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Comparative toxicity of the pesticides carbofuran and malathion to the freshwater flagellate *Euglena gracilis*

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Abstract Pesticides are toxic chemicals used for agricultural as well as non-agricultural purposes. The toxicity of pesticides does not remain limited to the site of application but they also cause toxicity to non-target organisms in terrestrial as well as in aquatic environments. This study discusses the comparative toxicity of a carbamate (carbofuran) and an organophosphorus (malathion) pesticide to the freshwater flagellate *Euglena gracilis* during short- and long-term exposures. To evaluate the toxicity of the pesticides, different parameters of the flagellate, like cell density, motility, swimming velocity, cell shape, gravitactic orientation, photosynthetic efficiency, and concentration of light harvesting pigments, were used as end points. Carbofuran was found to be more toxic to *E. gracilis* than malathion and adversely affected almost all the tested parameters in short- and long-term experiments. The only significant adverse effect by malathion could be demonstrated on the swimming velocity of cells in short-term experiments. The adverse effects of the pesticides were more pronounced during short-term than during long-term exposure.

Keywords *Euglena gracilis* · Carbofuran · Malathion · Pesticides · Toxicity

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Introduction

The use of pesticides has been an integral part of modern agricultural practices. In addition, pesticides are also used for non-agricultural purposes. The worldwide annual consumption of pesticides has been estimated to be about two million tons (Abhilash and Singh 2009). Of the total pesticides applied, an estimated quantity of only 0.1% reaches the target organisms and the remaining 99.9% disperse through air, soil and water, thus resulting in the pollution of natural ecosystems (Pimentel 1995). The toxicity of pesticides is usually not limited to the target organisms for which they are applied but they can adversely affect non-target organisms in terrestrial and aquatic ecosystems (Dobsikova 2003; Ma et al. 2002; Rachid et al. 2008). Pesticides can interfere with various processes like cell growth, photosynthesis, respiration, biosynthetic reactions, and molecular composition in microorganisms (DeLorenzo et al. 2001).

Carbofuran is a broad-spectrum systemic carbamate pesticide and is used as an insecticide, acaricide and nematicide throughout the world (Dobsikova 2003). Due to its wide spread applications, relatively high water solubility (320 mg l^{-1} at 20°C) and minimal adsorption to soil, carbofuran has been frequently reported in ground, surface and rain waters (Dobsikova 2003; Trotter et al. 1991). The compound degrades within 1–8 weeks in neutral and moderate alkaline water depending upon water temperature (Anton et al. 1993) but is stable in acid waters (Dobsikova 2003). Carbofuran has been found to be toxic to various non-target organisms in the aquatic and terrestrial environment (Anton et al. 1993; Dobsikova 2003; Megharaj et al. 1993).

Malathion is a non-systemic, wide spectrum organophosphate insecticide, used for agricultural as well as

non-agricultural purposes (Patil and David 2008). There are various reports regarding its occurrence in water as well as in air (Newhart 2006; Patil and David 2008). Like carbofuran, the degradation of malathion in aquatic environments is also pH dependent i.e., the compound degrades sooner at alkaline pH than at acidic pH (Newhart 2006). Malathion is toxic to aquatic organisms and can cause severe metabolic disturbances in non-target species (Newhart 2006; Patil and David 2008). For example, it was found to inhibit hatching in *Macrobrachium lamarrei* and *Danio rerio* (Ali et al. 2007; Nguyen and Janssen 2001) and caused behavioural and respiratory dysfunctions in *Labeo rohita* (Patil and David 2008). Malathion can affect aquatic organisms at environmental concentrations lower than 100 $\mu g\ l^{-1}$, and concentrations at these levels have been routinely detected at presently recommended application rates (Newhart 2006).

Both carbofuran and malathion cause their toxic effects by inhibiting the acetylcholinesterase (AChE) activity in the nervous system. Apparently, they are not expected to be toxic to aquatic plant and algae as these organisms do not have the target site for these chemicals (i.e., AChE). However, there are reports that both these pesticides can adversely affect various processes in different groups of algae (Anton et al. 1993; Dobsikova 2003; Kar and Singh 1978; Megharaj et al. 1989; Poorman 1973; Tandon et al. 1988; Tiwari et al. 2001). Different algae have different sensitivity to pesticides (Ma et al. 2002). The mechanism of toxicity or mode of action of pesticides in non-target microorganisms may not be the same as in the target organisms (DeLorenzo et al. 2001).

Euglena gracilis is a freshwater unicellular flagellate found in many aquatic habitats, especially shallow eutrophic ponds. This flagellate has characteristic features of both animals and plants. The organism has a fast growth and can be cultured easily and economically both under autotrophic and heterotrophic conditions. These characteristics make *Euglena* a model organism intermediate between plants and animals for use in ecotoxicology (Tahedl and Häder 2001). *Euglena* has extensively been used as a test organism for toxicity assessment of various chemicals like heavy metals and other biologically active compounds (Danilov and Ekelund 2001; Einicker-Lamas et al. 2002; Ekelund 1993; Gajdosova and Reichrtova 1996; Navarroa et al. 1997; Tahedl and Häder 2001). However, data regarding toxicity of pesticides to *Euglena* can hardly be found. Therefore, such a study was needed to address the applicability of *E. gracilis* for toxicity assessment of pesticides in aquatic environment.

Euglena gracilis is powered by a single flagellum inserted at the front end and responds to light and gravity (Häder 1987; 1991). *E. gracilis* orientation in the water column depends on external physical and chemical parameters like light and gravity in search of a region optimal for growth and reproduction (Häder 1987; Häder and Liu 1990; Häder and Vogel 1990; Lebert and Häder 1999; Richter et al. 2003). Both phototaxis and gravitaxis are based on active physiological mechanisms (Häder 1997; Kamphuis 1999; Lebert et al. 1996; Machemer and Bräucker 1996; Richter et al. 2001; Tahedl et al. 1998). The species of the genus *Euglena* are capable of changing their cell shape in response to various chemicals in the water (Conforti 1998). Motility, cell shape and gravitactic orientation in *E. gracilis* have been successfully applied for the bioassessment of various water pollutants like heavy metals, organic pollutants, and wastewaters (Ahmed and Häder 2010; Pettersson and Ekelund 2006; Richter et al. 2007; Tahedl and Häder 1999).

The introduction of PAM (pulse amplitude modulation) fluorometry (using chlorophyll fluorescence) has provided a simple and rapid technique for measurement of photosynthetic efficiency in algae and green plants. Studies reveal that fluorescence yields of plants were closely related to the photosynthetic efficiency measured by other methods like carbon metabolism and gas exchange measurements (Genty et al. 1989; Walker et al. 1983). The fluorescence parameters in photosynthetic organisms change in response to various environmental factors and can be used as sensitive biomarkers in ecotoxicology studies (Conrad et al. 1993; Dewez et al. 2008; Dorigo and Leboulanger 2001; Ekelund and Aronsson 2007; Gonzáles-Moreno et al. 1997; Juneau and Popovic 1999). Photosynthesis and pattern of light harvesting pigments in *Euglena* were reported to be sensitive to environmental stressors and can be used as biomarkers in evaluating the toxicity of different substances (Ahmed and Häder 2010; Azizullah et al. 2010; Gonzáles-Moreno et al. 1997; Nass and Ben Shaul 1973).

The aim of this study was to evaluate the effects of the pesticides carbofuran and malathion on various parameters like motility, swimming velocity, gravitactic orientation, cell shape, photosynthetic efficiency, light harvesting pigments, and cell growth (during long-term exposure) of *E. gracilis* during short- and long-term exposure and to compare their toxicity towards this flagellate.

Materials and methods

Cell culture and growth conditions

Axenic cultures of the freshwater flagellate *E. gracilis* KLEBS, strain *Z*, obtained from the algal culture collection at the University of Göttingen, Germany (Schlösser 1994), were used in this study. Cultures were grown in mineral medium (Starr 1964) inoculated from a static culture grown in organic medium (Checcucci et al. 1976). All cultures

were grown under continuous light of 20 W m^{-2} from mixed cool white and warm tone fluorescent lamps at a temperature of about 20°C.

Exposure to pesticides

External standard solutions of the pesticides carbofuran (98%) (Sigma-Aldrich, CAS-NO. 1563-66-2) and malathion (96.1%) (Sigma-Aldrich, CAS-NO. 121-75-5) were used in this study.

(a) Already grown (1–2 week old) cultures of *E. gracilis* were exposed to different concentrations (0, 6, 12, 21, 34, and 50 mg l^{-1}) of both the pesticides and the effects on various parameters were evaluated at five different incubation times (0, 1, 6, 24, and 72 h). All treatments (except 0 h measurements with ECOTOX; see "Motility and orientation analysis" section) were done in three independent replicates in 100-ml Erlenmeyer flasks with a total culture volume of 50 ml. Cultures were incubated under the same conditions as described in "Cell culture and growth conditions" section. These experiments were termed as short-term experiments.

(b) The cultures of *E. gracilis* were grown in media having different concentrations of the pesticides (0, 0.75, 1.5, 3, 6, 12, 21, 34, and 50 mg l^{-1}) and the effect on various parameters was observed after 7 days of growth. Cultures were grown in three independent replicates in 100-ml flasks with 50 ml of total volume in each flask. All cultures were inoculated with an initial cell density of about 80,000 cells per ml. The other growth conditions were similar as described in "Cell culture and growth conditions" section. These experiments were termed as long-term experiments.

Concentrations above 50 mg l^{-1} could not be tested for pesticides due to the solubility limits of malathion in water (130 mg l^{-1}). We used a 100 mg l^{-1} solution of malathion which was further diluted (ECOTOX makes the lowest dilution (highest concentration) in 1:1) resulted in the highest tested concentration of 50 mg l^{-1}. To test higher concentration of malathion, the only option was to use some organic solvent (e.g., ethanol, acetone or ethyl ether) for making malathion solution. However, an organic solvent can not be used in these experiments because motility factors of *Euglena* (especially gravitactic orientation) are very sensitive to organic solvents. For example, ethanol strongly impaired the *r*-value (precision of gravitactic orientation) in *Euglena* giving an EC_{50} value of 0.0008% (Tahedl and Häder 1999). Due to such high sensitivity of *Euglena* to organic solvents, an organic solvent can not be used for making a solution of pesticides in these experiments; therefore, higher concentrations could not be tested for malathion. For ease in comparison, same concentrations of carbofuran as for malathion were tested.

Determination of growth rate

Cells were fixed with 70% ethanol in a ratio of 1:9 (ethanol:culture) and were counted with the help of a Thoma chamber under the light microscope with a ×25 objective and ×8 eye piece. Growth rates were calculated as $G = ((N_s–N_0)/N_0)/t)$, where G is the growth rate, N_s is the number of cells at day seven, N_0 is the initial number of cells (day zero) and t is time (7 days).

Motility and orientation analysis

The motility and orientation parameters of *E. gracilis* cells were measured by using the automatic biotest ECOTOX (Tahedl and Häder 1999). The hardware of the ECOTOX consists of a miniaturized microscope connected to a Firewire camera (for a schematic diagram of the device see Tahedl and Häder 1999). Three stepper motor pumps are used to pump the cell culture, rinsing water and toxin solution (test sample) into the observation cuvette through a mixing chamber. The microscope is fixed horizontally, so that gravitactic orientation of the organisms swimming in the vertical cuvette can be assayed. In order to avoid light-induced orientation and/or interference from photosynthetically produced oxygen by the monitoring light, an infrared diode ($\lambda = 875$ nm) is used as a light source. The system operates in real time and tracks a virtually unlimited number of cells in parallel. The software uses the vectors of the tracks to calculate various parameters like percent motility, swimming velocity, cell compactness, percentage of cells moving upwards and *r*-value. The motility parameter gives the percentage of cells moving at a speed equal to or faster than the minimum velocity set in the program. The parameter velocity gives the mean speed (swimming velocity) of cells in μm s^{-1}. The cell compactness or form factor describes the cell shape and has the lowest value of one when the outline of the object is a circle (absolutely round cell) and increases as the cell increases in length. The parameter upward gives the percentage of cells which are moving upward in the cuvette (±90° around the vertical direction upward). The *r*-value is a statistic parameter which describes the precision of gravitactic orientation of the cells and ranges from zero (when the cells are moving randomly) to one (when all the cells are moving in a single direction).

The ECOTOX system has three different operation modes; control, single toxin, and online mode. In the control mode the cell culture is pumped into the observation cuvette and the various parameters are measured. This

mode is used to examine cell behaviour of control cultures or cultures treated with some toxin solution. In the single toxin mode, a control sample (undiluted or diluted with water (1:1) depending on the setting of the software) is measured first followed by measurement of a culture mixed with a toxin (test solution). In this mode five dilutions (1:31, 1:15, 1:7, 1:3, and 1:1) of the toxin stock solution are made automatically and the effects on various parameters are assayed. The software plots the calculated parameters and compares the data with those of a previous control measurement, which is carried out immediately before the sample measurement using unpolluted water instead of the test sample. The resultant effects of the toxin are expressed as percent inhibition for all parameters. In the online mode control measurements are alternated with sample measurements at fixed time intervals.

For direct exposure (0 h) experiments with ECOTOX, the "single toxin mode" with the setting of automatically decreasing dilutions (1:31, 1:15, 1:7, 1:3, and 1:1) was used. A stock solution (100 mg l^{-1}) of each pesticide was provided which was diluted by the ECOTOX automatically into five dilutions resulting in five concentrations 6, 12, 21, 34, and 50 mg l^{-1}. All measurements were performed in three independent replicates. For further measurements (1, 6, 24, and 72 h) cultures were treated with the same five concentrations of pesticides as described in "Exposure to pesticides" section, and measurements were made with the option "one control" for all concentrations and controls and percent inhibition of all parameters was calculated.

The filling time of the cuvette was 75–100 s and the rinsing time was 45 s. In each experiment the cells were tracked for 3 min. Minimum areas for objects to be included in the vector analysis were set to 400 μm^2 and maximum to 2,000 μm^2. Minimum speed, at which the cells were considered motile, was set to 15 $\mu m\ s^{-1}$. In order to avoid the effect of light, the cells of *E. gracilis* were incubated in darkness for 30 min before making measurements (Aronsson and Ekelund 2005; Azizullah et al. 2010).

Photosynthesis measurement

The fluorescence parameters of cultures were measured using a portable pulse amplitude modulated (PAM) fluorometer (PAM 2000, Walz, Germany). A complex fiber optics probe is used for guiding the measuring light, the actinic light and the saturating pulse to the sample. Fluorescence is measured by applying μs-long light pulses of measuring light for fluorescence excitation, and the measurement of photosynthetic yield is carried out by application of a saturating light pulse. Various photosynthetic parameters can be measured by the instrument using different settings of the software. In this study, the single saturating pulse method was used for measuring the quantum yield of photosystem II.

Pigments determination

Chlorophyll was extracted in 80% acetone (Osafune and Sumida 2006; Sumida et al. 2007). Aliquots of cultures were centrifuged at 6,000 g for 10 min at 4°C. An appropriate volume of acetone (80%) was added to the precipitated cells and kept at 4°C for 60 min to extract the pigments. The mixtures were then centrifuged at 6,000 g for 10 min at 4°C to pellet the cell debris. The absorbance spectrum of the supernatant (extract of pigments) was measured from 400 to 750 nm using a spectrophotometer (UV-2550, Shimadzu). Chlorophyll *a*, *b* and total carotenoids were calculated according to Lichtenthaler and Wellburn (1983).

Data treatment

All the tests were performed in three independent replicates. Statistical analysis of data (calculation of means and standard deviation) was done using Microsoft Excel. The significance of differences between the treatments and the control was calculated using one-way ANOVA (Dunnett's test as post hoc test). The difference was considered to be significant if the *P* value was smaller than 0.05 ($P < 0.05$) for a given parameter. For motility and orientation parameters the effect was considered significant if the inhibition values of all three replicates exceeded the threshold value of the ECOTOX for a given parameter (11.4% for motility, 12.3% for *r*-value, 3.1% for upward, 3.4% for compactness, and 6.8% for velocity) (Tahedl 2000).

Results

Short-term effects

The effect of pesticides on motility, orientation, and morphological parameters were measured using the ECOTOX. The ECOTOX device measures all these parameters in parallel, analyses the data automatically and expresses the effects of the test substance on various parameters as percent inhibition (in comparison to the control). The data of control measurements for all parameters are shown in Table 1. The effects of carbofuran on the various parameters measured in *E. gracilis* in short-term experiments are shown in Table 2. Immediately upon exposure to carbofuran the motility, orientation, and shape of the cells were disturbed, but the observed effects were not significant due to large variation in response except for gravitactic

Table 1 Motility, velocity, cell compactness, upward swimming, and *r*-value of control cultures

	Motility (%)	Velocity (μm s^{-1})	Compactness	Upward (%)	*r*-value
Carbofuran					
0 (h)	91.73 ± 6.34	60.01 ± 1.8	5.39 ± 0.27	80.07 ± 1.27	0.53
1 (h)	79.36 ± 3.89	54.18 ± 2.88	4.38 ± 0.16	77.0 ± 4.56	0.49
6 (h)	77.67 ± 9.41	54.31 ± 0.87	4.20 ± 0.09	73.53 ± 3.85	0.43
24 (h)	87.76 ± 3.60	71.04 ± 1.86	4.67 ± 0.06	76.46 ± 6.22	0.49
72 (h)	85.18 ± 2.12	60.65 ± 1.11	4.40 ± 0.09	80.77 ± 2.18	0.55
Malathion					
0 (h)	87.14 ± 5.12	71.30 ± 3.17	6.67 ± 0.62	79.71 ± 6.97	0.54
1 (h)	91.49 ± 2.25	65.59 ± 3.41	5.55 ± 0.21	73.03 ± 0.95	0.41
6 (h)	87.68 ± 3.41	60.42 ± 1.34	5.55 ± 0.24	73.29 ± 3.04	0.43
24 (h)	82.99 ± 5.28	62.80 ± 3.34	6.72 ± 0.32	78.25 ± 3.07	0.51
72 (h)	87.63 ± 2.32	77.06 ± 1.09	6.39 ± 0.63	82.22 ± 3.33	0.58

Values given are means ± standard deviation of three independent replicates

Table 2 Effects of carbofuran on motility, swimming velocity, cell compactness (cell shape), and gravitactic orientation (upward swimming and *r*-value) in *E. gracilis* after 0–72 h exposure

Carbofuran (mg l^{-1})	Motility	Velocity	Cell compactness	Upward swimming	*r*-value
0 (h)					
6	1.00 ± 4.80	−4.40 ± 1.87	3.30 ± 14.87	8.02 ± 5.48	10.13
12	2.58 ± 6.44	−1.94 ± 4.54	4.55 ± 11.91	1.55 ± 0.67	3.55
21	11.60 ± 8.91	−1.35 ± 0.57	12.99 ± 5.32	5.44 ± 10.08	9.87
34	12.29 ± 6.13	1.39 ± 6.52	11.64 ± 9.90	5.70 ± 6.11	12.02
50	12.60 ± 12.30	4.37 ± 10.25	11.51 ± 8.43	10.46 ± 2.36*	24.97*
1 (h)					
6	−2.37 ± 9.42	−0.47 ± 2.23	2.31 ± 1.03	0.53 ± 5.86	1.76
12	−0.45 ± 2.93	−2.84 ± 3.42	2.64 ± 3.10	5.69 ± 4.70	14.24*
21	2.38 ± 3.22	−1.14 ± 0.71	1.60 ± 3.89	0.96 ± 8.97	2.54
34	2.40 ± 7.72	−2.46 ± 3.36	0.51 ± 3.62	9.29 ± 8.32	22.52*
50	−4.33 ± 9.81	−4.44 ± 1.64	−3.80 ± 4.32	0.19 ± 7.80	−0.48
6 (h)					
6	2.31 ± 15.12	−0.45 ± 1.08	−1.88 ± 3.32	−1.98 ± 6.60	−7.81
12	8.15 ± 18.14	−0.56 ± 0.15	−3.43 ± 3.03	−2.50 ± 2.15	−9.11
21	5.37 ± 18.97	−1.21 ± 2.06	−4.73 ± 4.04	2.87 ± 6.07	8.20
34	7.54 ± 15.30	1.91 ± 5.52	−5.31 ± 4.75	4.64 ± 8.85	13.34*
50	−8.64 ± 11.17	−2.28 ± 2.89	−8.26 ± 2.33	6.40 ± 4.56	16.13*
24 (h)					
6	3.17 ± 6.72	−1.03 ± 1.71	1.47 ± 5.69	4.98 ± 9.30	12.58*
12	2.01 ± 4.40	−0.93 ± 0.58	2.14 ± 0.34	6.79 ± 3.13*	18.43*
21	4.47 ± 7.74	5.51 ± 1.31	2.55 ± 3.37	9.16 ± 6.23*	22.61*
34	13.02 ± 14.11	16.50 ± 7.42*	4.44 ± 4.92	9.88 ± 7.94	27.08*
50	24.54 ± 15.34	30.11 ± 6.54*	10.31 ± 1.99*	13.34 ± 10.50*	36.86*
72 (h)					
6	−2.68 ± 4.13	−4.61 ± 3.12	0.68 ± 2.66	5.10 ± 3.70	13.05*
12	−1.98 ± 8.21	−3.00 ± 5.06	−2.04 ± 1.70	4.15 ± 1.11	8.99
21	11.32 ± 4.06	12.24 ± 10.70	0.91 ± 4.27	4.21 ± 0.49*	8.57
34	20.54 ± 10.94	16.00 ± 3.52*	9.58 ± 4.51*	11.08 ± 8.00	26.30*
50	32.94 ± 7.00*	24.86 ± 1.19*	10.46 ± 1.12*	17.64 ± 6.32*	41.98*

Data are presented as percent inhibition compared to the control. Values given are means ± standard deviation of three independent replicates. *Asterisks* indicate significant effects as compared to the control

orientation (upward swimming and *r*-value). Both upward swimming of cells and *r*-value were significantly affected at 50 mg l^{-1} of carbofuran immediately upon exposure. Motility of the cells was not affected by carbofuran at incubation up to 6 h. However, after 24 and 72 h exposure, the motility was inhibited by more than 20% at the highest

two concentrations tested. Swimming velocity of the cells was not inhibited up to 6 h exposure to carbofuran but afterward (24 and 72 h); a significant decrease in cell velocity was measured at concentrations above 21 mg l^{-1}. Carbofuran was also shown to affect cell shape of *E. gracils* and to cause a significant rounding of the cells after 24 and 72 h of exposure at concentrations above 34 and 21 mg l^{-1}, respectively. After 1 and 6 h incubation, the upward swimming of cells (gravitaxis) was only slightly affected by carbofuran. However, after 24 and 72 exposures, upward swimming of cells was significantly inhibited by carbofuran at concentrations exceeding 12 mg l^{-1}. Since *r*-value (precision of gravitactic orientation) is a statistic parameter, error bars/standard deviation can not be calculated and the effect was considered significant if the mean inhibition was above the threshold value of the ECOTOX (12.3%). After 1 h incubation the effect of carbofuran on *r*-value was not very dose-dependent. However, afterward a strong inhibition of *r*-value was observed which was significant even at the lowest tested concentration (6 mg l^{-1}) after 24 h and thereafter. A 50 mg l^{-1}, carbofuran caused $\approx$37 and 42% inhibition of *r*-value after 24 and 72 h, respectively.

The data regarding the effect of malathion on motility, velocity, cell shape, and gravitactic orientation of the cells are shown in Table 3. The effects of malathion were less pronounced as compared to carbofuran. The only significant inhibition caused by malathion was that of swimming velocity after 24 and 72 h at the highest concentrations. The motility and cell shape of the cells were not significantly affected by malathion irrespective of concentrations and incubation times. Cultures treated with malathion showed slightly more precise gravitactic orientation than the control cultures as evident from inhibition data obtained for upward swimming cells and *r*-value (the negative sign of inhibition value indicates that the effect on the parameter was positive). However, this effect was not dose-dependent and no regular trend was observed.

Table 3 Effects of malathion on motility, swimming velocity, cell compactness (cell shape), and gravitactic orientation (upward swimming and *r*-value) in *E. gracilis* after 0–72 h exposure

Malathion (mg l^{-1})	Motility	Velocity	Cell compactness	Upward swimming	*r*-value
0 (h)					
6	4.32 ± 7.75	−2.85 ± 3.25	5.94 ± 2.93	−1.37 ± 7.44	−5.66
12	5.70 ± 9.45	−0.72 ± 4.47	4.36 ± 6.44	0.96 ± 8.43	−1.49
21	10.68 ± 8.27	2.42 ± 4.41	6.44 ± 5.08	−6.00 ± 5.39	−18.77*
34	12.54 ± 16.74	6.25 ± 7.89	7.48 ± 12.71	−2.88 ± 17.54	−17.12*
50	3.58 ± 10.60	6.51 ± 3.79	4.58 ± 6.78	−2.83 ± 9.05	−11.72
1 (h)					
6	−0.75 ± 4.21	−0.50 ± 2.42	1.06 ± 1.27	−0.38 ± 1.86	−0.69
12	−2.68 ± 2.93	−2.22 ± 3.92	−0.49 ± 1.61	−0.54 ± 0.63	−2.11
21	−1.36 ± 3.11	−2.69 ± 5.09	−1.58 ± 1.94	0.44 ± 3.47	2.41
34	−3.04 ± 3.76	−3.23 ± 5.64	−4.17 ± 5.02	−1.42 ± 4.03	−4.50
50	−2.27 ± 2.75	−3.94 ± 4.96	−5.60 ± 5.63	1.46 ± 6.62	4.63
6 (h)					
6	2.71 ± 3.52	2.62 ± 3.69	4.96 ± 5.49	−4.37 ± 9.29	−11.78
12	0.11 ± 12.13	2.09 ± 4.17	1.42 ± 5.62	2.91 ± 9.32	8.03
21	−1.15 ± 6.10	0.55 ± 2.73	0.58 ± 4.01	−0.53 ± 4.66	−3.64
34	−2.95 ± 5.35	1.67 ± 2.75	−0.31 ± 1.87	−2.85 ± 9.16	−9.36
50	4.21 ± 10.04	3.91 ± 4.15	0.03 ± 3.32	2.15 ± 8.90	3.86
24 (h)					
6	4.38 ± 5.24	3.12 ± 2.63	9.33 ± 3.54	2.01 ± 5.52	4.29
12	−2.70 ± 2.11	4.91 ± 5.26	1.84 ± 7.06	0.78 ± 6.64	2.41
21	11.49 ± 7.09	14.15 ± 8.37	2.82 ± 14.43	−1.59 ± 11.99	−6.91
34	4.56 ± 2.08	16.58 ± 4.27*	−2.71 ± 7.35	−8.60 ± 5.08	−25.18*
50	14.59 ± 6.07	19.76 ± 2.62*	1.28 ± 8.80	−5.14 ± 7.70	−16.79*
72 (h)					
6	−3.92 ± 5.18	−1.58 ± 0.71	−0.29 ± 6.97	−3.35 ± 2.89	−8.38
12	−3.08 ± 3.07	−3.03 ± 1.97	−1.92 ± 8.51	−1.68 ± 2.84	−5.48
21	−1.42 ± 2.62	2.74 ± 3.21	1.07 ± 10.39	−0.67 ± 2.39	−2.09
34	−1.68 ± 1.80	5.02 ± 4.08	0.56 ± 10.41	−2.75 ± 2.21	−6.87
50	1.76 ± 3.43	11.85 ± 1.78*	1.64 ± 9.37	0.28 ± 1.51	1.04

Data are presented as percent inhibition as compared to the control. Values given are means ± standard deviation of three independent replicates. *Asterisks* indicate significant effects as compared to the control

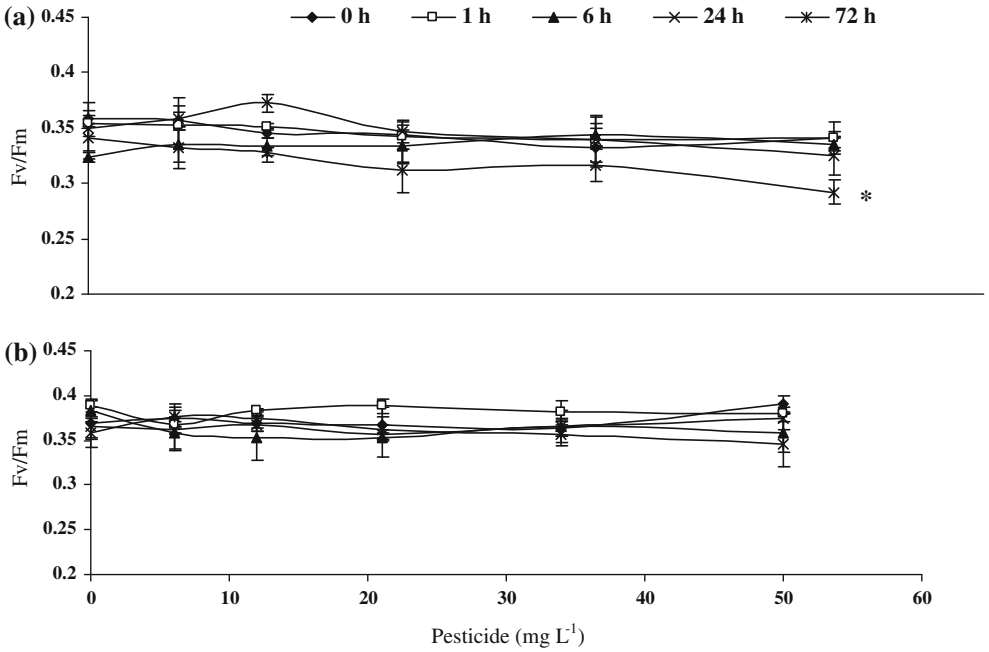

Fig. 1 Quantum yield of photosystem II (Fv/Fm) of *E. gracilis* cultures after exposure to carbofuran (**a**) and malathion (**b**). Each point represents the mean value of three independent replicates and the *error bars* represent standard deviation. *Asterisk* indicates a significant difference as compared to the control

Table 4 Concentrations (μg ml^{-1}) of chlorophyll *a* (Chl *a*), chlorophyll *b* (Chl *b*), and total carotenoids (total carot.) in *E. gracilis* cultures after 6, 24, and 72 h of exposure to carbofuran

Carbofuran (mg l^{-1})	6 (h)			24 (h)			72 (h)		
	Chl *a*	Chl *b*	Total carot.	Chl *a*	Chl *b*	Total carot.	Chl *a*	Chl *b*	Total carot.
0	5.15 ± 0.16	0.65 ± 0.03	1.22 ± 0.05	5.35 ± 0.11	0.59 ± 0.04	1.36 ± 0.02	5.45 ± 0.49	0.57 ± 0.07	1.51 ± 0.13
6	5.35 ± 0.07	0.64 ± 0.02	1.28 ± 0.02	5.45 ± 0.03	0.63 ± 0.01	1.36 ± 0.02	5.48 ± 0.16	0.59 ± 0.03	1.51 ± 0.05
12	5.27 ± 0.27	0.63 ± 0.02	1.27 ± 0.08	5.27 ± 0.26	0.59 ± 0.06	1.34 ± 0.04	5.65 ± 0.06	0.58 ± 0.02	1.57 ± 0.03
21	4.99 ± 0.17	0.63 ± 0.03	1.18 ± 0.04	5.37 ± 0.23	0.61 ± 0.02	1.36 ± 0.08	5.92 ± 0.26	0.65 ± 0.05	1.65 ± 0.04
34	5.01 ± 0.13	0.62 ± 0.03	1.20 ± 0.02	5.05 ± 0.46	0.54 ± 0.06	1.31 ± 0.12	5.43 ± 0.33	0.59 ± 0.04	1.56 ± 0.08
50	4.87 ± 0.18	0.62 ± 0.02	1.15 ± 0.05	5.42 ± 0.34	0.63 ± 0.04	1.39 ± 0.08	5.58 ± 0.17	0.61 ± 0.01	1.60 ± 0.05

Values given are means ± standard deviation of three independent replicates

The results for the quantum yield of photosystem II (Fv/Fm) are shown in Fig. 1 (a for carbofuran, b for malathion). No significant effect was observed on Fv/Fm except by the highest concentration of carbofuran (50 mg l^{-1}) after 72 h of incubation.

In short-term experiments, the light harvesting pigments (Chl *a*, *b* and total carotenoids) were determined after three incubation times (6, 24, and 72 h). Both pesticides did not significantly affect the pigment concentration irrespective of concentration and incubation time (Tables 4 and 5).

Long-term effects

After 7 days of growth of *E. gracilis* in media with pesticides carbofuran significantly reduced cell growth at 50 mg l^{-1} but malathion did not affect cell growth (Fig. 2a,b). Both carbofuran and malathion did not affect motility, velocity, and cell compactness in long-term exposure (Table 6). The significant inhibition of gravitactic orientation was shown at a concentration of 21 and 34 mg l^{-1} of carbofuran. Malathion induced a slight positive effect on gravitactic orientation of the cells just as in short-term experiments (Table 6). The quantum yield of photosystem II (Fv/Fm) was affected neither by carbofuran nor malathion (Fig. 3). A slight decrease in the concentrations of light harvesting pigments was shown by the pesticides but this effect was not significant. The only significant decrease was observed in the concentration of total carotenoids at 50 mg l^{-1} of carbofuran (Table 7).

Table 5 Concentrations (μg ml^{-1}) of chlorophyll *a* (Chl *a*), chlorophyll *b* (Chl *b*), and total carotenoids (total carot.) in *E. gracilis* cultures after 6, 24, and 72 h of exposure to malathion

Malathion (mg l^{-1})	6 h			24 h			72 h		
	Chl *a*	Chl *b*	Total carot.	Chl *a*	Chl *b*	Total carot.	Chl *a*	Chl *b*	Total carot.
0	7.71 ± 0.13	0.88 ± 0.01	1.74 ± 0.04	7.75 ± 0.38	0.94 ± 0.05	1.84 ± 0.08	8.23 ± 0.43	1.00 ± 0.05	2.09 ± 0.10
6	7.88 ± 0.32	0.94 ± 0.03	1.77 ± 0.09	8.18 ± 0.26	1.00 ± 0.04	1.92 ± 0.07	8.40 ± 0.19	0.97 ± 0.03	2.14 ± 0.07
12	7.52 ± 0.09	0.88 ± 0.04	1.69 ± 0.03	8.13 ± 0.31	1.00 ± 0.04	1.94 ± 0.10	8.55 ± 0.42	1.03 ± 0.07	2.18 ± 0.13
21	7.74 ± 0.20	0.90 ± 0.03	1.74 ± 0.03	8.14 ± 0.25	1.05 ± 0.03	1.91 ± 0.03	8.53 ± 0.04	0.99 ± 0.03	2.20 ± 0.04
34	7.55 ± 0.35	0.90 ± 0.05	1.68 ± 0.08	7.89 ± 0.35	1.00 ± 0.08	1.87 ± 0.09	7.63 ± 1.01	0.91 ± 0.17	1.94 ± 0.25
50	7.73 ± 0.20	0.89 ± 0.09	1.74 ± 0.02	8.14 ± 0.06	1.05 ± 0.04	1.92 ± 0.03	7.93 ± 0.18	0.94 ± 0.03	2.01 ± 0.07

Values given are means ± standard deviation of three independent replicates

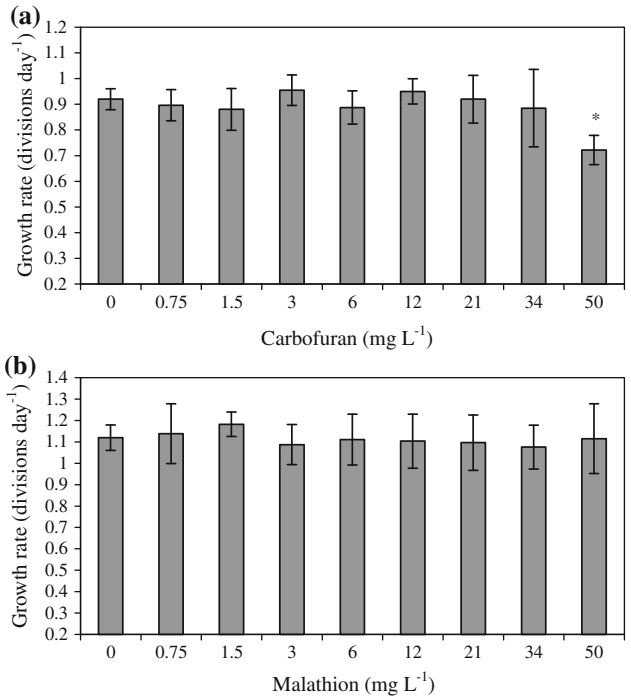

Fig. 2 Growth rates of *E. gracilis* cultures after 7 days of growth in carbofuran (**a**) and malathion (**b**). Each *bar* represents the mean value of three independent replicates and the *error bars* represent standard deviation. *Asterisk* indicate a significant difference as compared to the control

Discussion

Motility is a characteristic feature of many organisms and is an important physiological factor for their survival (Tahedl and Häder 2001). In the short-term exposure to pesticides the motility factors (% motility and swimming velocity) of *E. gracilis* were adversely affected by the pesticides after incubation of 24 h or longer. The motility of *E. gracilis* is powered by a single flagellum. How the toxic pollutants/chemicals affect motility in flagellates is not fully known and can not be explained by a general mechanism because different substances may affect motility in different ways. A toxic substance may affect motility of flagellates by interacting directly with the flagellum/flagella of the cell. According to Aizdaicher and Markina (2006), the impairment of motility in algae can be attributed to a blockage of ATP synthesis, depletion of ATP resources in the cell as well as to a disturbance of ionic homeostasis of cells.

It was also observed that carbofuran significantly affected the cell compactness (treated cells became rounder) after 24 or 72 h of incubation. Numerous studies have reported that the species of the genus *Euglena* change their cell shape in response to increasing concentrations of water pollutants and other physical or chemical stressors (Azizullah et al. 2010; Conforti 1998; Mikolajczyk and Diehn 1978; Murray 1981; Takenaka et al. 1997). Many freshwater algae are known to change their shape to a globular form in response to osmotic stress, and the globular form is therefore considered to be the stress-adapted form (Takenaka et al. 1997). A chemical may affect the cell shape by exerting osmotic stress or interacting with the plasma membrane of the cell. In the present case, the interaction of carbofuran with membrane properties (Megharaj et al. 1993) can be a possible reason for the observed effect on cell shape.

Gravitactic orientation is an important physiological phenomenon which helps *Euglena* in finding a place in the water column suitable for growth and reproduction (Häder 1987; Häder et al. 1999; Häder and Griebenow 1988; Häder and Vogel 1990; Lebert et al. 1997). The cells of *Euglena* show a precise negative gravitaxis, i.e., the cells swim upward in the water column, particularly in the absence of light stimulus (Häder and Vogel 1990). In this study, carbofuran was shown to strongly impair gravitactic orientation of the cells. For example, after 72 h incubation, it caused ≈42% impairment of the *r*-value at the highest concentration. Many environmental stressors like heavy metals, herbicides, industrial wastewaters, and increased UV radiation have been shown to invert the negative gravitaxis in *E. gracilis* into a positive gravitaxis

Table 6 Inhibition of motility, swimming velocity, cell compactness (cell shape), and gravitactic orientation (upward swimming and *r*-value) in *E. gracilis* by the pesticides carbofuran and malathion after 7 days of growth

Pesticides (mg l^{-1})	Motility	Velocity	Cell compactness	Upward swimming	*r*-value
Carbofuran					
0.75	1.64 ± 2.63	0.45 ± 1.01	0.75 ± 12.22	3.08 ± 3.33	8.22
1.5	0.26 ± 1.98	−1.06 ± 1.38	−2.02 ± 2.93	1.59 ± 2.31	2.80
3	1.01 ± 0.63	−0.39 ± 2.00	1.61 ± 3.48	0.63 ± 3.41	2.79
6	0.24 ± 0.33	−2.75 ± 4.08	−2.79 ± 1.65	0.66 ± 4.27	−0.01
12	−0.80 ± 1.42	−2.28 ± 3.28	−3.84 ± 13.61	2.85 ± 2.07	5.87
21	−1.09 ± 0.99	−1.48 ± 0.38	0.87 ± 11.22	5.58 ± 2.15	15.91*
34	−0.08 ± 1.83	−5.41 ± 2.33	−0.62 ± 10.70	6.15 ± 2.04	13.41*
50	1.74 ± 0.65	−5.42 ± 3.01	−3.20 ± 4.45	2.79 ± 1.45	7.35
Malathion					
0.75	1.35 ± 1.60	−3.39 ± 1.47	−3.05 ± 3.54	−4.36 ± 9.01	−13.16*
1.5	0.11 ± 1.45	−2.93 ± 4.84	−3.78 ± 5.28	−4.86 ± 7.13	−11.65
3	2.16 ± 0.50	−3.55 ± 6.14	−3.78 ± 4.11	−1.66 ± 3.56	−3.69
6	2.26 ± 1.86	−3.78 ± 6.80	−3.90 ± 1.97	−4.77 ± 11.21	−14.72*
12	2.22 ± 0.96	−2.59 ± 6.13	−2.58 ± 3.62	−6.17 ± 7.49	−17.50*
21	1.17 ± 0.08	−1.80 ± 7.93	−2.64 ± 3.90	−4.89 ± 7.01	−9.14
34	2.57 ± 2.04	−2.64 ± 7.40	1.26 ± 1.40	−3.09 ± 10.19	−3.43
50	2.11 ± 0.53	−0.82 ± 7.49	0.19 ± 1.10	−6.56 ± 9.23	−9.07

Data are presented as percent inhibition compared to the control. Values given are means ± standard deviation of three independent replicates. *Asterisks* indicate significant effects as compared to the control

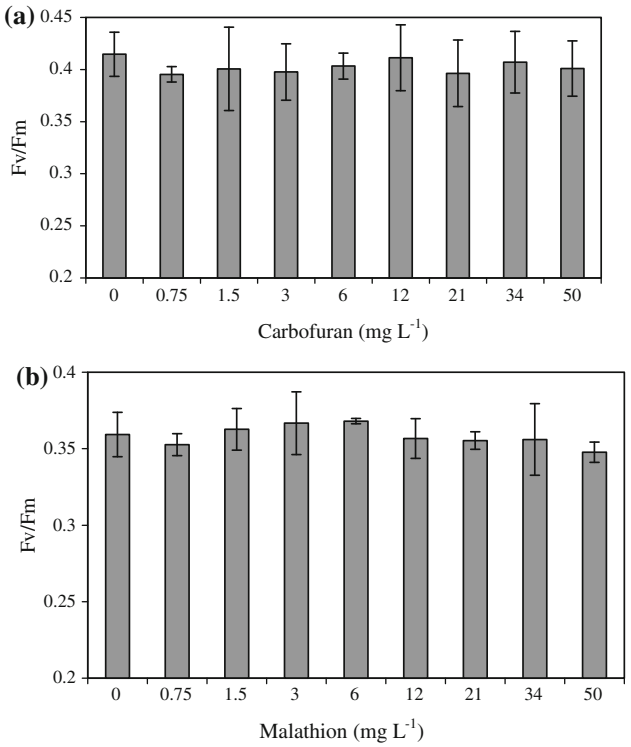

Fig. 3 Quantum yield of photosystem II (Fv/Fm) of *E. gracilis* cultures after 7 days of growth in carbofuran (**a**) and malathion (**b**). Each *bar* represents the mean value of three independent replicates and the *error bars* represent standard deviation

(Ahmed and Häder 2010; Ahmed and Häder 2011; Pettersson and Ekelund 2006; Richter et al. 2007; Tahedl and Häder 2001). On the other hand, malathion-treated cultures were shown to have slightly more precise gravitactic orientation as compared to the controls. However, this effect of malathion was not very pronounced and no regular or dose-dependent trend was observed. A previous study, however, showed that micromolar concentrations of some heavy metals like cadmium or lead clearly inverted the positive gravitaxis into a negative gravitaxis in young cultures of *E. gracilis* (Stallwitz and Häder 1994). Studies reveal that the mechanism of mechano-sensitive ion channels in the cell membrane is involved in gravitaxis (Häder et al. 2003; Lebert et al. 1999). Toxic chemicals and water pollutants can directly interfere with these channels and thus impair the orientation of cells in the gravity field (Tahedl and Häder 1999).

The quantum yield of photosystem II (Fv/Fm) in *E. gracilis* was significantly reduced at 50 mg l^{-1} of carbofuran after 72 h of incubation, but malathion did not affect the quantum yield. Megharaj et al. (1993) reported that carbofuran slightly reduced photosynthetic activity (determined by $^{14}CO_2$ uptake) in *Chlorella vulgaris* and *Nostoc linckia* at concentrations above 5 mg l^{-1} and the observed effect was very pronounced at 50 mg l^{-1}. Hammouda (1999) observed that carbofuran did not affect photosynthetic oxygen evolution in *Anabaena doliolum* after 18 and 24 h incubation. However, after 48 h incubation they observed a decrease in photosynthetic oxygen evolution at 50 and 100 mg l^{-1} of carbofuran. These observations are in agreement with ours showing that carbofuran needs some time to affect the photosynthetic functions of the algae.

The inhibition of growth rate has been a very common and standard endpoint for the toxicity assessment of

Table 7 Concentrations (μg ml^{-1}) of chlorophyll *a* (Chl *a*), chlorophyll *b* (Chl *b*), and total carotenoids (total carot.) in *E. gracilis* cultures after 7 days of growth at different concentrations of carbofuran and malathion

Pesticides (mg l^{-1})	Carbofuran			Malathion		
	Chl *a*	Chl *b*	Total carot.	Chl *a*	Chl *b*	Total carot.
0	6.74 ± 0.66	0.92 ± 0.11	2.23 ± 0.04	6.32 ± 0.34	0.90 ± 0.07	2.30 ± 0.07
0.75	6.21 ± 0.37	0.91 ± 0.06	2.12 ± 0.20	6.10 ± 0.24	0.86 ± 0.06	2.18 ± 0.05
1.5	5.86 ± 0.30	0.79 ± 0.04	1.95 ± 0.29	6.19 ± 0.24	0.87 ± 0.04	2.30 ± 0.09
3	6.05 ± 0.19	0.84 ± 0.09	2.00 ± 0.12	6.08 ± 0.22	0.91 ± 0.09	2.17 ± 0.05
6	6.20 ± 0.18	0.87 ± 0.05	2.00 ± 21	6.12 ± 0.05	0.89 ± 0.02	2.21 ± 0.03
12	6.29 ± 0.21	0.82 ± 0.04	1.94 ± 0.15	6.00 ± 0.33	0.88 ± 0.05	2.17 ± 0.07
21	6.27 ± 0.46	0.88 ± 0.05	2.07 ± 0.10	5.92 ± 0.03	0.85 ± 0.04	2.18 ± 0.07
34	6.84 ± 0.60	0.97 ± 0.10	2.00 ± 0.17	5.71 ± 0.14	0.81 ± 0.01	2.17 ± 0.10
50	5.97 ± 0.10	0.87 ± 0.12	1.75 ± 0.16*	5.87 ± 0.40	0.82 ± 0.09	2.18 ± 0.10

Values given are means ± standard deviation of three independent replicates

chemicals. We observed that after 7 days of growth, malathion did not affect the cell growth in *E. gracilis* while carbofuran caused a significant decrease in cell growth at 50 mg l^{-1}. Carbofuran has been shown to affect cell growth in diverse groups of algae. For example, at the same concentration of carbofuran as ours (50 mg l^{-1}), slight inhibition of cell growth has been reported in *Nostoc muscorum* (Kar and Singh 1978) and *Chlorella pyrenoidosa* (Anton et al. 1993), and above 50 mg l^{-1} strong inhibition of cell growth was shown for both organisms. Similarly, 5 mg l^{-1} of carbofuran was found to be lethal for *Synechococcus elongatus* (Megharaj et al. 1989), and a decrease in cell numbers in *Chlorella vulgaris* was observed at concentrations of carbofuran exceeding 5 mg l^{-1} (Megharaj et al. 1993). In contrast, a concentration of 20 mg l^{-1} of carbofuran significantly increased the cell number of *Scenedesmus bijugatus* (Megharaj et al. 1989). Dobsikova (2003) calculated a 72 h IC_{50} for growth inhibition in *Raphidocelis subcapitata* to be 0.1582 mg l^{-1} of carbofuran in comparison to 96 h IC_{50} of 208.48 mg l^{-1} for *Chlorealla pyrenoidosa* (Anton et al. 1993). All these reports led to the conclusion that carbofuran affects growth in diverse groups of algae; however, different organisms differ in their sensitivity to carbofuran regarding their growth efficiency.

In our experiments light harvesting pigments in *Euglena* were not very sensitive to the pesticides studied, and the only significant decrease was observed in total carotenoids at the highest concentration of carbofuran after 7 days of growth. Contrarily, literature reveals that carbofuran at concentrations above 10 or 20 mg l^{-1} caused a decrease in chlorophyll *a* in some species of green algae and cyanobacteria (Hammouda 1999; Megharaj et al. 1993). This difference in response of light harvesting pigments in *Euglena* as compared to the cited studies can be attributed to differential sensitivity of different algal species to insecticide stress.

Comparing the results of short- and long-term tests, it is evident that the toxic effects of pesticides were more pronounced in short-term tests. For example, in short-term tests, carbofuran significantly inhibited swimming velocity at concentrations above 21 mg l^{-1} after 24 and 72 h but in long-term test velocity was not affected even at the highest tested concentration (50 mg l^{-1}). Similarly, 50 mg l^{-1} of carbofuran caused 24–32% inhibition of motility after 24 and 72 h in short-term tests but the same concentration caused only 2% inhibition of motility in long-term experiment. In addition, carbofuran caused a decrease in cell compactness and photosynthesis in short-term tests after 24 and/or 72 h but no inhibition of these parameters was caused by carbofuran during long-term tests. Similarly, in short-term experiments, malathion significantly inhibited swimming velocity after 24 h at a concentration of 34 mg l^{-1} or above but no inhibition of velocity was observed in long-term tests even at the highest tested concentration of malathion. The low toxicity in long-term exposure suggests that the cells of *Euglena* may have some adaptation mechanism which allows them to tolerate pesticide stress. Similar adaptation has been reported for *Euglena* upon exposure to some heavy metals. For example, the photosynthetic efficiency in *Euglena* treated with aluminum for a long time was significantly higher than in *Euglena* treated with the same concentrations of aluminum for a short time (Danilov and Ekelund 2002). Similarly, Stallwitz and Häder (1993) observed that the short-term inhibitory effects of lead on motility in *Euglena* were stronger than the inhibitory effects after long time. This behaviour was attributed to the adaptation/acclimatisation capacity of *E. gracilis* to long-term stress (Danilov and Ekelund 2002; Stallwitz and Häder 1993).

The overall results of this study show that malathion caused a significant inhibition of only swimming velocity in short-term experiments and did not inhibit any other parameter in *Euglena*. In comparison, carbofuran was found to inhibit motility, velocity, cell shape, photosynthesis as well as cell growth. The observed low toxicity of malathion to *E. gracilis* in this study is in agreement with

previous studies which reported low toxicity of malathion to various species of algae. For example, in a disc and spot test assays by Mallison and Cannon (1984), at a concentration of 2,000 mg l^{-1} of malathion, no inhibitory effect was noticed on the lawns of *Plectonema boryanum*. Comparing the toxicity of malathion and endosulfan Tandon et al. (1988) observed that *Anabaena* survived up to 500 mg l^{-1} of malathion but was completely bleached at 50 mg l^{-1} of endosulfan. Other organophosphorus pesticides, like dichlorovos, diazinon phosphomidon and quinolphos were also found to pose low toxicity to algae (Singh 1973; Subramanian et al. 1994). However, in a study by Tiwari et al. (2001) various species of non-heterocystous cyanobacteria isolated from rice fields were found to be very sensitive to malathion. In their study, about half out of a total of 28 different isolates could tolerate up to 25 mg l^{-1} of malathion but some species like *Oscillatoria annae*, *Oscillatoria agardhii*, *Limnothrix redeckei*, and *Phormidium angustissimum* could not tolerate malathion above 5 mg l^{-1}. Their results show that the non-heterocystous cyanobacteria are more sensitive to malthion than other groups of algae. Some studies also support the higher sensitivity of some cyanobacterial species than green algae to other groups of insecticides. For example, carbofuran, after 32 days of exposure, caused 100% inhibition of growth and survival in cyanobacterium *Synechococcus elongatus* at a concentration of 5 mg l^{-1} but the green alga *Scenedesmus bijugatus* survived and grew in up to 50 mg l^{-1} of carbofuran (Megharaj et al. 1989). The same study reported that after 32 days of exposure *S. bijugatus* could survive and grow up to 20 mg l^{-1} of carbaryl but *S. elongatus* could not survive above 2 mg l^{-1} of the same insecticide. Contrarily, the same group of authors in their other studies reported that *S. bijugatus* was more sensitive than *S. elongates* to synthetic pyrethroids, fenvalerate, and cypermethrin and organophosphorous insecticides like monocrotophos and quinalphos (Megharaj et al. 1986; Megharaj et al. 1987). Similarly, Ma et al. (2006) reported that green algae were more sensitive than cyanobacteria to the pesticides carbosulfan and propoxur. Literature survey reveals that algal species show differential sensitivities to insecticides toxicity and it is difficult to make a generalized conclusion which group of algae is more sensitive to insecticides. It is also not known what makes algae differentially sensitive to pesticides; however, metabolic capability can be the main factor (Megharaj et al. 1993).

Conclusion

Carbofuran was found to be more toxic than malathion to *E. gracilis*. In short-term exposure, carbofuran adversely affected cell shape, motility, velocity, gravitactic orientation and photosynthesis in *E. gracilis* while in long-term exposure it affected cell growth, gravitaxis and concentration of total carotenoids. Malathion did not inhibit any parameter except swimming velocity in short-term experiments. Malathion has slightly increased the precision of negative gravitaxis both during short- and long-term exposure. The inhibitory effects of pesticides in short-term were stronger than in long-term experiments.

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