.75, 1.5 and 3.75 mg DM/ml; *LCA 1*) and all target species. In the second LCA (*LCA 2*) lower extract concentrations (0, 0.5, 1.0 and

1.5 mg DM/ml) and only some target species were used. In this way we determined which extract concentration and which test organisms should be used in the following assays. The two most sensitive species of cyanobacteria and green algae were chosen as target organisms for the next LCAs.

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

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 *Stratiotes* extract. *LCAs 3-5* were conducted with nutrient (K or P) limited and non-limited target cells.

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

Statistical analysis

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

Results

Allelopathic activity of *Stratiotes* extracts

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

M. aeruginosa V131 was the most sensitive cyanobacterium, exhibiting a significant ($P < 0.001$) growth inhibition in *LCA 1* of 32% already at 1 mg DM/ml (Fig. 1a). *S. elongatus* was inhibited (56%) by extract concentrations above 1.5 mg DM/ml (Fig. 1b). Other cyanobacteria showed no significant response (*LCA 1*) or even a significant growth stimulation (*LCA 2*), depending on the experiment (Table 1). 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 (Fig. 1c) with *N. limnetica* and a stimulation of all chlorophytes at 0.5 or 1 mg DM/ml in *LCA 2* (Table 1).

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

Fractionation of crude extracts

The ADA with *A. variabilis* and SPE fractions of crude extract showed strong inhibitory effects of the 80 and 90% methanol SPE fractions (Table 2). An additional LCA with crude extract

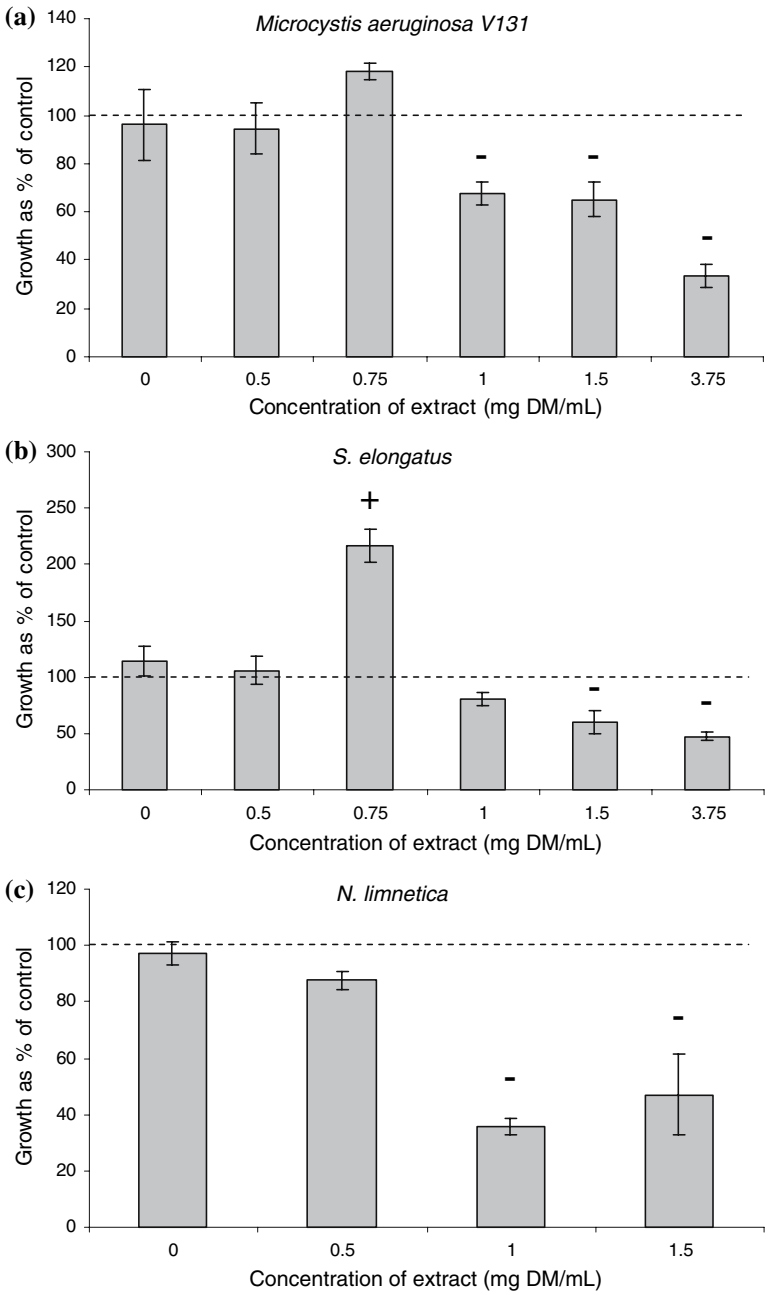

Fig. 1 Growth relative to the control of *Microcystis aeruginosa* (**a**), *Synechococcus elongatus* (**b**), and *Nannochloropsis limnetica* (**c**) at different concentrations of *Stratiotes* extract in the first two liquid assays. Bars indicate average values ($n = 3$), error bars are 1 SE. + and – indicate significant inhibitory and stimulatory effects on the growth of the phytoplankton species

and its SPE fractions (*LCA SPE*) showed significant ($P < 0.001$) effects on the growth of *S. elongatus* (Fig. 2b). 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. 2b). The 50% SPE fraction, on the contrary, showed significant stimulatory effects at concentrations of 1 and 3 mg DM/ml (Fig. 2b).

Heat stability and polyvinylpolypyrrolidone (PVPP) test

ADAs showed that *Stratiotes* extract remains allelopathically active after heating. The clearing

Table 1 Results statistical analyses of liquid culture assays 1 and 2 (*LCA 1 & LCA 2*). Effects of extract addition were either stimulatory (+), inhibitory (–) or not clear (0) with threshold values indicated in parenthesis

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

The one-way ANOVA of the growth as percentage of control, was carried out on a series of 24 values per phytoplankton species (3 replicates, 2 treatments: control and Stratiotes, and four extract concentrations: 0, 0.5, 1.0 and 1.5 mg DM/ml or 0, 0.75, 1.5 and 3.75 mg DM/ml)

* Criterion for significance was 0.01, see *Statistical analysis*

zone of untreated or heat-treated extracts did not differ. Further, three independent ADAs showed that PVPP treatment of *Stratiotes* 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* (Fig. 2a versus b).

Phosphorus and potassium limitation of S. elongatus and S. obliquus

LCA 5 with *S. elongatus* showed significant ($P < 0.001$) stimulatory effects of P-limited cultures above extract concentrations of 2.5 mg DM/ml (Fig. 3a), while non-limited cultures were significantly ($P < 0.001$) inhibited (45%) by concentrations above 4.0 mg DM/ml (Fig. 3a). P-limited and non-limited *S. obliquus* cultures, were significantly ($P < 0.001$) stimulated (up to 1600%) by *Stratiotes* extract at concentrations above 0.5 mg DM/ml (Fig. 3b), with the pronounced effects for non-limited cultures (Fig. 3b).

The short term *LCA 4* showed significant stimulatory effects of *Stratiotes* 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.

K-limited cultures of *S. elongatus* 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. 2b), no significant inhibitory effects were observed with K-limited cultures (Fig. 2c). The

Table 2 Algicidal activity of SPE-fractions of *Stratiotes* extract (50% acetone) on *A. variabilis*

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

Aliquots were applied in two concentrations in the agar diffusion assay

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

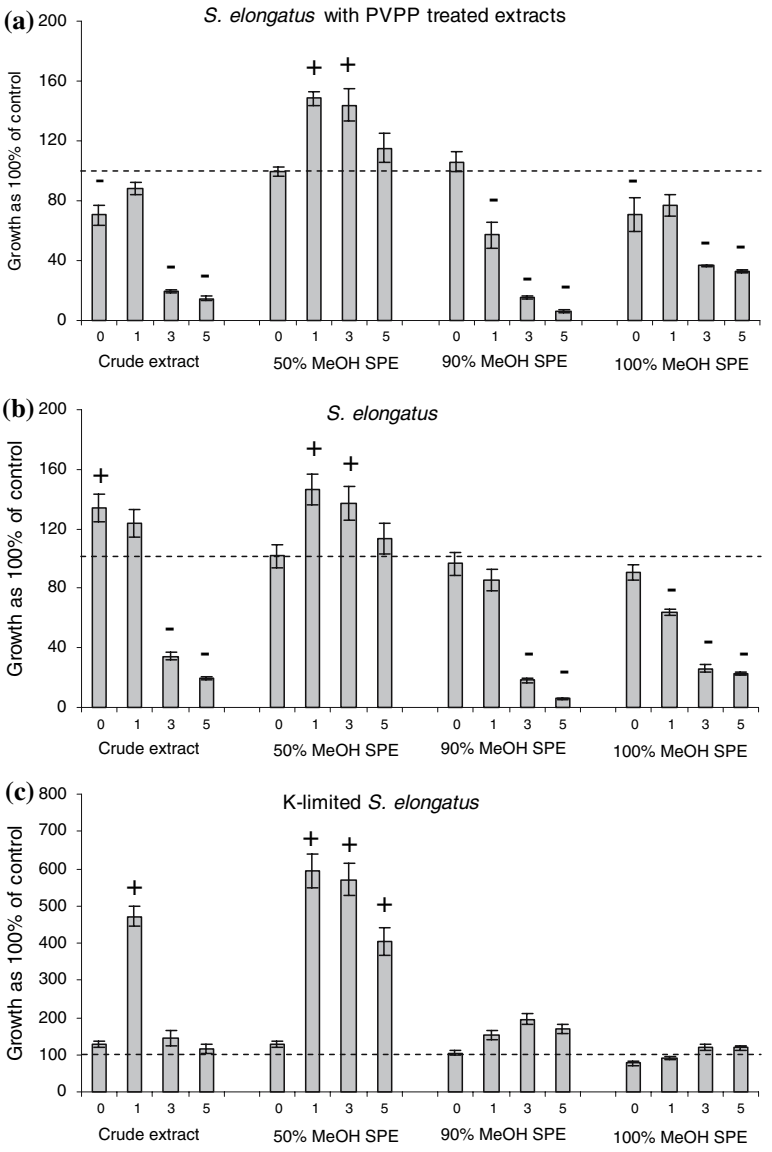

Fig. 2 Growth relative to the control in non-limited (**a, b**) and K-limited cultures (**c**) of *Synechococcus elongatus* when exposed to different concentrations (0, 1, 3 and 5 mg DM/ml) of *Stratiotes* extract (crude extract) or SPE fractions (50-, 90-, and 100% Methanol) either treated (**a**) or not treated (**b, c**) with polyvinylpolypyrrolidone (PVPP). Bars indicate average values ($n = 3$), error bars are 1 SE. + and – indicate significant inhibitory and stimulatory effects on the growth of the phytoplankton species

50% SPE fraction exhibited a significant growth stimulation on both non-limited and K-limited cultures of *S. elongatus*.

Exudation

The ADA with *A. variabilis* showed 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). An other 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 (Table 3), 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 4), with the strongest inhibitory effect found at 20 April (when *Stratiotes* plants were still submerged). Controls, however, also showed inhibitory effects, but generally less strong than the exudates.

Discussion

In general, our results showed both stimulatory and inhibitory effects of *Stratiotes* extract and exudates on phytoplankton growth, with more

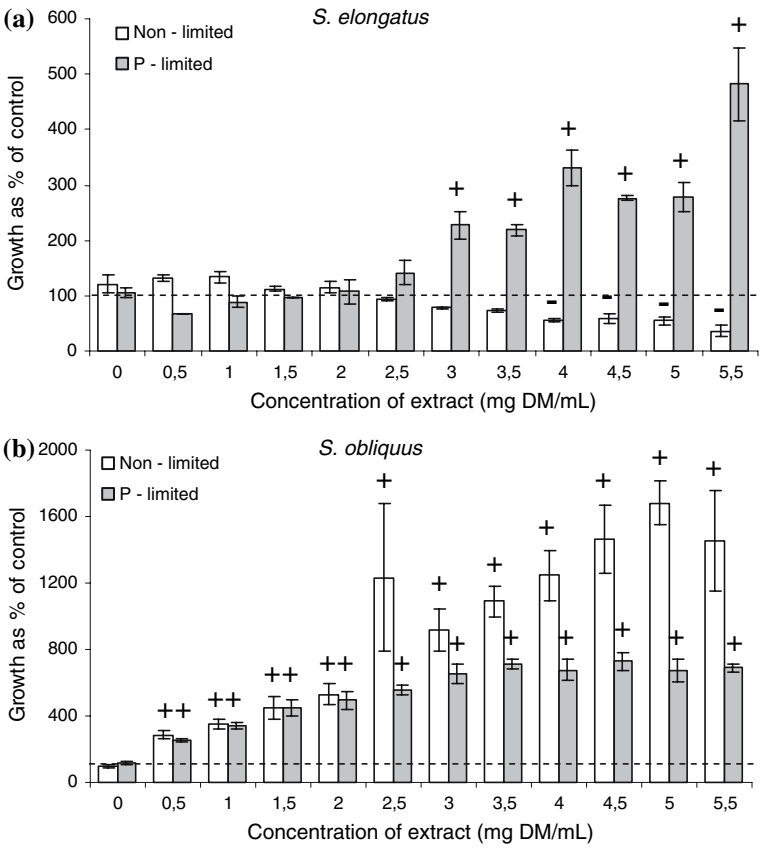

Fig. 3 Growth relative to the control in non-limited (white bars) and P-limited cultures (grey bars) of *Synechococcus elongatus* (**a**) and *Scenedesmus obliquus* (**b**) over a range of *Stratiotes* extract concentrations. Bars indicate average values ($n = 3$), error bars are 1 SE. + and – indicate significant inhibitory and stimulatory effects on the growth of the phytoplankton species

cyanobacteria than green algae being sensitive to allelopathic substances. Jasser (1994) and Körner & Nicklisch (2002) showed similar results, but Mulderij et al. (2005b) observed no significant differences in the sensitivity of cyanobacteria and green algae to allelopathic exudates from *Stratiotes*.

Table 3 Results agar diffusion assays with *Stratiotes* exudates from microcosms (set-up 2)

Incubation time [day]	Methanol fraction (%)	Clearing area diameter [cm]	
		Exudate	Control
1	50	(+)/(+)	0.6
	90	0.8/–	0.7
	100	–/+	–
2	50	–/+	–
	90	–/–	1
	100	–/–	–
3	50	(+)/–	(+)
	90	1.1/–	–
	100	–/–	–

SPE-enriched aliquot equivalent to 600 ml water (= 3.96 g FM) from the microcosms

( ), Inhibition less strong. Algal cells not completely absent in the clearing areas, only lower densities observed; –, no inhibition; +, slight inhibition

Similar to Fitzgerald (1969), P-limitation of *S. elongatus* did not increase the sensitivity of this cyanobacterium to *Stratiotes* 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 *Stratiotes* extract. The stimulatory effects on K-limited target cells may have occurred because the extract acted as K-source. The extract might have been 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

Table 4 Results agar diffusion assay with *A. variabilis* and *Stratiotes* exudates from three sites in ditches near Lake Naardermeer (C18 fractions)

SPE-enriched aliquot equivalent to 600 ml water from the ditch

[a] plants were still submerged

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

phytoplankton growth. Brammer (1979) and Brammer & Wetzel (1984) stated that the absence of phytoplankton in the presence of *Stratiotes* is caused by nutrient limitation rather than allelopathy. Recent measurements in situ, however, did not reveal differences in nutrient content between *Stratiotes* stands and *Stratiotes*-free ditch water (Mulderij et al., unpublished data). 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 *Stratiotes* exudates from laboratory cultures showed inhibitory effects after an incubation period of at least 3 days. Furthermore, exudates from a natural *Stratiotes* stand in Lake Naardermeer showed inhibitory effects, but some controls exhibited also a slight activity, which was, however, in general less strong than the exudates. Inhibitory effects were strongest when *Stratiotes* was still submerged, indicating that 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 photoautrophs is probably strongest. There are more indications for higher allelopathic activity in younger *Stratiotes* plants than in older ones (Mulderij et al., 2005a).

Compared to other studies (e.g., Gross et al., 1996; Gross et al., 2003) and based on the biomass of *Stratiotes* needed in both extract and exudate to achieve an inhibition, the allelopathic effects of *Stratiotes* shown in our study, are relatively weak. Mulderij et al. (2005a, b), on the contrary, observed inhibitory effects of *Stratiotes* exudates on phytoplankton growth ranging between 8 and 51%. The allelopathic activity of plant extracts does not reflect the potential allelopathic 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 *Stratiotes* exudates showed significant inhibitory effects of SPE-enriched *Stratiotes* medium on the cyanobacterium *A. variabilis*, indicating that *Stratiotes* may excrete the allelopathic substances. However, exudation experiments overall did not yet show a clear effect and differed from previous experiments using macrophyte filtrates (Mulderij et al., 2005a, b). 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., unpublished data).

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 *Stratiotes* are most likely not of phenolic nature. Smolders et al. (2000) showed that the phenolic content of *Stratiotes* leaves is relatively low compared to other macrophytes. Our results contradict those of Usenko et al.

(2002) who proposed that the algicidal activity in *Stratiotes* is based on phenolic acids. Our further characterization of the allelopathic compounds in *Stratiotes* indicated that the allelopathic substance is heat-stable and moderately lipophilic.

Conclusion

Our assays showed both stimulatory and inhibitory effects of *Stratiotes* 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 *Stratiotes*. The substance(s) responsible for allelopathic inhibition of phytoplankton are moderately lipophilic and most likely not phenolic compounds.

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

References

Alahari, A. & S. K. Apte, 2004. A novel potassium deficiency-induced stimulon in *Anabaena torulosa*. Journal of Biosciences 29: 153–161.

Anthoni, U., C. Christophersen, J. Madsen, S. Wium-Andersen & N. Jacobsen, 1980. Biologically active sulphur compounds from the green alga *Chara globularis*. Phytochemistry 19: 1228–1229.

Bloemendaal, F. H. J. L. & J. G. M. Roelofs, 1988. Waterplanten en Waterkwaliteit [in Dutch]. Stichting Uitgeverij KNNV, Utrecht, The Netherlands.

Brammer, E. S., 1979. Exclusion of phytoplankton in the proximity of dominant water soldier (*Stratiotes aloides*). Freshwater Biology 9: 233–249.

Brammer, E. S. & R. G. Wetzel, 1984. Uptake and release of K^+, Na^+ and Ca^{2+} by the water soldier, *Stratiotes aloides* L. Aquatic Botany 19: 119–130.

Burks, R. L., G. Mulderij, E. M. Gross, J. I. Jones, L. Jacobsen, E. Jeppesen & E. Van Donk (in press). Center stage: The crucial role of macrophytes in regulating trophic interactions in shallow lake wetlands. In Bobbink, R., B. Beltman, J. T. A Verhoeven & D. F. Whigham (eds), Wetlands: functioning, biodiversity, conservation and restoration. Ecological Studies.

Cook, C. D. K. & K. Urmi-König, 1983. A revision of the genus *Stratiotes* (Hydrocharitaceae). Aquatic Botany 16: 213–249.

De Geus-Kruyt, M. & S. Segal, 1973. Notes on the productivity of *Stratiotes aloides* in two lakes in the Netherlands. Polish Archive for Hydrobiology 20: 195–205.

Fitzgerald, G. P., 1969. Some factors in the competition or antagonism among bacteria, algae and aquatic weeds. Journal of Phycology 5: 351–359.

Flores, E. & C. P. Wolk, 1986. Production by filamentous, nitrogen-fixing cyanobacteria of a bacteriocin and of other antibiotics that kill related strains. Archives of Microbiology 145: 215–219.

Fowler, J., L. Cohen & P. Jarvis, 1998. Practical Statistics for Field Biology. John Wiley and Sons Ltd. Chichester, UK.

Fowler-Walker, M. J. & S. D. Connell, 2002. Opposing states of subtidal habitat across temperate Australia: consistency and predictability in kelp canopy-benthic associations. Marine Ecology Progress Series 240: 49–56.

Godmaire, H. & D. Planas, 1986. Influence of *Myriophyllum spicatum* L. on the species composition, biomass and primary productivity of phytoplankton. Aquatic Botany 23: 299–308.

Gross, E. M., 2003. Allelopathy of aquatic autotrophs. Critical Reviews in Plant Sciences 22: 313–339.

Gross, E. M., D. Erhard & E. Ivanyi, 2003. Allelopathic activity of *Ceratophyllum demersum* L. and *Najas marina* ssp. *intermedia* (Wolfgang) Casper. Hydrobiologia 506/509: 583–589.

Gross, E. M., H. Meyer & G. Schilling, 1996. Release and ecological impact of algicidal hydrolysable polyphenols in *Myriophyllum spicatum*. Phytochemistry 41: 133–138.

Gross, E. & R. Sütfeld, 1994. Polyphenols with algicidal activity in the submerged macrophyte *Myriophyllum spicatum* L. Acta Horticultura 381: 710–716.

Gross, E. M., C. P. Wolk & F. Jüttner, 1991. Fischerellin, a new allelochemical from the freshwater cyanobacterium *Fischerella muscicola*. Journal of Phycology 27: 686–692.

Guillard, R. R. L. & C. J. Lorenzen, 1972. Yellow-green algae with chlorophyllide *c*. Journal of Phycology 8: 10–14.

Jasser, I., 1995. The influence of macrophytes on a phytoplankton community in experimental conditions. Hydrobiologia 306: 21–32.

Jasser, I, 1994. Influence of *Ceratophyllum demersum* on phytoplankton community in experimental conditions. Verhandlungen der Internationalen Vereinigung für Theoretische und Angewandte Limnologie 25: 2291–2295.

Jüttner, F., J. Leonhardt & S. Mohren, 1983. Environmental factors affecting the formation of mesityloxide, dimethylallylic alcohol and other volatile compounds excreted by *Anabaena cylindrica*. Journal of General Microbiology 129: 407–412.

Kornatowski, J., 1938/84. Morphological forms of the water soldier. Acta Hydrobiologica 25/26: 145–156.

Körner, S. & A. Nicklisch, 2002. Allelopathic growth inhibition of selected phytoplankton species by submerged macrophytes. Journal of Phycology 38: 862–871.

Loomis, W. D. & Battaile J., 1966. Plant phenolic compounds and the isolation of plant enzymes. Phytochemistry 5: 423–438.

Lürling, M., 1999. The smell of water. Grazer-induced Colony Formation in Scenedesmus. PhD-thesis, University of Wageningen, The Netherlands.

Molisch, H., 1937. Der Einfluss einer Pflanze auf die andere - Allelopathie, Fisher, Jena.

Mulderij, G., W. M. Mooij & E. van Donk, 2005a. Allelopathic growth inhibition and colony formation of the green alga *Scenedesmus obliquus* by the aquatic macrophytes *Stratiotes aloides*. Aquatic Ecology 39: 11–21.

Mulderij, G., W. M. Mooij, A. J. P. Smolders & E. van Donk, 2005b. Inhibition of phytoplankton by allelopathic substances from *Stratiotes aloides*. Aquatic Botany 82: 284–296.

Mulderij, G., E. van Donk & J. G. M. Roelofs, 2003. Differential sensitivity of green algae to allelopathic sub-stances from *Chara*. Hydrobiologia 491: 261–271.

Planas, D., F. Sarhan, L. Dube, H. Godmaire & C. Cadieux, 1981. Ecological significance of phenolic compounds of *Myriophyllum spicatum*. Verhandlungen der Internationalen Vereinigung für Theoretische und Angewandte Limnologie 21: 1492–1496.

Reigosa, M. J., A. Sánchez-Moreiras, L. González, 1999. Ecophysiological approach in allelopathy. Critical Reviews in Plant Sciences 18: 577–608.

Scheffer, M, 1998. Ecology of shallow lakes. Chapman & Hall, London. 357 pp.

Schrader, K. K., M. Q. DeRegt, C. S. Tucker & S. O. Duke, 1997. A rapid bioassay for selective algicides. Weed Technology 11: 767–774.

Serrano, L., 1992. Leaching from vegetation of soluble polyphenolic compounds and their abundance in temporary ponds in the Donana National Park (SW Spain). Hydrobiologia 229: 43–50.

Smolders, A. J. P., L. H. T. Vergeer, G. van der Velde & J. G. M. Roelofs, 2000. Phenolic contents of submerged, emergent, and floating leaves of aquatic and semi-aquatic macrophyte species: why do they differ? Oikos 91: 307–310.

Suikkanen, S., G. O. Fistarol & E. Granéli, 2005. Effects of cyanobacterial allelochemicals on a natural plankton community. Marine Ecology Progress Series 287: 1–9.

Usenko, O. M., A. I. Sakevich & V. D. Palamarchuk, 2002. Influence of phenol acids of hydrophytes on the development of plankton algae. International Journal on Algae 4: 20–28.

van Donk, E. & W. J. van de Bund, 2002. Impact of submerged macrophytes including charophytes on phyto- and zooplankton communities: allelopathy vs other mechanisms. Aquatic Botany 72: 261–274.

Willis, R. J., 1985. The historical basis of the concept of allelopathy. Journal of the History of Biology 18: 71–102.

Wium-Andersen, S., U. Anthoni, C. Christophersen & G. Houen, 1982. Allelopathic effects on phytoplankton by substances isolated from aquatic macrophytes (Charales). Oikos 39: 187–190.

Wium-Andersen, S., 1987. Allelopathy among aquatic plants. Archiv für Hydrobiologie, Beiheft Ergebnisse der Limnologie 27: 167–172.