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SHALLOW LAKE ECOSYSTEMS

Laboratory and microcosm experiments testing the toxicity of chlorinated hydrocarbons on a cyanobacterium strain (*Synechococcus* PCC 6301) and on natural phytoplankton assemblages

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Abstract In the last few years, halogenated hydrocarbons have been detected in the soil, in the aquatic environment, in organisms, and even in drinking water. The toxic effects of three chlorinated aliphatic hydrocarbons (trichloroethylene, tetrachloroethylene and tetrachloroethane) were studied in laboratory experiments (using the cyanobacterium *Synecococcus elongatus* PCC 6301 as test organism) and in field-like circumstances (natural phytoplankton assemblages enclosed in microcosms). The results of the laboratory experiments showed that all of the tested compounds significantly inhibited the growth of the cultures within the first 4 h. Enzymatic changes of the treated cultures suggested that oxidative stress occured—all of the three compounds caused an increase in the activity of peroxidases and superoxide dismutase, and also increased the levels of lipid peroxidation. Observed changes in microcosms were comparable with the results of the laboratory experiments: the number of individuals and chlorophyll contents decreased in the treated assemblages. The elevated levels of peroxidation on the second day in the assemblages treated with tetrachloroethane and tetrachloroetylene suggest that oxidative stress could occur in field conditions. One of the most important findings is the decrease in species number. Our results showed that cryptomonads, some green algae species and the cyanobacterium *Limnothrix* gradually disappeared from the treated beakers during the experiment.

Keywords Chlorinated hydrocarbons · Growth inhibition · Oxidative stress · Laboratory experiments · Phytoplankton assemblages

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Introduction

Chlorinated ethanes and ethylenes are used in a wide range of industries: Tetrachloroethane has the highest solvent power of any chlorinated hydrocarbon, it has been used as a component of paints and pesticides, and as a refrigerant (Lewis, 2001). Tetrachloroethylene (Perchloroethylene, PCE) is used as a degreaser and in the dry cleaning of clothes (Verschueren, 1983), and

trichloroethylene (TCE)—one of the most widely produced chlorinated hydrocarbons—is applied as a degreaser and as a solvent during the production of other chemicals (Kuney, 1986). There is currently increasing evidence that chlorinated hydrocarbon contamination appears in soil and groundwater, because of the unsuitable storage of industrial waste. The latest studies show that there is an increasing risk of appearance of these contaminants in surface waters by the movement of polluted groundwater (Lorah & Voytek, 2004).

The half-life of tetrachloroethane, tetrachloroethylene and trichloroethylene in surface waters depends on the speed of their chemical decomposition or metabolism by microorganisms. Because of their low water solubility and high volatility, these contaminants are mainly lost through volatilization. The volatilization half-life of tetrachloroethane (assuming first-order decay kinetics) is estimated to be 6.1 days from a model lake 1 m deep flowing 0.05 m/s with a wind of 0.5 m/s (Thomas, 1990). Tetrachloroethylene rapidly volatilizes from water. One study found that only 2.7% of the initial mass of tetrachloroethylene remained in stagnant water with a surface-to-volume ratio of 81 m^2 m^{-3} after 4.5 h (Zytner et al., 1989). Authors did not find a direct relationship between the concentration of the chemical in water and rate of volatilization (Zytner et al., 1989). Measured volatilization half-lives in a mesocosm, which simulated ranged from 12 to 25 days (Wakeham et al., 1983). The Henry's law constant value of 2.0×10^{-2} atm m^3/mol at 20°C suggests that trichloroethylene rapidly partitions to the atmosphere from surface water (Chodola et al., 1989). Although volatilization is rapid, actual volatilization rates are dependent on temperature, water movement and depth, associated air movement, and other factors. According to a mathematical model based on Fick's diffusion law, the trichloroethylene volatilization rate constant was found to be inversely proportional to the square of the water depth (Peng et al., 1994). Measured volatilization half-lives in a mesocosm ranged from 13 days in summer conditions to 28 days in spring conditions (Wakeham et al., 1983).

Because their density is higher than the density of water, the rest of the chlorinated hydrocarbons generally sink to the bottom and from there they may continuously dissolve into the water body and damage the aquatic organisms (Leighton & Calo, 1981; Thomas, 1990).

Tetrachloroethane undergoes base-catalyzed hydrolysis in water at environmental pH to form trichloroethylene (Cervini-Silva, 2003). The anaerobic biodegradation of tetrachloroethane in the sediment can proceed through hydrogenolysis, dichloroelimination or dehydrochlorination (Ferguson & Pietari, 2000). Tetrachloroethylene does not readily transform in water (Roberts et al., 1986); previous results show that biodegradation may be the most important transformation process in natural waters (Wakeham et al., 1983; Parsons et al., 1984, 1985). Trichloroethylene is relatively stable in aquatic environments: Oxidation does not appear to be a significant fate process. The rate of hydrolysis is also too slow to be an important transformation process (EPA, 1979). Chemical hydrolysis appeared to occur only at high temperature and pH, even then, at a very slow rate. Microcosm studies of trichloroethylene biotransformation in aquifers have indicated that reductive dehalogenation is the primary degradation reaction (Parsons et al., 1985; Wilson et al., 1986).

The physical and chemical characteristics of these compounds (small molecule mass, volatility and considerable lipofility) lead to the fact that all of these chemicals quickly absorbed after their contact with organisms. Studies on vertebrates showed that these compounds cause oxidative stress in the different organisms (Yllner 1971; Ikeda & Ohtsuji, 1972; Halpert & Neal, 1981; Mitoma et al., 1985). Oxidative stress is the changing of oxygen metabolism, when one-electron transmission processes become dominant instead of the two-electron transmission processes, so free radicals, reactive oxygen species (ROS) will be generated (Hohman & Mager, 2003). ROS are produced as byproducts during membrane-linked electron transport activities as well as by a number of metabolic pathways (Shah et al., 2001). The intracellular accumulation of ROS, free radicals, oxidants cause damage in the normal functioning of cells, or threaten their survival and initiate oxidative stress responses (Hohman & Mager, 2003). The antioxidant defence system of cells comprises different enzymes. Peroxidases are a large family of enzymes that typically catalyze the reaction of hydrogen peroxide elimination. Others are more active with organic hydroperoxides such as lipid peroxides. Superoxide dismutases (SOD) are enzymes that catalyze the dismutation of superoxide into oxygen and hydrogen peroxide (Shah et al., 2001).

Although tetrachloroethane, tetrachloroethylene and trichloroethylene were or are widely distributed, relatively less data are available in the literature about

their effects in aquatic ecosystems. It is known that these compounds affect phytoplankton species: Their effects were studied in marine environment (Pearson & McConnell, 1975; Ward et al., 1986) and in the case of freshwater cyanobacteria and eukaryotic algal species (Bringmann & Kühn, 1980; Ando et al., 2003; Lukavsky et al., 2011). Some other types of halogenated hydrocarbons are produced by cyanobacteria themselves, which could be important biomarkers (Summons et al., 1999).

Comparatively, not much is known about how the halogenated hydrocarbon contaminants affect phytoplankton assemblages in shallow lake ecosystems. Berglund et al. (2001) found positive correlation between the trophic state of a lake and the amount of polychlorinated bifenyls. They concluded that this is a result of higher sedimentation rates in shallow, eutrophic lakes. Recently, the effect of the water-soluble fraction of hydrocarbons both on physiological parameters and community composition was studied in microcosm experiments (González et al., 2009). Their results demonstrated that the water-soluble fraction of hydrocarbons caused a transitory, short-term, negative effect on phytoplankton. Later, this effect caused changes in the structure of the plankton community.

The aim of this study was to identify the effects of tetrachloroethane, tetrachloroethylene and trichloroethylene on the growth and oxidative stress responses of a test organism, *Synechococcus elongatus* (Cyanobacteria) in the laboratory and to compare laboratory results with the results of a microcosm experiment. Both the laboratory experiments and the microcosms were "open" systems from the point of view of the halogenated hydrocarbons, to study that the effects appear or not among conditions not favourable for dissolving of the tested compounds. The study site chosen for the microcosm experiments (an artificial shallow lake) is a good representative of a typical and frequent surface water type of Hungary.

Materials and methods

Culturing conditions of *Synechococcus*, growth of the cultures

The strain *Synechococcus* PCC 6301 is in axenic culture, without mucilage layer around cells. The cultures were grown in 300 ml Erlenmeyer flasks in Allen medium (250 ml culture per flask) bubbled with 5% CO_2 containing sterile air, at 39°C, under continuous irradiation (80 µmol m^{-2} s^{-1}). The treatments were achieved with adding chlorinated hydrocarbon to the cultures in the 20th hour of the experiment. About 450 µl tetrachloroethane, 23 µl tetrachloroethylene and 234 µl tricholoethylene required to reach the theoretically saturated solvent state. The growth of the control cultures (without addition of chlorinated hydrocarbons) and treated cultures was monitored by measuring chlorophyll-*a* content and by counting cell numbers. A volume of 200 µl sample were collected in every second hour for chlorophyll-*a* content measurement, the absorbance was measured in 80% acetone at 663 nm (Bendall et al., 1988). Spectroquant Pharo 300 spectrophotometer was used for the spectrophotometric measurements, and Assistant Bürker chamber for the cell number count.

Enzymological experiments

The changes in peroxidase and superoxide dismutase enzyme activities and in the amount of thiobarbituric acid reactive substances were regularly measured in hour 0, 4, 8, 12 and 24. The samples (5 ml in volume) were centrifuged (6,000×*g*, 10 min, Beckman Avanti J-25), the cells were disrupted by freezing in liquid nitrogen and grinding with a glass pestle in Eppendorf tubes after the removal of supernatants. The disrupted cells were resuspended in 50 mM potassium phosphate buffer (pH 6.0 in the case of peroxidase and pH 7.8 in the case of superoxide dismutase). The cell-free crude extracts were used in the enzyme assays.

Peroxidase activity was measured as described by Lin et al. (2007) with minor modifications. The reaction mixture contained 50 mM potassium phosphate buffer (pH 6.0), 5 mM H_2O_2 and 0.02 mM pyrogallol and the different samples. The increase in absorbance was measured at 420 nm for up to 2 min. The specific activity was calculated to 1 mg protein, the protein contents of the samples were determined according to Bradford method (Bradford, 1976).

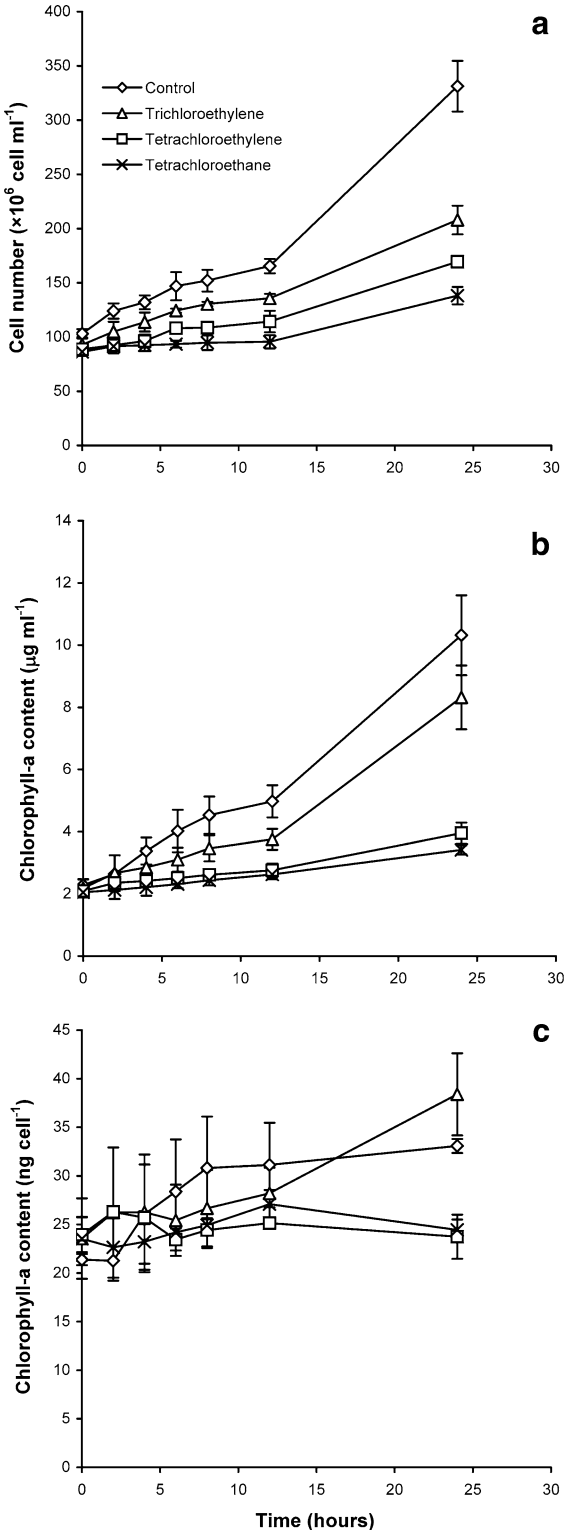
Lipid peroxidation levels were expressed as thiobarbituric acid reactive substances (TBARS) following the method of Verma & Dubey (2003). About 5 ml of samples was centrifuged (6,000×*g*, 10 min, Beckman Avanti J-25), the pellet was resuspended in 0.25% 2-thiobarbituric acid (TBA) in 10% trichloroacetic acid (TCA). The mixtures were incubated at 95°C for

30 min, and then quickly cooled in an ice bath and centrifuged (6,000×*g*, 10 min, Beckman Avanti J-25). The absorbance of the supernatant was measured at 532 and 600 nm (for correction of unspecific turbidity). The TBA solution in TCA served as blank. The amounts of TBARS were calculated in units of cell number using the extinction coefficient of 155 mM × (l cm)$^{-1}$. The results are given in percentage, 100% is the value measured in the control culture at the beginning of the treatments. The same method was applied in the case of microcosm experiments, the amounts of TBARS were calculated in units of number of individuals.

Superoxide dismutase (SOD) activities were measured using the nitro blue tetrazolium (NBT) photoreduction method (Lin et al., 2007). The assay in total volume of 1 ml contained 13 mM metionine, 10 μM riboflavine, 75 μM NBT, 10 μM EDTA and the different samples in 50 mM potassium phosphate buffer (pH 7.8). The absorbance was recorded at zero time and after 5 min illumination (colour development) at 560 nm. The difference between the reduction of NBT with and without enzyme extract was used to calculate SOD activity: one unit of activity is the amount of protein required to inhibit 50% initial reduction of NBT under light.

Microcosm experiment

The location of the microcosm experiment was the Botanical Garden Pond of the University of Debrecen. This is a shallow artificial pond (0.7 m average depth, about 100 m^2 area). The experimental design was as follows: 16 l water sample was filled into 12 plastic (PMP) beakers (1.3 l to each one). At the start of the experiment, 300 ml aliquots were collected from each beaker and from the pond (zero time samples). Sampling the pond was necessary to be able to see which changes occur because of the enclosing and which could be related to the effects of organic solvents. Three of these beakers were treated as controls (without addition of chlorinated hydrocarbons) and the other nine were utilized to test three experimental treatments: 1.8 ml tetrachloroethane was added to three beakers, 92 μl tetrachloroethylene to another three beakers and 935 μl trichloroethylene to the last three beakers (theoretically saturated solvents). The beakers were placed in a plastic basket and the basket was placed into the pond 15 cm deep. The basket had a thin translucent top to allow photosynthesis and gas exchange (the distance between the water surface of the beakers and the translucent top was at least 8 cm), but avoid the beakers from damage. There was no contact between the beakers contents and the pond water but the basket containing the beakers allowed the pond water to flow in and surround the beakers, allowing to develop a comparable temperature with that in pond. A volume of 300 ml samples were collected from each beaker and from the pond on the 24th, 48th and 96th hour of the experiment. The 20-ml aliquots of these samples were stored in Lugol's solution for phytoplankton identification and counting the number of algal individuals (definition of an algal individual is given in the following). The phytoplankton samples were processed by the Utermöhl method (1958) and European Standard EN 15204 (2006) standard. Briefly, the samples were decolorized with 1 M solution of sodium thiosulphate, after that they were placed into a sedimentation chamber with a final volume of 5 ml. Samples were left to settle down for a minimum of 8 h. An Olympus CKX31 inverted microscope and 400× magnification was used for counting and species identification. During quantitative analysis, 400 algal objects (individuals) were counted in transects. Algal object/algal individual is a unit/cluster of one or more algal cells that is discrete from (liable to settle independently of) other particles in the sample. According to this, coenobia, filaments and colonies were considered as an individual, their cell number was not separately recorded. The chlorophyll-*a* content of the samples (1 ml) was spectrophotometrically measured according to the method of Felföldy (1987). A volume of 1 ml samples were centrifuged (6,000×*g*, 10 min, Beckman Avanti J-25), the supernatants were removed and 500 μl of methanol was added to the pellets. The mixtures were heated in a water bath (Bandelin Sonorex RK 103 H) to the first boiling (~65°C). After cooling, another 500 μl of methanol was added to the samples, the mixtures were centrifuged again (6,000×*g*, 10 min, Beckman Avanti J-25) and the supernatants were measured at 653, 666 and 750 nm. The chlorophyll contents were calculated as follows: Chl $a = (17.12 \times x_1 - 8.68 \times x_2) \times m \times 1{,}000/M$ (μg/l), where $x_1 = \mathrm{Abs}_{666} - \mathrm{Abs}_{750}$; $x_2 = \mathrm{Abs}_{653} - \mathrm{Abs}_{750}$; M = centrifuged sample volume; m = volume of methanol used for the extraction. The rest 279 ml of the samples were centrifuged (6,000×*g*,



◀ **Fig. 1** Changes of the cell number (**a**), chlorophyll-*a* content on culture volume basis (**b**) and chlorophyll-*a* content of the cells (**c**) in control and chlorinated hydrocarbon treated *Synechococcus* cultures. Mean value ($n = 3$) and standard deviations are reported

10 min, Beckman Avanti J-25), and the lipid peroxidation levels were determined from the pellets as described earlier.

Statistical analyses

All experiments were done in triplicate. Two-way ANOVA and Tukey test as multiple comparison procedure were used to determine the significances among the control and treated cultures. For analysing response of biological communities to toxic stress and changes of species composition in microcosms, RDA was used. RDA is a powerful multivariate technique that can be used to analyse the response of biological communities to toxic stress (van Wijngaarden et al., 1995). The RDA was calculated by CANOCO 4.5 (ter Braak & Šmilauer, 2002; Lepš & Šmilauer, 2003).

Results

Effects of chlorinated hydrocarbons on the growth of *Synechococcus* cultures

There was a decrease in cell number in all of the treated cultures (Fig. 1a). The strongest inhibition was noticeable in culture treated with tetrachloroethane: the cell number at the 12th hour was 42% lower than in the control culture. The inhibition was less in cultures treated with tetrachloroethylene: the cell number was 69% of the control culture at the 12th hour. Trichloroethylene seemed to be the least toxic: the cell number of trichloroethylene treated cultures was only 18% lower than in the control cultures at the 12th hour. All treatments significantly differed from control ($P < 0.001$). The inhibitory effects of chlorinated hydrocarbons appeared at the 4th hour after the start of experiment in all cases. The differences between tetrachloroethane and tetrachloroethylene treated cultures were not significant at the 4th hour ($P = 0.061$), but later these differences became significant ($P < 0.01$).

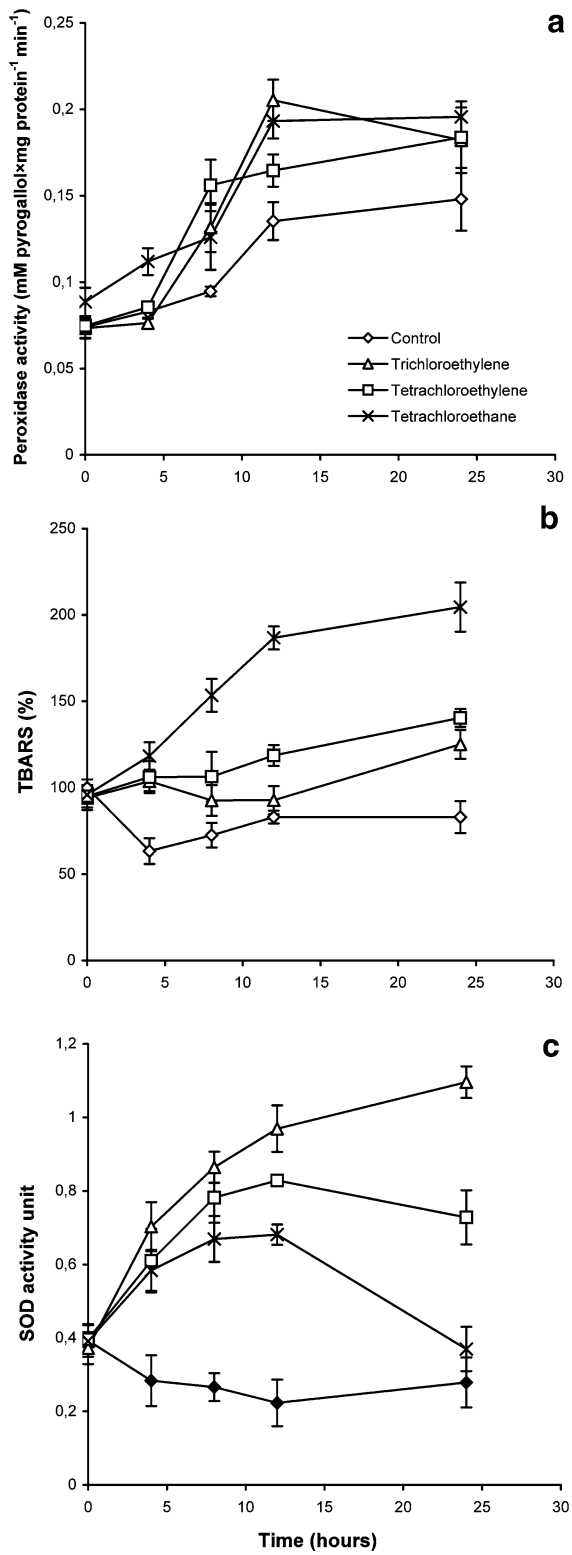


Fig. 2 Changes of peroxidase activities (**a**), lipid peroxidation levels (amount of thiobarbituric acid reactive substances—**b**) and superoxide dismutase activities (**c**) in control and chlorinated hydrocarbon treated *Synechococcus* cultures. Mean value ($n = 3$) and standard deviations are reported

The tetrachloro derivatives also seemed more toxic than trichloroethylene on the basis of chlorophyll-*a* content (the chlorophyll contents of the treated cultures were on average 46% lower than those of the control cultures). The cultures treated with tetrachloro derivatives significantly differed from the control culture already at the 4th hour ($P = 0.007$ and 0.033), but did not differ from one other at this time or later during the treatments. The differences between the trichloroethylene treated culture and control culture were not significant at the 4th hour ($P = 0.427$; Fig. 1b), later the chlorophyll contents of trichloroethylene treated cultures were only 25% lower than in control cultures (Fig. 1b). The lower toxicity of trichloroethylene was also indicated by the phenomenon that the growth of the trichloroethylene-treated cultures regenerated after 24–28 h, whereas there was either weaker or no regeneration in the case of cultures treated with the tetrachloro derivatives (data not shown).

Studying the chlorophyll-*a* contents on a per cell basis provided different information: The chlorophyll-*a* content did not significantly change in either the control or the treated cultures of *Synechococcus* cells (Fig. 1c). Significant differences between control and tetrachloro-treated cultures were notable only at the end of the experiments (Fig. 1c). Thus, the changes in concentration of chlorophyll-*a* per ml were because of the reduction of cell number, and not because of decreasing chlorophyll-*a* content of the cells.

Enzymological experiments

All three chlorinated hydrocarbons caused an increase in peroxidase activity (Fig. 2a). A certain order in the enzyme stimulating effect of the studied compounds could not be established, but it can be said that the activity increased in the first 12 h. Statistical analysis showed that the treated cultures significantly differed from the control from the 8th hour onward. After the 12th hour, the difference in peroxidase activity between treated and control cultures decreased (Fig. 2a).

The levels of lipid peroxidation were higher in the case of tetrachloro derivatives than for trichloroethylene (Fig. 2b). Tetrachloroethylene caused a 43% increase, and tetrachloroethane >100% increase in the amount of TBA reactive substances compared with that in the control cultures in 12 h. The amount of TBA reactive substances was still higher in the treated than in control cultures after 24 h (Fig. 2b). All treated cultures significantly differed from controls ($P < 0.001$) from the 4th hour onwards and from each other from the 8th hour onwards.

The SOD activity showed the highest increase in the trichloroethylene-treated cultures: The activity at the 12th hour was four-times higher than the activity measured in control (Fig. 2c). The SOD activity scores were 3.7-times higher in tetrachloroethylene and three-times higher in tetrachloroethane-treated cultures than measured in control cultures. These activity levels decreased in cultures treated with tetrachloro derivatives after the 12th hour, but they remained higher than in the control cultures at the 24th hour of the treatment (Fig. 2c). The SOD activity in cultures treated with tetrachloro derivatives did not significantly differ from each other until the 8th hour ($P = 0.133$), later statistical analysis showed that they significantly differed ($P < 0.001$).

Microcosm experiments

The numbers of algal individuals decreased in the pond, control and in treated assemblages in the first 24 h (Fig. 3), but there were significantly more individuals in the control assemblages than in the treated ones ($P = 0.025$–<0.001), whereas the number of individuals in control did not significantly differ from the pond ($P = 0.384$). There were increases in the numbers of individuals to the second day, but the numbers of individuals were significantly lower in the case of tetrachloro derivatives. The treatments did not significantly differ from one other; the number of individuals was significantly lower in the case of tetrachloroethane treatment than in control to the end of the experiment (Fig. 3a).

A stronger effect was detected for chlorophyll-*a* content than for the number of individuals (Fig. 3b). As in the number of individuals, reduction also occurred in chlorophyll-*a* contents of the assemblages treated with the chlorinated hydrocarbons in the first 24 h. This reduction was >50% even in the case of the

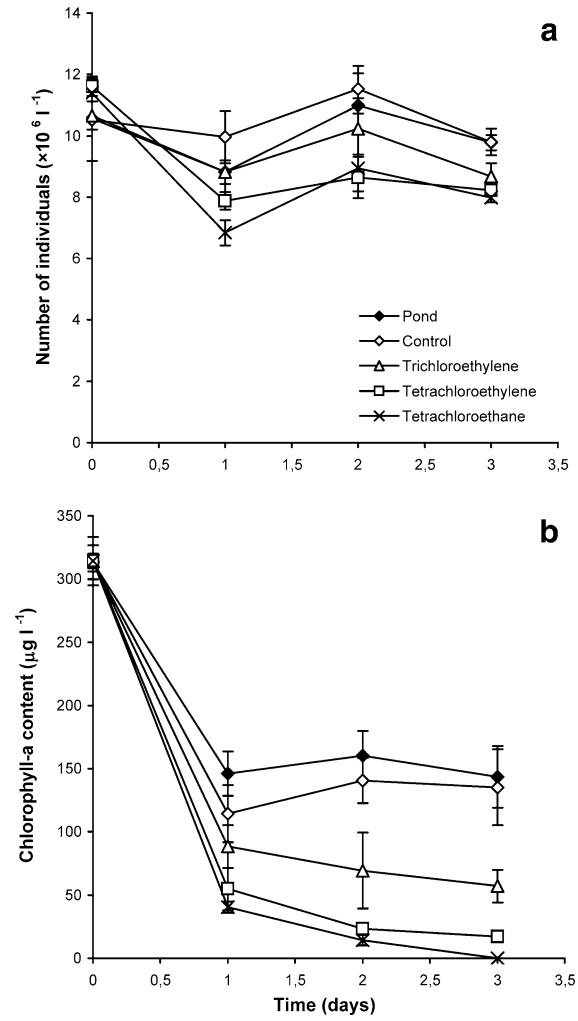


Fig. 3 Changes of the number of individuals (**a**) and chlorophyll-*a* content per volume (**b**) in the pond, control and chlorinated hydrocarbon treated beakers during microcosm experiments. Mean value ($n = 3$) and standard deviations are reported

pond and control assemblages (Fig. 3b). This reduction was greater in assemblages treated with tetrachloroethane and tetrachloroethylene (87 and 82.5%, respectively; Fig. 3b). The chlorophyll content of the pond and the control assemblages increased by the second day and decreased only slightly by the third day, whereas it decreased further in the treated assemblages. This decrease was greater in the case of tetrachloro derivatives. There were significant differences among control and treated assemblages ($P = 0.008$–<0.001). The effects of tetrachloro derivatives on chlorophyll content did not significantly

differ ($P = 0.113$–0.808), which was also observed during the laboratory experiments.

The oxidative stress–causing effects of the tested chemicals were not as clear as the effects observed in laboratory experiments. There was an average decrease in the amount of thiobarbituric acid reactive substances in all treatments from the start of the experiments to the third day (Fig. 4). Elevated TBARS levels were observable in the case of tetrachloro derivatives on the second day comparing with the control values and with the values that were measured in the pond or on the previous day (Fig. 4, crosses and open squares). On the second day, the TBARS levels were significantly higher ($P < 0.01$) in assemblages treated with tetrachloro derivatives than in control assemblages, or than in the pond. There was an increased lipid peroxidation in assemblages treated with tetrachloroethane on the third day, but the differences compared with controls and with other treatments were no more significant. The amount of TBARS in the pond significantly differed from the assemblages on the third day ($P = 0.05$–<0.01). Trichloroethylene did not cause detectable oxidative stress in microcosm experiments (Fig. 4).

The species richness decreased in the first 2 days, but it was more pronounced in the treated assemblages than in the pond and in controls, especially for the tetrachloro derivatives (Fig. 5). The differences

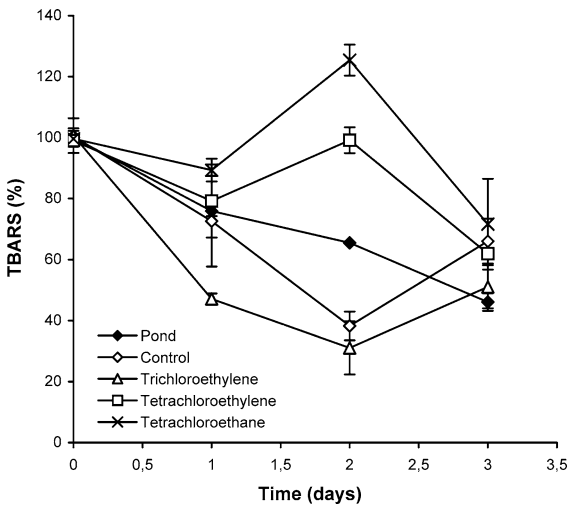


Fig. 4 Changes of lipid peroxidation levels (amount of thiobarbituric acid reactive substances) in the pond, control and chlorinated hydrocarbon treated beakers during microcosm experiments. Mean value ($n = 3$) and standard deviations are reported

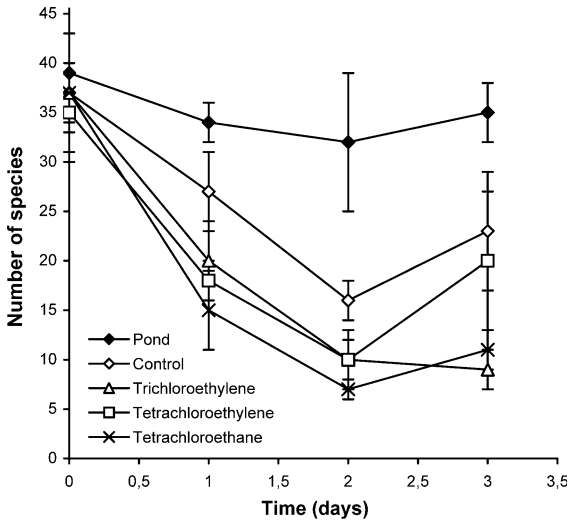


Fig. 5 Changes in number of species in the pond, control and chlorinated hydrocarbon treated beakers during microcosm experiments. Mean value ($n = 3$) and standard deviations are reported

between pond and control assemblages were not significant on the first day, but those between the pond and treated assemblages and between control and tetrachloroethane treated assemblages were significant ($P = 0.008$–<0.001; Fig. 5). The pond and the control assemblages appeared to significantly differ throughout the experiment, but the treatments and controls also significantly differed on the second and third days. The total number of algal species increased by the third day in all cases, it reached the control level in tetrachloroethylene-treated assemblages (Fig. 5).

Multivariate analysis (RDA) displays the changes in phytoplankton assemblages during microcosm experiments (Fig. 6). Symbols represented a certain assemblage (species list) of a treatment in a given sampling time. Time was represented by the first, while treatment by the second axis. The individual number of most species decreased within study period, especially to the first day, but this decrease was more pronounced in the case of tetrachloro derivatives, than trichloroethylene (Fig. 6). The composition of the assemblages did not change, and the individual number of some species further decreased in assemblages treated with tetrachloro derivatives to the second day (Fig. 6), whereas the phytoplankton composition remained more similar to each other in control and trichloroethylene-treated assemblages on each day of the experiment (Fig. 6). The average decrease

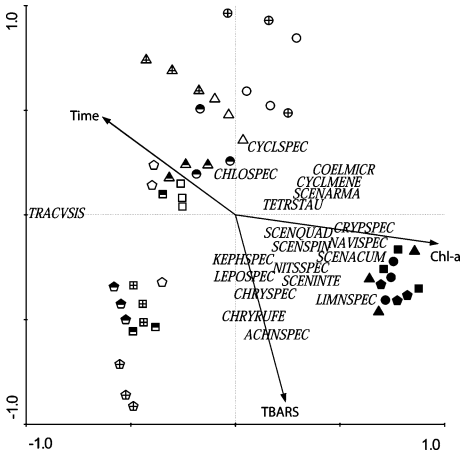


Fig. 6 Temporal change of species composition in the control and treated beakers plotted by an RDA. Figure legends: controls, day 0: ●, day 1: ◒, day 2: ⊕, day 3: ○. Tetrachloroethane treatments day 0: ⬟, day 1: ⬟, day 2: ⊕, day 3: ⬠. Tetrachloroethylene treatments, day 0: ■, day 1: ▬, day 2: ⊞, day 3: □. Trichloroethylene treatments, day 0: ▲, day 1: ▲, day 2: ⧋, day 3: △. Species were abbreviated using four letters of genus and four letters of species names (for meanings of the abbreviations, see Table 1). The 20 most frequent species were plotted only. Notations: *Chl-a* concentration of chlorophyll-*a*, *TBARS* amount of thiobarbituric acid reactive substances. *Symbols* represented a certain assemblage (species list) of a treatment in a given sampling time. Time was represented by the first axis and treatment by the second axis

in chlorophyll-*a* content and in lipid peroxidation levels are also shown (Fig. 6).

Individual numbers of the 20 most frequent phytoplankton species on certain days in the pond and in the different assemblages are presented in Tables 1 and 2. The euglenid *Trachelomonas volvocinopsis* and the diatoms *Cyclotella* species were dominant during the whole experiment, but the other diatoms (*Navicula* and *Nitzschia* species), green algae (*Chlorella*, *Scenedesmus* spp., *Coelastrum* and *Tetrastrum* species), cryptomonads, chrysophytes (*Chrysococcus* and *Kephyrion* species) and the cyanobacterium genus *Limnothrix* were present in notable number. The euglenid *T. volvocinopsis* appeared to be the most resistant species, as its number barely changed during the experiment (Fig. 6; Tables 1 and 2). The three green algae (*Chlorella*, *Coelastrum* and *S. armatus*) and the diatom *Cyclotella* also appeared to be less sensitive to the treatments than other species (Fig. 6; Tables 1 and 2). *Cryptomonas*, *Chrysococcus* and *Kephyrion* were the most sensitive genera, they disappeared from the tetrachloroethane- and trichloroethylene-treated assemblages (Table 2). Other *Scenedesmus* spp., the euglenid *Lepocynclis*, the diatoms *Nitzschia* and *Navicula* and the cyanobacterium *Limnothrix* can be considered as sensitive species, because they already disappeared in treated assemblages on the first and second day, but reappeared at the end of the experiment (Table 2).

Discussion

Effects of chlorinated hydrocarbons on the growth of Synechococcus cultures

Our results of the growth inhibition of the *Synechococcus* strain by chlorinated hydrocarbons correspond with the data of the literature on the toxic effects of chlorinated hydrocarbons and other organic compounds. Wang et al. (1996) have shown the growth inhibitory effect of tetrachloroethylene on two marine algal species, *Heterosigma akashiwo* and *Skeletonema costatum*. The negative effects of trichloroethylene on growth are known for several algal species, *Microcystis aeruginosa, Scenedesmus quadricauda* (Bringmann & Kühn, 1978), *Skeletonema* sp. (EPA, 1978) and *Chlamydomonas reinhardtii* (Brack & Rottler, 1994). Recent studies also show the inhibitory effects of trichloroethylene on green algae and a *Synechococcus elongatus* cyanobacterium strain (Lukavsky et al., 2011).

In laboratory cultures, chlorophyll content of the cells significantly changed only at the end of the experiments (Fig. 1c). Thus, the changes in the concentration of chlorophyll-*a* were because of the decrease in cell number. It is not clear if the toxicity of tetrachloro derivatives is higher. The membrane-damaging effects of these lipophilic compounds, i.e., because of their metabolism and oxidative stress can cause reduced chlorophyll-*a* levels. Such chlorophyll loss (decomposition of antenna pigments) was shown under oxidative stress in the green alga, *Chlorella vulgaris* (Qian et al., 2009).

Effects of chlorinated hydrocarbons on the oxidative stress enzymes of *Synechococcus* cultures

That toxic substances (metals, organic contaminants or natural toxins) can cause oxidative stress in the exposed organisms is well known. Oxidative stress in

Table 1 Individual number of the 20 most frequent phytoplankton species in the pond and in control beakers in microcosms during the time span of the experiment

Species	PDay0	PDay1	PDay2	PDay3	CDay0	CDay1	CDay2	CDay3
1. *Achnantes* sp. (ACHNSPEC)	7.41 ± 1.95	5.75 ± 4.71	0	5.03 ± 0.07	9.10 ± 0.14	6.01 ± 2.01	0	0
2. *Chlorella* sp. (CHLOSPEC)	3.99 ± 0.92	11.71 ± 0.52	12.72 ± 2.77	6.61 ± 2.73	16.29 ± 6.05	2.49 ± 0.22	11.6 ± 3.08	11.16 ± 5.00
3. *Chrysococcus rufescens* (CHRYRUFE)	3.06 ± 0.06	2.20 ± 0.10	3.71 ± 1.45	3.32 ± 1.44	3.00 ± 0.09	3.32 ± 1.45	5.98	3.72 ± 1.67
4. *Chrysococcus* sp. (CHRYSPEC)	3.10	2.34 ± 0.1	0	2.49 ± 0.07	3.00 ± 0.07	2.49	0	2.54
5. *Coelastrum microporum* (COELMICR)	8.96 ± 4.38	9.78 ± 0.77	7.55 ± 1.88	18.27 ± 1.48	17.95 ± 3.69	8.87 ± 3.38	6.78 ± 1.84	18.05 ± 4.44
6. *Cryptomonas* sp. (CRYPSPEC)	54.95 ± 3.26	43.83 ± 1.02	25.09 ± 7.34	23.99 ± 7.1	33.29 ± 18.00	26.89 ± 13.38	38.99 ± 12.51	6.03 ± 1.86
7. *Cyclotella meneghiniana* (CYCLMENE)	23.73 ± 3.73	16.79 ± 8.24	10.32 ± 1.70	79.87 ± 12.45	88.12	0	8.62 ± 2.53	73.9281 ± 23.29
8. *Cyclotella* sp. (CYCLSPEC)	30.47 ± 8.50	46.04 ± 5.35	202.09 ± 98.71	99.79 ± 46.8	37.83 ± 14.45	31.5 ± 12.87	232.63 ± 81.71	49.42 ± 7.14
9. *Kephyrion* sp. (KEPHSPEC)	7.41 ± 1.95	8.85 ± 3.52	2.69	5.08	2.97 ± 0.05	6.55 ± 2.22	2.74	0
10. *Lepocynclis* sp. (LEPOSPEC)	18.37 ± 4.67	8.22 ± 2.08	7.55 ± 1.88	13.34 ± 4.09	3.00 ± 0.07	2.49 ± 0.22	7.83 ± 4.41	2.54
11. *Limnothrix* sp. (LIMNSPEC)	13.68 ± 6.21	8.93 ± 2.38	5.75 ± 0.17	2.52 ± 0.04	3.00 ± 0.07	2.49 ± 0.02	5.82 ± 4.35	2.54
12. *Navicula* sp. (NAVISPEC)	3.01 ± 0.12	5.29 ± 2.66	4.73 ± 1.77	3.32 ± 1.44	4.57 ± 2.22	7.47 ± 0.92	4.36 ± 2.29	2.54
13. *Nitzschia* sp. (NITSSPEC)	13.73 ± 1.89	5.29 ± 1.34	2.81 ± 0.17	2.44	4.54 ± 2.26	4.54	0	3.30 ± 1.55
14. *Scenedesmus acuminatus* (SCENACUM)	9.04 ± 0.35	3.83 ± 1.49	4.73 ± 1.77	5.03 ± 0.07	4.47 ± 1.98	2.49 ± 0.22	4.47 ± 2.13	4.90
15. *Scenedesmus armatus* (SCENARMA)	2.97 ± 0.06	10.58 ± 2.13	22.31 ± 9.75	2.49	2.97 ± 0.05	7.69 ± 3.21	26.67	2.54
16. *Scenedesmus intermedius* (SCENINTE)	9.17 ± 0.18	11.25 ± 4.09	9.25 ± 3.86	6.31 ± 1.87	10.41 ± 2.26	2.49 ± 0.22	5.79 ± 2.96	7.35

Table 1 continued

Species	PDay0	PDay1	PDay2	PDay3	CDay0	CDay1	CDay2	CDay3
17. *Scenedesmus quadricauda* (SCENQUAD)	11.93 ± 4.44	3.25 ± 1.32	2.88 ± 0.09	13.55 ± 1.55	22.23 ± 5.95	4.98	2.99	10.79 ± 1.40
18. *Scenedesmus spinosus* (SCENSPIN)	5.90 ± 4.08	5.34 ± 1.66	4.73 ± 1.77	11.29 ± 5.18	7.47 ± 1.89	4.97	5.48	3.58 ± 1.6
19. *Tetrastrum staurogeniaeforme* (TETRSTAU)	12.05	10.63 ± 1.90	4.34 ± 2.16	2.49 ± 0.05	2.97 ± 0.05	9.27 ± 4.18	2.99	2.50
20. *Trachelomonas volvocinopsis* (TRACVSIS)	811.26 ± 60.84	658.51 ± 38.35	790.72 ± 155.64	645.09 ± 65.79	824.76 ± 131.82	828.69 ± 40.81	892.38 ± 173.28	717.24 ± 86.53

Data are given in individual per ml × 10^6 and presented as mean values ($n = 3$) ± SD. Abbreviations plotted on Fig. 6 are also indicated. *P* pond, *C* control

Synechococcus strains exposed to toxic conditions has also been demonstrated in several cases. Perelman et al. (2004) showed that high light intensity causes oxidative stress in *Synechococcus* PCC 7942 strain. Hu et al. (2004) observed the increase of glutathione peroxidase activity and lipid peroxidation levels in microcystin-RR-treated *Synechococcus elongatus* cultures. Vassilakaki and Pflugmacher (2008) studied the increase of glutathione peroxidase, superoxide dismutase and glutathione reductase enzymes in microcystins-LR-treated *Synechocystis* PCC 6803 cultures. The studied chlorinated hydrocarbons caused growth inhibition on the cyanobacterium *Synechococcus elongatus* and furthermore, the tested chemicals caused oxidative stress in the photosynthetic prokaryotes (similary to the observations in the case of mammalian cells—Gavino et al., 1983; Sano & Tappel, 1990; and also similarly to the observations related to toxic effects of exposed cyanobacteria discussed earlier). These effects may be correlated with the metabolism of the compounds in cyanobacterial cells, which probably can lead to the formation of a great number of reactive derivatives (Yllner, 1971; Halpert & Neal, 1981). If such reactive metabolites can cause other toxic effects than oxidative stress, it needs to be demonstrated by further experiments.

Effects of chlorinated hydrocarbons on the phytoplankton density and chlorophyll content in microcosms

The number of algal individuals decreased during the treatments in microcosm experiments (Fig 3a). The treatments did not significantly differ from each other, but the number of individuals was significantly lower than in controls at the end of the experiment.

The effects of the tested hydrocarbons on chlorophyll content were similar in the both microcosm experiments and laboratory observations: The chlorophyll-*a* content significantly decreased in the presence of the chlorinated hydrocarbons (Figs. 1b and 3b). Further reduction of chlorophyll levels in the treated assemblages could be clearly attributed to the presence of the chlorinated hydrocarbons. We compared the changes in chlorophyll content and show that tetrachloro derivatives cause greater decrease than trichloroethylene: There were no differences between the effects of tetrachloro derivatives in the laboratory experiments, and there were no differences between

Table 2 Individual number of the 20 most frequent phytoplankton species in treated beakers in microcosms during the time span of the experiment

Sp.	T1 Day0	T1 Day1	T1 Day2	T1 Day3	T2 Day0	T2 Day1	T2 Day2	T2 Day3	T3 Day0	T3 Day1	T3 Day2	T3 Day3
1.	6.95 ± 1.57	3.32 ± 0.15	3.73 ± 1.31	5.32 ± 1.16	7.01 ± 1.75	5.92 ± 0.31	0	4.15 ± 0.14	6.86 ± 3.30	2.25 ± 0.28	7.43 ± 2.07	0
2.	10.56 ± 1.97	6.89 ± 2.02	7.04	3.99 ± 0.11	30.05 ± 8.24	15.8 ± 0.58	3.62 ± 1.31	24.92 ± 4.54	22.73 ± 1.85	16.90 ± 7.39	20.97 ± 8.03	25.43 ± 4.00
3.	7.40 ± 2.21	4.98 ± 0.22	0	3.32 ± 1.11	7.55 ± 2.00	9.95 ± 3.31	3.62 ± 1.31	6.22 ± 0.21	3.01 ± 0.06	2.25 ± 0.4	0	0
4.	2.92	1.71	0	0	15.05 ± 0.14	9.87 ± 0.52	0	10.24 ± 0.18	7.5 ± 1.98	0	0	0
5.	2.99 ± 0.10	2.82 ± 0.87	0	1.98 ± 0.03	12.02 ± 4.15	5.92 ± 0.31	2.17 ± 0.26	11.10 ± 3.40	16.75 ± 4.30	11.83 ± 5.58	2.56 ± 0.51	2.94 ± 1.41
6.	22.43 ± 2.83	0	0	0	21.30 ± 8.43	25.96 ± 12.51	10.20 ± 3.83	4.15 ± 0.14	36.42 ± 9.56	2.11 ± 0.2	0	0
7.	16.29 ± 6.53	17.63 ± 9.00	0	7.32 ± 1.16	25.49 ± 18.92	7.90 ± 0.29	2.08 ± 1.32	26.96 ± 0.93	9.91 ± 4.58	5.92 ± 0.85	0	3.21 ± 1.62
8.	47.39 ± 17.47	51.56 ± 27.42	8.91 ± 2.10	23.73 ± 0.32	40.86 ± 1.4	9.92 ± 4.22	0	39.66 ± 16.02	35.93 ± 18.2	25.92 ± 7.97	19.32 ± 3.88	3.21 ± 1.31
9.	5.97 ± 0.19	0	0	0	4.52 ± 2.06	7.90 ± 0.29	2.17 ± 0.19	5.21 ± 1.65	2.97	0	0	0
10.	12.05 ± 4.08	0	0	1.99 ± 0.05	55.97 ± 5.41	0	47.32 ± 19.14	54.46 ± 19.78	23.64 ± 3.21	0	0	3.59 ± 1.14
11.	12.99 ± 3.67	0	0	8.93 ± 4.32	10.54 ± 2.22	0	0	2.07 ± 0.07	8.77 ± 0.18	0	2.85 ± 0.17	0
12.	3.96 ± 1.63	1.66 ± 0.07	0	3.99 ± 0.11	6.02 ± 0.05	2.05	0	0	2.97	0	0	2.12 ± 0.08
13.	2.95 ± 0.05	4.34 ± 1.58	2.35	2.00 ± 0.05	9.03 ± 0.08	5.81 ± 0.15	0	12.55 ± 6.29	7.46 ± 2.40	6.34 ± 0.6	0	0
14.	2.99	1.71 ± 0.15	0	0	9.09 ± 0.17	1.94 ± 0.05	6.24 ± 0.39	2.05 ± 0.04	3.92 ± 1.59	2.25 ± 0.28	5.93	0
15.	2.99 ± 0.1	0	2.34	0	6.02 ± 4.17	0	0	3.09 ± 1.50	7.94 ± 1.89	6.34 ± 0.60	2.73	0
16.	4.54 ± 2.21	1.66 ± 0.07	0	0	3.03 ± 0.06	9.73 ± 3.00	2.17 ± 0.26	4.10 ± 0.07	3.95 ± 1.71	2.11 ± 0.20	0	2.18 ± 0.15
17.	5.93 ± 2.85	2.86 ± 1.01	0	5.31 ± 1.08	3.03 ± 0.04	1.97 ± 0.1	2.08 ± 0.13	14.16	2.97 ± 0.09	2.25 ± 0.28	2.85 ± 0.17	2.18 ± 0.11
18.	3.02 ± 0.05	0	2.35	5.93 ± 0.08	6.04 ± 4.31	5.24 ± 0.1	0	2.07	8.87 ± 2.80	6.76 ± 0.85	0	0
19.	4.51 ± 2.06	2.82 ± 0.87	2.24	0	3.01 ± 0.03	1.97 ± 0.07	0	2.05 ± 0.04	3.98 ± 1.84	3.76 ± 1.42	2.47 ± 0.69	2.18 ± 0.15
20.	946.22 ± 36.52	604.94 ± 39.03	869.26 ± 39.82	740.33 ± 35.59	921.17 ± 71.54	679.15 ± 36.21	802.25 ± 88.48	641.75 ± 49.92	802.34 ± 23.79	783.53 ± 115.80	959.55 ± 183.93	823.65 ± 42.94

Data are given in individual per ml × 10^6 and presented as mean values ($n = 3$) ± standard deviations (SD). For species names, see Table 1. *T1* Treatment 1 (tetrachloroethane), *T2* Treatment 2 (tetrachloroethylene), *T3* Treatment 3 (trichloroethylene)

the effects of tetrachloro derivatives in the microcosm experiments. The magnitude of differences that occurred from one day to the next was greater in microcosms: This may be because of many reasons, e.g., lower temperature allowed longer persistence of the chemicals.

Effects of chlorinated hydrocarbons on lipid peroxidation levels in microcosms

The results of enzymological experiments are similar in that tetrachloro derivatives cause greater increase in TBARS levels, implying increased lipid peroxidation both in laboratory cultures and in microcosms, but the magnitude of these changes is hardly comparable. The amount of TBARS in microcosm experiments does not show such a tendency as predicted on the basis of laboratory results. The decreasing lipid peroxidation levels can be attributed to the decreasing number of algal individuals. The elevated amounts of TBARS on the second day in the assemblages treated with tetrachloroethane and tetrachloroethylene suggest that oxidative stress occurs in field conditions (Fig. 4).

Effects of chlorinated hydrocarbons on species composition in microcosms

Exposure to the hydrocarbons causes significant changes in species richness (Fig. 5) and in the composition of phytoplankton assemblages. Multivariate analysis (RDA) depicts the changes in phytoplankton assemblages during microcosm experiments (Fig. 6). The initial species composition of all assemblages (both treated and control) was very similar to each other. The individual numbers of most species decreased within the study duration, especially compared with the first day, but this decrease was more clear for tetrachloro derivatives. The phytoplankton composition remained more similar to control in trichloroethylene-treated assemblages than in tetrachloro derivates treated assemblages during the experiment (Fig. 6).

The effects of the tested halogenated hydrocarbons on phytoplankton assemblages strongly depend on the initial species composition of plankton of water studied. In this study, it seems both dominant species, the euglenid *Trachelomonas volvocinopsis* and the diatoms *Cyclotella* species, are not sensitive to the tested compounds, because despite their decreasing densities, they persisted and their relative abundant increased in the assemblages during the experiments. Some green algal species (*Chlorella* sp., *Coelastrum microporum* and *Scenedesmus armatus*) were less sensitive to the exposure to hydrocarbons than species from other groups (Fig. 6; Tables 1 and 2). The relative insensitivity of these algae could be because of the special covering of their cells: the presence of so-called lorica in *Trachelomonas*, mucilage sheath covering the cells of *Chlorella* and the colony formation in *Coelastrum*, or the thick cell wall of *Scenedesmus armatus*. Other *Scenedesmus* species, the euglenid *Lepocynclis*, the diatoms *Nitzschia* and *Navicula* and the cyanobacterium *Limnothrix* can be considered as sensitive species, because they disappeared in treated assemblages already on the first and second day, but they were present again at the end of the experiment (Table 2). *Cryptomonas*, *Chrysococcus* and *Kephyrion* were the most sensitive genera, they disappeared from the tetrachloroethane- and trichloroethylene-treated assemblages (Table 2). The phenomenon that the species diversity increased until the third day in the tetrachloroethylene-treated beakers can be explained by the insolubility of the chemical in water—a significant amount of it may be lost by volatilization to the third day. These results showed that the tested compounds have an impact on the species composition of phytoplankton assemblages. The presence of these contaminants could disturb the species composition in shallow ponds, despite their poor water solubility and high volatility.

Despite the fact that *Synechococcus* strains are general photoautotroph test organisms, because of their short generation time, easy culturing methodology and their known DNA sequences (Rojickova-Padrtova and Marsalek, 1999), it is hard to assess how valid the toxicology results are on *Synechococcus* for other cyanobacteria or to eukaryotic algae. Lukavsky et al. (2011) showed that the EC_{50} values for trichloroethylene depend not only on the tested species but also on the applied methods (plates or enclosures) as well. For example, *Synechococcus leopoliensis* seems to be more sensitive than *S. elongatus* on plates, but not in enclosures. *Microcystis aeruginosa* is the most sensitive among the tested cyanobacteria and among the tested phytoplankton species including eukaryotic algae (Lukavsky et al., 2011). Concerning the effects of trichloroethylene, the literature data widely varying concentrations from 0.008 to 1 g l^{-1}, for species of cyanobacteria, green algae and diatoms

(Bringmann & Kühn, 1980; Ward et al., 1986; Tadros et al., 1994; Ando et al., 2003). Despite these observations, the data of laboratory experiments and microcosms are quite comparable, at least for tested hydrocarbons and the extent of the toxicity of these compounds.

Conclusions

In general, it can be concluded that all the three tested compounds (tetrachloroethane, tetrachloroethylene and trichloroethylene) strongly affect the density and chlorophyll content of phytoplanton. The tetrachloro derivatives seemed to have stronger effects in both laboratory and in field experiments (microcosms). The changes in species composition in microcosm experiments showed that cryptomonads, some chrysophytes and some green algae react more sensitively to the presence of the tested hydrocarbons than planktonic diatoms or euglenids, although the possible effects on species composition of phytoplankton assemblages need to be determined more exactly.

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