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Table: 1, Figs : 5

Toxicity of allelochemicals released by submerged macrophytes on phytoplankton

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ABSTRACT

The allelopathic mechanism of submerged macrophytes was investigated on the cyanobacterium *Microcystis aeruginosa* and the green alga *Pseudokirchneriella subcapitata*. The effect of the allelochemicals pyrogallic acid (PA), gallic acid (GA), (+)-catechin (CA), caffeic acid (CFA), protocatechuic acid (PCA), nonanoic acid (NA) and cis-9-octadecenoic acid (COA) was evaluated by flow cytometry on esterase activity, membrane integrity, chlorophyll *a* fluorescence, cell volume and internal cell complexity. The highest sensitivity was observed for esterase activity in both test species. NA damaged the cell membrane and severely altered the internal cell complexity of both species. PA changed the esterase activity of both species and significantly decreased the fluorescence of chlorophyll *a* in *M. aeruginosa* cells ($P < 0.05$). Both GA and PCA inhibited the fluorescence of chlorophyll *a* and caused morphological changes in *M. aeruginosa* ($P < 0.05$). CFA inhibited the esterase activity, fluorescence of chlorophyll *a* and cell volume in *M. aeruginosa* at 50 and 100 mg L^{-1} concentrations; In *P. subcapitata*, the fluorescence of chlorophyll *a* and cell volume was inhibited at 50 mg L^{-1} but was stimulated at 100 mg L^{-1}. COA changed the esterase activity. CA at 100 mg L^{-1} slightly inhibited the fluorescence of chlorophyll *a* in *M. aeruginosa* and stimulated the esterase activity in *P. subcapitata*. The responses observed for each parameter investigated were highly dependent on the specific inhibitory mechanism of each allelochemical. These allelochemicals act on multiple target sites.

Key words: Allelochemical, cell volume, chlorophyll *a* fluorescence, esterase activity, flow cytometry, internal cell complexity, membrane integrity, phytoplankton.

INTRODUCTION

Eutrophication of water bodies results in frequent occurrence of algal blooms. This situation is of concern because some algal bloom-forming species release toxins that cause ecological and health problems. Most methods proposed for the control these algal species are expensive, generate pollution or are not feasible (1). In this situation, submerged macrophytes may be an effective control strategy as they excrete secondary metabolites that inhibit the growth of phytoplankton species (11,16,18,12,20).

Myriophyllum spicatum L. (Haloragaceae) is highly competitive submerged macrophyte with strong allelopathic potential (13). It contains high concentrations of

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phenolics, especially gallo- and ellagitannins (9,10). Its secretions contained 14 kinds of phenols and polyphenols, which may significantly inhibit the phytoplankton growth (24). Nakai *et al*. (21,23) reported 4 polyphenols (pyrogallic acid, (+)-catechin, ellagic and gallic acids) and 3-fatty acids (nonanoic, cis-6-octadecenoic, and cis-9-octadecenoic acids) in the culture solution of *M. spicatum,* which inhibited the growth of *M. aeruginosa*. Nevertheless, little information is available about the physiological and biochemical responses of phytoplankton to allelochemicals stress. The concentrations of polyphenols and fatty acids excreted by *M. spicatum* were 5.2-76.6 µg L^{-1} at the density of 100 g fw L^{-1} after 3 days cultivation in laboratory (21,23). Lower concentrations of allelochemicals might be released by submerged macrophytes in the natural waters where the biomass density of submerged macrophytes was only about 10 g fw L^{-1} (4,15). The range of EC_{50} values of individual polyphenols and fatty acids were from 0.5 to 9.77 mg L^{-1} for different strains of *M. aeruginosa* (21,22,23,36). Thus, concentrations of allelochemicals secreted into natural environments by submerged macrophytes are much lower than EC_{50} values of individual allelochemicals (21,22,23). The real impact of these low concentrations on phytoplankton growth in natural waters needs to be elucidated.

Flow cytometry is a powerful tool for rapid and multiparameter analysis of individual cells in moving fluids (32) and has the ability to gather information simultaneously on the morphological, biochemical, and physiological effects of a toxicant. Therefore, it may provide a better insight into the mechanisms of toxicity (6,25).

In this study, we selected 5 polyphenols and 2 long-chain fatty acids excreted by *M. spicatum* with EC_{50} values < 10 mg L^{-1} (21,22,23,36). The physiological effects of these compounds on the cyanobacterium *M. aeruginosa* and the green alga *P. subcapitata* were measured by flow cytometry.

MATERIALS AND METHODS

I. Culture conditions

M. aeruginosa (toxic FACHB 942) and *P. subcapitata* (FACHB 271) were purchased from Freshwater Algae Culture Collection, Institute of Hydrobiology, Chinese Academy of Sciences. The unialgal *M. aeruginosa* were axenically cultivated in BG11 medium (29) under an irradiance of 22.5 µmol m^{-2} s^{-1}. *P. subcapitata* cells were grown axenically in ISO 8692 medium (17) under an irradiance of 60 µmol m^{-2} s^{-1}; all cultures were cultivated at 25 ± 2 ºC in an incubator with a photoperiod of 12 h light/12 h dark and shaken twice daily by hand at 12-h intervals. Exponentially growing cultures were used in all experiments.

II. Chemicals

To investigate toxic action of allelochemicals on *M. aeruginosa* and *P. subcapitata* cells, five polyphenols and two long-chain fatty acids were purchased: pyrogallic acid (PA, 99.5%, Chem Service, West Chester, PA, USA), gallic acid (GA, >96.0%, Acros Organics, Morris Plains, NJ, USA), (+)-catechin (CA, 98%, TCI, Tokyo, Japan), caffeic acid (CFA, Sigma-Aldrich, St. Louis, MO, USA), protocatechuic acid (PCA, 97%, Alfa Aesar, Heysham, Lancashire, UK), nonanoic acid (NA, Alfa Aesar, Heysham, Lancashire, UK) and cis-9-octadecenoic acid (COA, Sigma-Aldrich, St. Louis,

MO, USA). Dimethyl sulfoxide (DMSO >99.5%), fluorescein diacetate (FDA) and propidium iodide (PI) were obtained from Sigma-Aldrich (St. Louis, MO, USA).

III. Method of exposure

Polyphenol allelochemicals suffer fast autoxidation by dissolved oxygen in culture medium (21). For this reason high exposure concentrations and a short exposure time (4 h) were selected. For all allelochemicals, the concentrations used were: 10, 50, 100 mg L^{-1}. Exponentially growing cells of the two species were centrifuged (5000 rpm, 5 min), washed once and resuspended in respective culture medium as appropriate. Each flask was inoculated with 1×10^6 cells mL^{-1} and 4×10^5 cells mL^{-1} for *M. aeruginosa* and *P. subcapitata*, respectively. Samples were incubated with allelochemicals under an irradiance of 22.5 and 60 µmol m^{-2} s^{-1} for *M. aeruginosa* and *P. subcapitata*, respectively, at 25 ± 2 °C for 4 h. Then, 10 mL cultures were harvested by centrifugation and resuspended in 1 mL of phosphate buffered saline solution (50 mM, pH 7.8) for flow cytometry analysis. Cultures without allelochemicals were included as control cultures. DMSO concentration did not exceed 0.25% (v/v) in controls and treated flasks. Preliminary assays showed that the DMSO concentrations added had no effect on the test organisms. All experiments were carried out in triplicate.

IV. Staining protocol

The cells collected after exposure were stained with FDA and PI to determined esterase activity and membrane integrity, respectively. FDA dye diffuses into cells where it is cleaved by non-specific esterases to yield the fluorescent product fluorescein (8). The fluorescence signal detected is proportional to the esterase activity. The staining protocols for FDA developed by Geary et al. (8) and Regel et al. (26) were followed. Initial FDA stock solutions were prepared by dissolving FDA (Cat No.F-7378) in DMSO at 5 mg mL^{-1} and were stored at −20 °C.

Collected cells were incubated with FDA solution at room temperature for 7 min and 9 min for *M. aeruginosa* and *P. subcapitata*, respectively. The FDA concentration remained consistent at 40 µM (8). Negative controls were incorporated in each test to ensure that the esterase activities of both healthy and unhealthy phytoplankton cells were easily distinguishable from each other based on their FDA fluorescence intensity. One control consisted of healthy cells stained with FDA and another control contained the unhealthy cells subjected to heat-inactivation of esterases (100°C, 5 min for *M. aeruginosa*; 10 min for *P. subcapitata*) and then stained with FDA.

To determine whether inhibition of FDA fluorescence was mainly due to membrane disruption (reduced uptake of dye/reduced retention inside the cell) or inhibition of intracellular esterases (5), cells were also stained with PI, a fluorescent dye of nucleic acid. PI cannot enter the fully intact cell membranes, but enters and stains the nucleic acids in dead cells or with damaged/broken membranes (5,7). In this way, PI can be used to discriminate between live non-fluorescent cells and non-viable fluorescent cells (7). A stock solution of 1.5 mM PI (P-4170 in Milli-Q water) was added to allelochemicals/H_2O_2-exposed *M. aeruginosa* and *P. subcapitata* to give final concentration of 7.5 µM PI. After 5 min incubation, the samples were analysed by flow cytometry. Negative controls (i.e., heat-treated cells and unstained cells) were also included in each experiment.

V. Flow cytometric analysis

The fluorescence of stained cells was analysed using a Cytomics FC 500 flow cytometer (Beckman Coulter, Inc., Fullerton, CA, USA) equipped with a 488 nm argon laser. Green fluorescence from cells stained with FDA was collected in the FL1 channel (525 nm) and orange fluorescence from cells stained with PI was collected in the FL3 channel (610 nm). Chlorophyll *a* was detected by its red fluorescence in the FL4 channel (675 nm). The Beckman-Coulter FC-500 instrument also has two light-scatter detectors, which identify the cell morphology. The forward angle light scatter (FS <15°) detector provides information about the cell volume/size, while the side angle light scatter (SS, 90°) detector provides information about the internal cell complexity/granularity (32). Cells were 'accepted' for analysis, according to cell volume and positive fluorescence of chlorophyll *a*, to prevent interference from cell fragments, bacteria and dust particles. For each cytometric parameter investigated, at least 10^4 gated cells were analysed per sample and fluorescence measurements were expressed in a logarithmic scale. The results for each parameter (except membrane integrity) were calculated by the following formula:

$$\text{Percentage of control} = \frac{\text{Mean fluorescence/scatter intensity}_{[\text{allelochemicals-added}]}}{\text{Mean fluorescence/scatter intensity}_{[\text{control}]}}$$

VI. Statistical analysis

The normality of data was verified using the Kolmogorov-Smirnov test and the homogeneity of variance was checked using Levene's test. One-way ANOVA followed by Dunnet's post-test was used to determine the significant differences between the control and exposure groups. *P*-Values less than 0.05 were considered statistically significant. All the statistical analyses were performed using SPSS 13.0 (SPSS, Chicago, IL).

RESULTS AND DISCUSSION

Validation of all parameters

Typical flow cytometric FL1, FL3, FL4, FS, SS histograms of unstained, FDA/PI-stained control cells and stained heat-treated phytoplankton cells are shown in Fig.1. A clear separation (< 5% overlap in FL1 fluorescence intensity) between the FDA-stained control and two types of negative controls (unstained control and heat-treated FDA exposed populations) can be seen in Fig. 1a, f and k. As for PI staining, the Fig. 1 b and g showed the discrimination among unstained (0.0%), stained (0.5%), and stained heat-treated controls (100.0%) of a *M. aeruginosa* sample. Chlorophyll *a* fluorescence of phytoplankton was collected simultaneously with FDA fluorescence, because there were no significant differences in the fluorescence of chlorophyll *a* between FDA-stained and unstained populations (Fig. 1c and h). FS and SS were determined together with PI fluorescence, as the staining of PI dye didn't alter the volume and complexity of cells (Fig. 1d, e, i and j). These experiments were also done for *P. subcapitata* (data not shown) and indicated that all parameters can provide a reliable assessment of allelochemical effects.

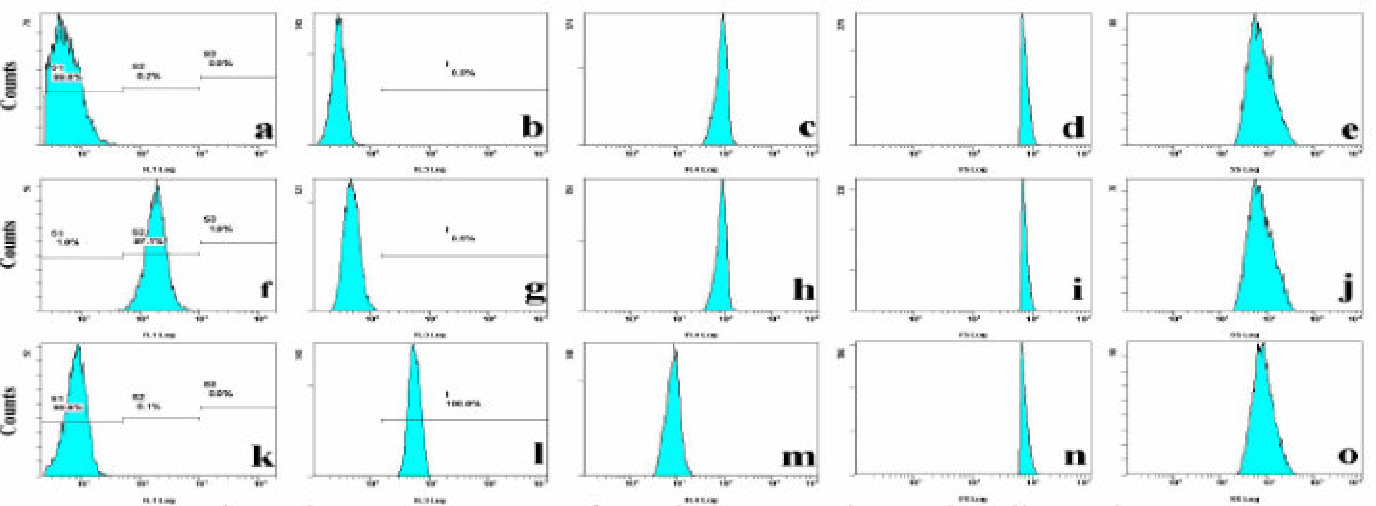

Figure 1. Flow cytometric diagrams showing representative examples of unstained and FDA/PI-stained control and stained heat-treated (100°C, 5min) *M. aeruginosa* sample. Flow cytometric FL1 histograms (a,f,k), FL3 histograms (b,g,l), FL4 histograms (c,h,m), FS histograms (d,i,n) and SS histograms (e,j,o) of unstained control sample (a–e), FDA/PI-stained control cells (f–j), and FDA/PI-stained and heat-treated control cells (k–o).

Table 1. Percentage of non-viable cells (stained with propidium iodide) of *M. aeruginosa* and *P. subcapitata* after 4 h of exposure to allelochemicals

Allelochemicals	Non-viable cells (%)							
	M. aeruginosa				*P. subcapitata*			
	Control	10 mg L^{-1}	50 mg L^{-1}	100 mg L^{-1}	Control	10 mg L^{-1}	50 mg L^{-1}	100 mg L^{-1}
Pyrogallic acid (PA)	0.6±0.2	0.4±0.3	0.6±0.1	0.6±0.1	0.6±0.3	0.5±0.2	0.7±0.1	0.8±0.1
Gallic acid (GA)	0.7±0.2	0.7±0.4	0.5±0.2	1.1±0.2	0.4±0.1	0.4±0.2	0.7±0.0	4.1±0.1*
(+)-Catechin (CA)	0.5±0.1	0.5±0.1	1.2±0.6	1.5±0.1	0.3±0.1	0.2±0.1	0.4±0.1	0.3±0.1
Protocatechuic acid (PCA)	1.3±0.8	-	1.1±0.5	0.5±0.0	0.3±0.1	0.4±0.1	0.7±0.1	6.1±0.4*
Caffeic acid (CFA)	1.0±0.4	-	0.2±0.1	0.8±0.5	0.4±0.1	0.5±0.4	0.5±0.1	4.1±0.1*
cis-9-Octadecenoic acid (COA)	0.6±0.3	-	0.7±0.1	0.4±0.2	0.4±0.1	0.7±0.3	0.4±0.0	0.5±0.1
Nonanoic acid (NA)	0.6±0.3	-	0.1±0.1	4.8±0.6*	0.4±0.1	0.4±0.1	0.6±0.1	91.5±0.3*

Significant differences over control at 0.05 ($P < 0.05$) are represented by an asterisk. Data are given as mean values ± standard error of the means.

Esterase activity

The PA, GA, PCA, CFA, COA, NA significantly decreased the levels of FDA fluorescence in cells of *M. aeruginosa* after an exposure of 4 h ($P < 0.05$) (Fig. 2). COA significantly decreased the FDA fluorescence at all concentrations assayed ($P < 0.05$). PA, GA, CFA inhibited the FDA fluorescence at 50 mg L^{-1} and higher concentrations. PCA and NA decreased the FDA fluorescence only at 50 mg L^{-1}. The decrease in fluorescence might be due to inhibition of intracellular esterase activity or membrane disruption (reduced uptake of the dye/reduced retention inside the cell) (5). However, Table 1 shows that only NA could significantly disrupt membrane integrity of *M. aeruginosa* cells at the highest concentration assayed (4.8% nonviable cells) compared to control (0.6% nonviable cells) ($P < 0.05$). Therefore, the decreased FDA fluorescence in *M. aeruginosa* after exposure to PA, GA, PCA, CFA, COA and NA likely resulted from the inhibition of intracellular esterase activity rather than from the loss of membrane integrity. In *P. subcapitata*, both an increase and a decrease in FDA fluorescence were observed (Fig. 2). Table 1 also shows that GA, PCA, CFA, NA significantly disrupted the membrane integrity of *P. subcapitata* at the highest concentration assayed ($P < 0.05$). PA inhibited the intracellular esterase activity at low concentrations and stimulated the esterase activity at higher concentrations. The FDA fluorescence in *P. subcapitata* gradually decreased with the increase in concentration of GA and NA. This was likely due to inhibition of esterase activity because the loss of membrane integrity was only observed at 100 mg L^{-1}. At high concentrations, the CA and COA increased FDA fluorescence in *P. subcapitata.* This situation may be due to an enhancement in esterase activity to adapt phytoplankton physiology to stress (25). The decrease in FDA fluorescence generated by CFA and PCA at the highest concentrations assayed may be due to either inhibiting esterase activity and/or changing in cell membrane permeability.

Membrane integrity

Table 1 showed that only NA disrupted the membrane integrity of *M. aeruginosa* cells with a 4.8% of nonviable cells at 100 mg L^{-1}. For *P. subcapitata*, GA, PCA, CFA and NA significantly altered membrane integrity ($P < 0.05$). NA had a strong effect on membrane integrity with about 91.5% of nonviable cells. This medium-chain monocarboxylic acid might affect normal physiology of plasma membrane by changing its spatial organization.(33,31).

Fluorescence of Chlorophyll *a*

PA, GA and NA inhibited the fluorescence of chlorophyll *a* in *M. aeruginosa* at all concentrations assayed (Fig. 3). PCA and CFA reduced the fluorescence at concentrations higher than 10 mg L^{-1} ($P < 0.05$). CA had a detrimental effect only at 100 mg L^{-1} and COA had no effect. For *P. subcapitata*, PA and CFA significantly changed the fluorescence of chlorophyll *a,* both with a slight reduction at low concentrations and an increase at high concentrations (Fig. 3). NA only showed a decreased effect on fluorescence of chlorophyll *a* in *P. subcapitata* at 50 mg L^{-1} after 4 h.

Fluorescence of chlorophyll *a* indicates the photochemical activity of photosystem II (PS II) in the phytoplankton (2,3,6). The maximum of fluorescence detected by flow cytometry corresponded to the PS II reaction centers locked in the Q_A

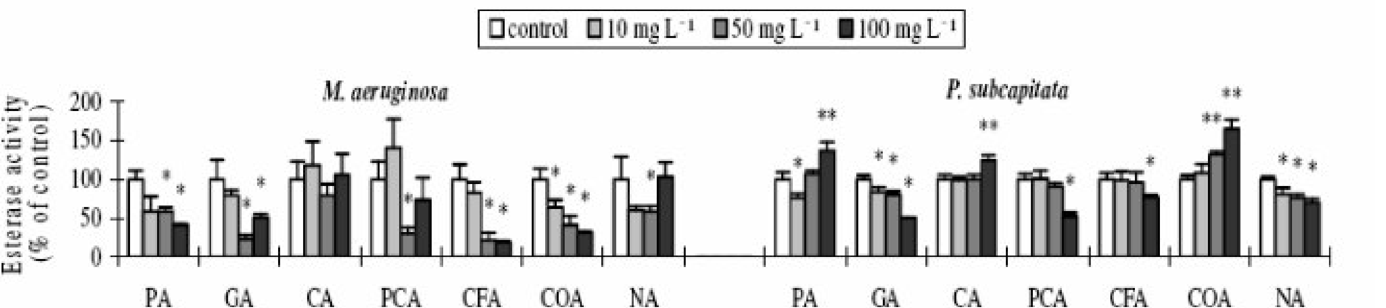

Figure 2. Effects of allelochemicals on esterase activity of *M. aeruginosa* and *P. subcapitata* cultures. The esterase activity of control cells was considered as 100%. *Significant decrease in esterase activity respect to controls ($P < 0.05$). **Significant increase in esterase activity respect to controls ($P < 0.05$). Each bar represents mean ± standard deviation (n=3-9). Abbreviations: Pyrogallic acid (PA), Gallic acid (GA), (+)- Catechin (CA), Caffeic acid (CFA), Protocatechuic acid (PCA), Nonanoic acid (NA), cis-9-Octadecenoic acid (COA).

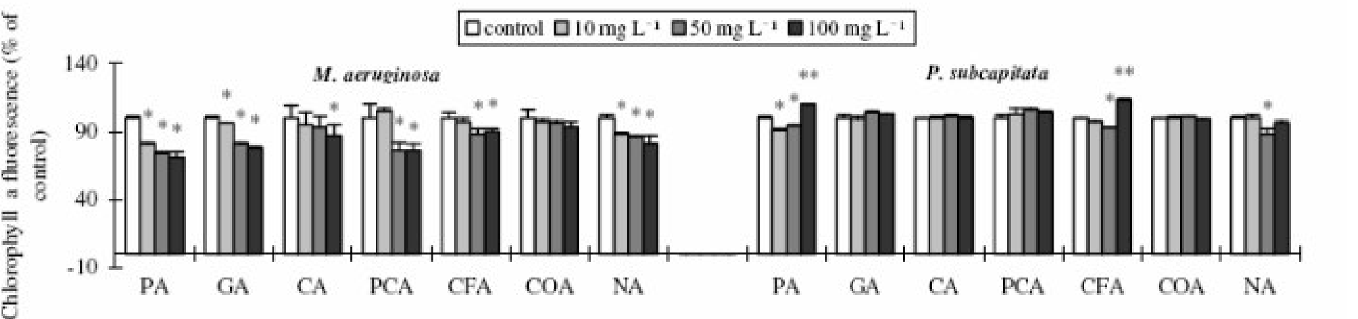

Figure 3. Effects of allelochemicals on fluorescence of chlorophyll *a* detected in cultures of *M. aeruginosa* and *P. subcapitata*. Intensity of fluorescence detected in control cells was considered as 100%. *Significant decrease in chlorophyll *a* fluorescence respect to controls ($P < 0.05$). **Significant increase in chlorophyll *a* fluorescence respect to controls; ($P < 0.05$). Each bar represents mean ± standard deviation (n=3-9). Abbreviations: Pyrogallic acid (PA), Gallic acid (GA), (+)- Catechin (CA), Caffeic acid (CFA), Protocatechuic acid (PCA), Nonanoic acid (NA), cis-9-Octadecenoic acid (COA).

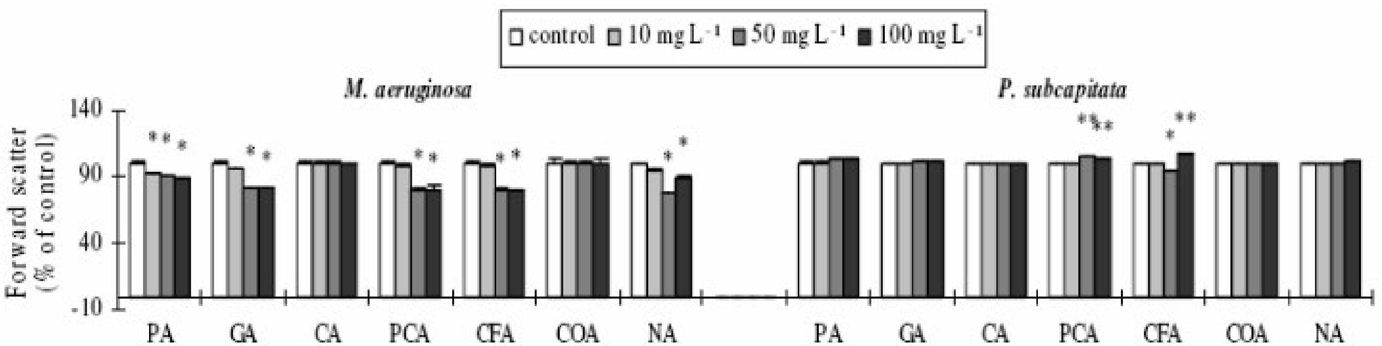

Figure 4. Effects of allelochemicals on forward scatter (related with cell volume) of *M. aeruginosa* and *P. subcapitata* cultures. Forward scatter intensity of control cells was considered as 100%. *Significant decrease in cell volume respect to controls ($P<0.05$) **Significant increase in cell volume respect to controls ($P < 0.05$). Each bar represents mean ± standard deviation (n=3-9). Abbreviations: Pyrogallic acid (PA), Gallic acid (GA), (+)- Catechin (CA), Caffeic acid (CFA), Protocatechuic acid (PCA), Nonanoic acid (NA), cis-9-Octadecenoic acid (COA).

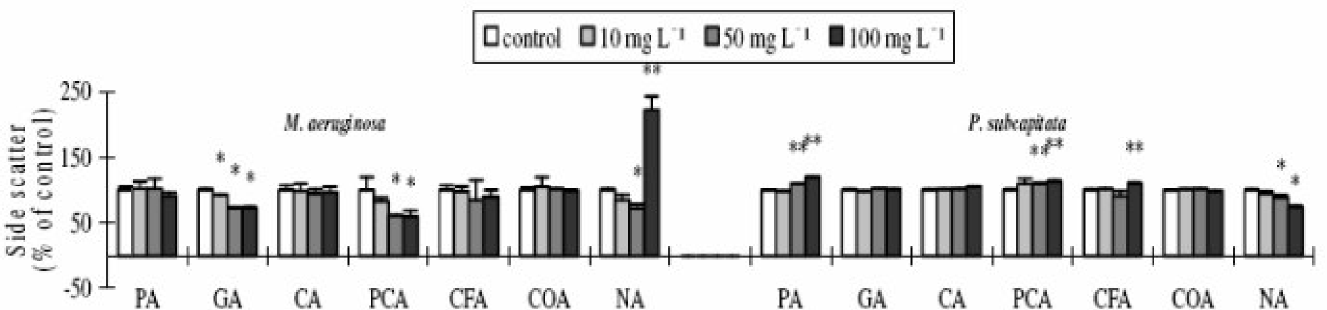

Figure 5. Effects of allelochemicals on side scatter (related with internal cell complexity) of *M. aeruginosa* and *P. subcapitata* cultures. The side scatter intensity of control cells was considered as 100%. *Significant decrease in internal cell complexity respect to controls ($P < 0.05$) **Significant increase in internal cell complexity respect to controls ($P < 0.05$). Each bar represents mean ± standard deviation (n=3-9). Abbreviations: Pyrogallic acid (PA), Gallic acid (GA), (+)- Catechin (CA), Caffeic acid (CFA), Protocatechuic acid (PCA), Nonanoic acid (NA), cis-9-Octadecenoic acid (COA).

state (35). Inhibition of the electron flow in this center decreases the fluorescence of chlorophyll *a* (30,7) as observed for *M. aeruginosa* cells affected by PA, GA, PCA, CFA and NA. Alternatively, inhibition of the acceptor side of PSII increases the fluorescence of chlorophyll *a* (30,6), as can be observed for *P. subcapitata* cultures with PA and CFA. Zhu et al. (36) also observed a significant decrease in PS II and whole electron transport chain activities of *M. aeruginosa* exposed to PA and GA by pulse amplitude modulated (PAM) fluorometry and the Clark-type oxygen electrode. Shao et al. (31) reported that NA could affect the photosynthetic processes of *M. aeruginosa* by inhibiting the oxygen evolution and decreasing the contents of phycobilisomes. Thus, fluorescence of chlorophyll *a* measured by flow cytometry is a reliable and convenient indicator for screening the toxic effects of allelochemicals on phytoplankton at PS II level.

Cell morphology

Cell volume/cell size of the two species exposed to several concentrations of the allelochemicals is shown in Fig. 4. PA, GA, PCA, CFA and NA significantly decreased cell volume of *M. aeruginosa* (Fig. 4) while CA and COA had no effect ($P > 0.05$). On *P. subcapitata*, a significant reduction in cell volume was only found after exposure to PCA (at 50 mg L^{-1} and 100 mg L^{-1}) and CFA (at 50 mg L^{-1}). Cell volume was less sensitive to allelochemicals than the other parameters investigated. Franklin et al. (6) evaluated the effect of copper on cell size and rate of cell division by flow citometry. Cell size was not as sensitive to copper as cell division after an exposure of 1- to 4-h. However, the former was sensitive and in good agreement with the latter at longer periods of exposure (48 and 72 h). Therefore, the parameter of cell size measured by flow cytometry to study toxicity of allelochemicals was suitable as a chronic toxicity test endpoint.

Changes in internal cell complexity/granularity of the two species were also detected (Fig. 5). GA and PCA decreased this parameter on *M. aeruginosa.* NA significantly decreased ($P < 0.05$) and strongly increased (2.23 times) internal cell complexity at 50 mg L^{-1} and 100 mg L^{-1}, respectively (Fig. 5). For *P. subcapitata*, PA and PCA caused a concentration-dependent increase in internal cell complexity. CFA could only increase internal cell complexity at 100 mg L^{-1} while NA caused a decrease at concentrations higher than 50 mg L^{-1} (Fig. 5). The strong increase in cell complexity observed for some allelochemicals may relate to an increase in membrane ultrastructure (5). Franklin et al. (6) reported an increase in the side scatter signal for *P. subcapitata* by flow cytometry over 72 h of copper exposure which was related with ultrastructural changes mainly in lysosomes and vacuoles. However, there is scarce information about the effects of these allelochemicals on phytoplankton.

CONCLUSIONS

The allelochemicals assayed exerted their action on multiple target sites of *M. aeruginosa* and *P. subcapitata*. The sensitivity of different parameters was highly dependent of the specific toxicological mechanism of each allelochemical (14). Nevertheless, further investigations at lower concentrations and in long-term exposures are needed to clearly understand the allelochemical impact in macrophyte allelopathy.

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