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# LIBERATION OF 5,8,11,14,17-EICOSAPENTAENOIC ACID AND OTHER POLYUNSATURATED FATTY ACIDS FROM LIPIDS AS A GRAZER DEFENSE REACTION IN EPILITHIC DIATOM BIOFILMS[1]

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**Acute grazer toxicity of freshwater diatom biofilms was determined using *Thamnocephalus platyurus* Packard, an anostracan grazer, as the bioassay organism. The diatoms exhibited toxicity when the cells of the biofilm were freeze–thawed before the assay. The toxicity could be extracted from the biofilms with methanol and acetone, and only minimal toxicity was left in the insoluble residue. Bioassay-guided HPLC separation of the methanolic extract was performed to trace the most toxic components. Analysis by UV spectrometry, gas chromatography, and mass spectrometry showed that 5,8,11,14,17-eicosapentaenoic acid was responsible for most grazer toxicity. The 24-h $LC_{50}$ of this polyunsaturated fatty acid was 34 μM in the *Thamnocephalus platyurus* bioassay. The concentrations of other free fatty acids were not high enough to contribute significantly to the toxicity. Procedures that affected the integrity of the cells (e.g. solvent extraction, freezing and thawing, osmotic stress by addition of 20% NaCl, or grinding the cells in a mortar) were taken as model reactions for grazing and had the common effect of resulting in a dramatic increase of free polyunsaturated and saturated fatty acids. Under these conditions, about 30% of the total fatty acids of the diatoms was transformed from the bound into the free form. The time necessary for liberation was very short. With the exception of 5,8,11,14,17-eicosapentaenoic acid, which continued to be liberated, the hydrolysis of the other fatty acids was terminated less than 1 min after initiating the reaction. The classical extraction procedures using methanol and other solvents led to the appearance of a high percentage of free fatty acids in live cells. Treatment of biofilms with these solvents did not stop the hydrolysis of lipids initiated by the disintegration of the cells. However, boiling acetone completely suppressed the hydrolytic reactions, and free polyunsaturated fatty acids were not detected in live biofilm organisms, although nontoxic saturated fatty acids were present in moderate concentrations. These results were interpreted as an indication that the frequently reported existence of free polyunsaturated fatty acids in live biomass is an analytical artifact.**

*Key index words:* **biofilm; diatom; *Diatoma*; 5,8,11,14,17-eicosapentaenoic acid; free polyunsaturated fatty acids; grazer toxicity; liberation**

*Abbreviations:* **DMSP, dimethylsulfoniopropionate; GC-MS, gas chromatography–mass spectroscopy; MTBSTFA, *N*-(*tert.*butyldimethylsilyl)-*N*-methyltrifluoroacetamide; TMSH, trimethylsulfonium hydroxide**

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Grazing is a major control on population density of planktonic and benthic photoautotrophic microorganisms. As a consequence, these organisms often deter grazers with morphological structures (filaments, spines, colony formation) that make their ingestion more difficult and/or by synthesizing molecules for chemical defense. The synthesis of toxins, which contribute to the observed vertebrate toxicity of cyanobacterial and algal blooms and are constitutively present in the cells, has been shown to be responsible for reduced grazing (Wolfe 2000). Eukaryotic algae have advanced systems by which the formation of deterrents and toxins is initiated by grazing. Two types of induced reactions have been studied in detail: the reaction of dimethylsulfoniopropionate (DMSP) to dimethylsulfide and acrylate, which is catalyzed by DMSP lyase, and the lipoxygenase cascade. The view that the DMSP lyase catalyzed reaction is a protection mechanism was supported by experiments with *Emiliania huxleyi* (Lohmann) Hay et Mohler. When strains containing low and high DMSP lyase concentrations were allowed to be grazed by the protozoon *Oxyrrhis marina* Dujardin, selectivity for strains with low DMSP lyase concentration was shown (Wolfe et al. 1997). This reaction, which has been described for many marine algae, may represent a chemical protection mechanism for these organisms. Another reaction sequence, the lipoxygenase cascade, is more complicated because it liberates a greater number of compounds. In organisms exhibiting these reactions, lipids are hydrolyzed upon disintegration to give unsaturated fatty acids, which are then readily oxidized to hydroperoxy fatty acids, and in a subsequent cleavage reaction unsaturated aldehydes and ω-oxo-fatty acids are formed. The cleavage of the hydroperoxides shows great variation, and a wide array of different products has been detected (Gerwick 1999, Gerwick et al. 1999). Polyunsaturated aldehydes isolated from *Thalassiosira rotula* Meunier and other marine diatoms have been shown to induce low hatching rates of the copepod *Acartia*

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*clausi* and *Calanus helgolandicus* and to contribute to the control of grazers through this antiproliferative reaction (Miralto et al. 1999). Another product, 9-oxo-nona-5,7-dienoic acid, isolated from *Gomphonema parvulum* Kützing (Pohnert and Boland 1996), was a deterrent against the marine amphipod *Amphitoe longimana* (Pohnert 2000). Although the degradation products of hydroperoxy polyunsaturated fatty acids have been shown to contribute to the control of grazers as deterrents or antiproliferative compounds, the present study shows in addition that the first reaction products, the polyunsaturated fatty acids, are also toxic to grazers.

## MATERIALS AND METHODS

*Harvest of diatom biofilms.* The diatom biofilms that covered the cobbles of the littoral zone of Lake Zürich during the cold season were scrapped off and diluted with artificial fresh water (EPA, moderately hard, containing 96 $mg \cdot L^{-1}$ $NaHCO_3$, 60 $mg \cdot L^{-1}$ $CaSO_4 \times 2\ H_2O$, 122 $mg \cdot L^{-1}$ $MgSO_4 \times 7\ H_2O$, and 4 $mg \cdot L^{-1}$ KCl) to give a 27-μM cell bound chl *a* concentration and were stored frozen at −196° C.

*Extraction of grazer toxins from the biomass.* The frozen biofilm suspension (240 mL) was thawed, and methanol was added to give a 60% methanolic suspension. After a 10-min treatment in an ultrasonic bath, the suspension was centrifuged for 15 min at 25,700*g*. The supernatant was removed, and 280 mL *tert.*butyl-methylether and 200 mL crushed ice in 100 mL water were added to the supernatant. The resulting ether phase was washed three times with 70 mL saturated $Na_2SO_4$ solution. The ether phase was dried by addition of 50 g of solid anhydrous $Na_2SO_4$ and then passed through 15 g anhydrous $Na_2SO_4$ to obtain a clear solution. The used sodium sulfate was extracted once with ether. The ether solution was dried in a rotary evaporator and the residue taken up in 5 mL methanol:acetonitrile (1:9; vol:vol) and passed through a syringe filter (Teflon, 0.2 μm, 13 mm, Whatman, Clifton, NJ). Samples of 900 μL were separated by isocratic elution on a C18 reversed-phase HPLC column (Gromsil ODS-4 HE; 5 μm, 120 Å, 10 × 250 mm) using the same solvent with a flow rate of 3 $mL \cdot min^{-1}$. To perform bioassays, fractions were taken every minute on an automatic fraction collector of an HPLC system with diode array detection (SPD-M10AVP, Shimadzu Corp., Kyoto, Japan).

*Bioassay-guided fractionation.* The fractions (fraction 1 contained the eluate between 0 and 1 min, and the other fractions were collected analogously) obtained by HPLC separation were dried in a stream of nitrogen. The residues taken up in 100 μL methanol were analyzed for toxicity in a bioassay with *Thamnocephalus platyurus.* The concentration of bioactive compounds present in each bioassay vial corresponded to an extract of diatom biofilm containing 113 μM chl *a*.

*Analysis of free fatty acids in the HPLC fractions.* The fractions obtained by HPLC separation were analyzed for free fatty acids. The solvents were removed in a stream of nitrogen and the residues taken up in 30 μL $CH_2Cl_2$, 10 μL internal standard (112 ng stearic acid-$d_{35}$ [98 atom % D] in 1 μL $CH_2Cl_2$), and 10 μL *N*-(*tert.*butyldimethylsilyl)-*N*-methyltrifluoroacetamide (MTBSTFA). After incubation for 1 h at ambient temperature, 1-μL portions were injected onto a gas chromatograph–mass spectrometer (Fisons MD 800, Carlo Erba/Fisons Instruments, Valencia, CA) equipped with a fused silica capillary column (DB 1301, 30 m × 0.32 mm i.d., 0.25-μm film thickness). Fractions with high concentrations of free fatty acids were diluted up to 1:10 to stay in the range of linearity of the signals. The injection port of the gas chromatograph was held at 270° C. Helium was used as the carrier gas (split ratio 1:15) and the gas flow (1 $mL \cdot min^{-1}$) was controlled by a head pressure of 50 kPa. Separation was performed with a temperature program (1 min 200° C, 10° $C \cdot min^{-1}$, 270° C). Full spectra were recorded between 65 and 420 amu in the EI ionization mode. The ions *m/z* [M-57] that were typically for the individual fatty acids (Table 1) were extracted from the chromatograms and the peaks integrated by area. The areas were calibrated by injection of known amounts of authentic compounds. The identity of the fatty acids was determined by co-chromatography and mass fragmentation analysis in comparison with authentic compounds. Reference compounds were not available for some minor fatty acids. In this case, typical fragment ions and the sequence of elution were used for tentative identification. For quantitative analysis of these minor fatty acids (16:2 and 16:3) not available as reference standards, the response was interpolated from the factors obtained for the corresponding unsaturated octadecanoic acids after adjustment for hexadecanoic acid. The same response factor as for 18:3Δ9,12,15 was used for the determination of 18:4.

*Gentle harvest of diatom biofilms for the analysis of natural free and total fatty acids.* For a gentle harvest that avoided any disintegration of diatoms that might initiate the liberation of free fatty acids, cobbles from the littoral zone of Lake Zürich covered with diatom biofilms were removed, and the loose part was wiped off by hand under lake water in a basket. The suspended biofilm particles were discarded several times to remove most of the sandy material. Concentration of the biofilm matter was achieved with a 150-μm net. The filtrate was passed through a net with a smaller mesh size (21 μm). Both residues were washed with particle-free lake water and suspended in lake water. The diatoms were filtered over a glass-fiber filter (GF6, Schleicher & Schuell) and immediately dipped in a beaker with 100 mL of boiling acetone. The extract was cleared with a folded filter paper and brought to dryness in a rotary evaporator. The residue was dissolved in acetone, supplemented with an internal standard, and divided into two parts. One part served for the determination of free fatty acids and the other after saponification for the determination of the total fatty acids by gas chromatography–mass spectroscopy (GC-MS) as stated above. Saponification was performed in 10 M NaOH at 80° C for 1 h. After acidification to pH < 1, the fatty acids were transferred into a $CH_2Cl_2$ phase and analyzed by GC-MS.

TABLE 1. Gas chromatographic (retention time) and mass spectrometric properties (electron impact ionization) of *tert.*butyl-dimethylsilyl derivatives (TBDMS) of fatty acids (molecular mass).

| TBDMS fatty acid | Mr (g/mol) | Rt (min) | Fragment ion (*m/z*) | Rel. intensity (%) |
|---|---|---|---|---|
| 12:0 | 314 | 3.15 | 257 | 100 |
| 13:0 | 328 | 4.06 | 271 | 100 |
| 14:0 | 342 | 5.63 | 285 | 100 |
| 15:0 | 356 | 6.75 | 299 | 100 |
| 16:0 | 370 | 8.00 | 313 | 100 |
| 16:1Δ9 | 368 | 7.82 | 311 | 95 |
| 16:2 | 366 | 7.90 | 309 | 100 |
| 16:3 | 364 | 7.72 | 307 | 12 |
| 16:Δ6,9,12,15 | 362 | 7.70 | 305 | 2 |
| 17:0 | 384 | 9.11 | 327 | 72 |
| 18:0 | 398 | 10.17 | 341 | 100 |
| 18:0 $d_{35}$ | 433 | 9.87 | 376 | 100 |
| 18:1Δ6 | 396 | 9.91 | 339 | 37 |
| 18:1Δ9 | 396 | 9.93 | 339 | 70 |
| 18:1Δ11 | 396 | 10.03 | 339 | 74 |
| 18:2Δ9,12 | 394 | 9.92 | 337 | 29 |
| 18:3Δ6,9,12 | 392 | 9.73 | 335 | 12 |
| 18:3Δ9,12,15 | 392 | 10.02 | 335 | 16 |
| 18:4 | 390 | 9.85 | 333 | 12 |
| 19:0 | 412 | 11.12 | 355 | 41 |
| 20:0 | 426 | 11.98 | 369 | 91 |
| 20:5Δ5,8,11,14,17 | 416 | 11.55 | 359 | 5 |
| 21:0 | 440 | 12.87 | 383 | 28 |
| 22:0 | 454 | 13.91 | 397 | 73 |

The fragment ions $[M\text{-}57]^+$ used for quantitative analysis and their relative intensities are given.

*Isolation of 6,9,12,15-hexadecatetraenoic acid.* About 1050 g wet weight of diatom biofilm was extracted with 1 L of acetone in an ultrasonic bath and filtered through folded filter paper. The residues were again extracted with four portions of 0.5 L of acetone and the extracts combined. The acetone was removed under reduced pressure in a rotary evaporator to give 700 mL of a dark brown turbid suspension in which 200 g NaOH was dissolved. The solution was refluxed for 2 h at 80° C for saponification. The solution was cooled to room temperature, and about 100 mL concentrated $H_2SO_4$ was added to obtain a solution of pH 13. This solution was extracted with $CH_2Cl_2$ until it became weakly yellow in color. The purified alkaline solution was acidified to pH 1 by addition of about 20 mL concentrated $H_2SO_4$ and then extracted twice with 400 mL $CH_2Cl_2$. The dichloromethane extract was dried with 50 g anhydrous $Na_2SO_4$ to obtain a clear solution.

The solvent was removed with a rotary evaporator, and 1.6 g of a dark green residue remained. This residue was dissolved in 25 mL of a mixture of acetonitrile:methanol:dichloromethane (9:1:0.1, vol:vol:vol) of which 2 mL portions were supplied to a preequilibrated cartridge (10 g Varian Mega bond RP C18, Varian, Harbor City, CA) and eluted with the same solvent. The fatty acids coeluted with the first pigments. The fatty acid fraction indicated by the pigments was collected. After removal of the solvent, this fraction gave a residue of 963 mg. A part of the residue (142 mg) was taken up in 1.3 mL solvent as stated above, and 50-μL portions were separated under isocratic conditions on the same HPLC column as used for separation of the grazer toxin. The 6,9,12,15-hexadecatetraenoic acid containing fraction, which eluted between 6.25 and 7.0 min, was dried in a rotary evaporator, and the residue was taken up in 1.5 mL acetonitrile:water:trifluoroacetic acid (79.95:20:0.05, vol:vol:vol) and further purified from traces of other fatty acids by HPLC under acid conditions. Portions of 50 μL were separated on the same column but with a gradient that contained 0.05% trifluoroacetic acid (in 20 min from 50% aq. acetonitrile to 100% acetonitrile). The eluate at 18.0–19.95 min was sampled, and most of the solvent was removed under reduced pressure. The rest was extracted three times with 50 mL $CH_2Cl_2$ and the extract dried with anhydrous $Na_2SO_4$. The yield of 6,9,12,15-hexadecatetraenoic acid was 10.3 mg and used for proton NMR spectrometry.

To remove traces of trifluoroacetic acid from the fatty acid, which was necessary for performing bioassays, another HPLC separation was conducted. The fatty acid (10.3 mg) was taken up in 2 mL acetonitrile:$H_2O$:trifluoroacetic acid (79.975:20:0.025, vol:vol:vol) and separated in a trifluoroacetic acid free solvent gradient starting with 20% aq. acetonitrile. In this chromatogram, the 16:4Δ6,9,12,15 was separated from 16:4Δ4,7,10,13, which was present at a much lower concentration. The fraction collected exhibited an HPLC purity of 99% as measured by the absorption at 203 nm. The yield of 6,9,12,15-hexadecatetraenoic acid using 5,8,11,14,17-eicosapentaenoic acid as a calibration standard was 0.8 mg.

*Determination of chl* a. The wet weight of the biofilm biomass was determined, and acetone was added to give a 90% solution. After a 10-min treatment in an ultrasonic bath, the suspension was passed through a syringe filter (0.2 μm, 13 mm, nylon, Semadeni, Ostermundingen, Switzerland) and filled up to a defined volume. The visible light absorption was read on a spectrophotometer (Cary 3). The equation of Jeffrey and Humphrey (1975) for diatoms was used to calculate the chl *a* concentration.

*Preparation of fatty acid methyl esters.* Methyl esters of fatty acids were obtained by applying trimethylsulfonium hydroxide (TMSH) as a less toxic methylation reagent (Yamauchi et al. 1979, Müller et al. 1990). The fatty acids were dissolved in 20 μL $CH_2Cl_2$, and 20 μL TMSH (0.25 M in methanol) was added and incubated at room temperature for 30 min. The solution was injected into a gas chromatograph without further treatment.

*Determination of grazer toxicity.* A bioassay with *Thamnocephalus platyurus*, conducted as described previously (Todorova and Jüttner 1996), was used to determine the 24-h acute grazer toxicity. Briefly, the wells of a 24-well microtiter plate were filled with 1 mL synthetic freshwater medium (EPA) and 10 μL of the methanolic extracts or fatty acids dissolved in ethanol. Methanol and ethanol served as the references. About five animals were added to each well, and four wells were analyzed for each concentration or fraction after a 24-h incubation time at 20° C in the dark.

*Growth of* Gomphonema parvulum. *Gomphonema parvulum* Kützing was obtained from the culture collection of the University of Göttingen (SAG 1032-1) and grown under the same conditions as stated by Wendel and Jüttner (1996), except the growth temperature was 20° C.

*Origin of chemicals.* Most fatty acids (99% purity), MTBSTFA, and TMSH were obtained from Fluka (Buchs, Switzerland); *cis*-vaccenic acid (97% purity) was from Sigma (Sigma-Aldrich, St. Louis, MO) and stearic acid-$d_{35}$ from Aldrich (Sigma-Aldrich, St. Louis, MO).

## RESULTS

Experiments to determine the acute grazer toxicity of diatom biofilms were conducted from January through April in 1998–2000. During the cold season in these years (average water temperature 4–5° C), dense epilithic diatom biofilms developed in the littoral zone of Lake Zürich. They consisted primarily of *Diatoma tenuis* Agardh (= *Diatoma elongatum* (Lyngbye) Agardh), *D. vulgaris* Bory, and *Gomphonema*, accompanied by *Fragilaria* and several small diatoms, such as *Navicula*, *Cymbella*, *Ceratoneis*, and *Nitzschia*. Green algae and cyanobacteria showed only low abundance. The loose part of the biofilm containing floating chains of *Diatoma* and stalked cells of *Gomphonema* could easily be removed by wiping by hand.

Previous experiments showed that high 24-h acute grazer toxicity for *Thamnocephalus platyurus* was obtained when the diatom biofilms were frozen once before conducting the bioassays. When the activated aqueous suspension was separated by centrifugation into a water-soluble and a particulate fraction, a minor part of the toxicity was found in the supernatant, whereas the major part of the toxicity was retained in the resuspended particulate matter (Jüttner 1999). To obtain more information on the chemical nature of toxicity, the particulate matter was analyzed by bioassay-guided HPLC and GC-MS.

The toxicity of the particulate matter could be extracted to a very high degree with methanol, and very little activity was left in the cell residue. The solvent of the methanolic extract was evaporated, and the residue was taken up in a small amount of solvent and then subjected to an HPLC separation with a water-free eluent. The development of this eluent was based on the work of Fuse et al. (1997) and was optimized for the separation of free fatty acids. The separations were traced by the UV absorption at 208 nm. The elution sequence of carotenoids and chls used as markers was followed by monitoring the 460-nm and 660-nm absorption (Fig. 1). Initially, it was not known which compounds were responsible for the absorption at 208 nm, but later it became evident that free fatty acids contributed primarily to this absorption.

Twenty-four fractions were collected and tested for 24-h acute grazer toxicity with *T. platyurus*. Two dilutions were used to trace high and low toxicity. Each

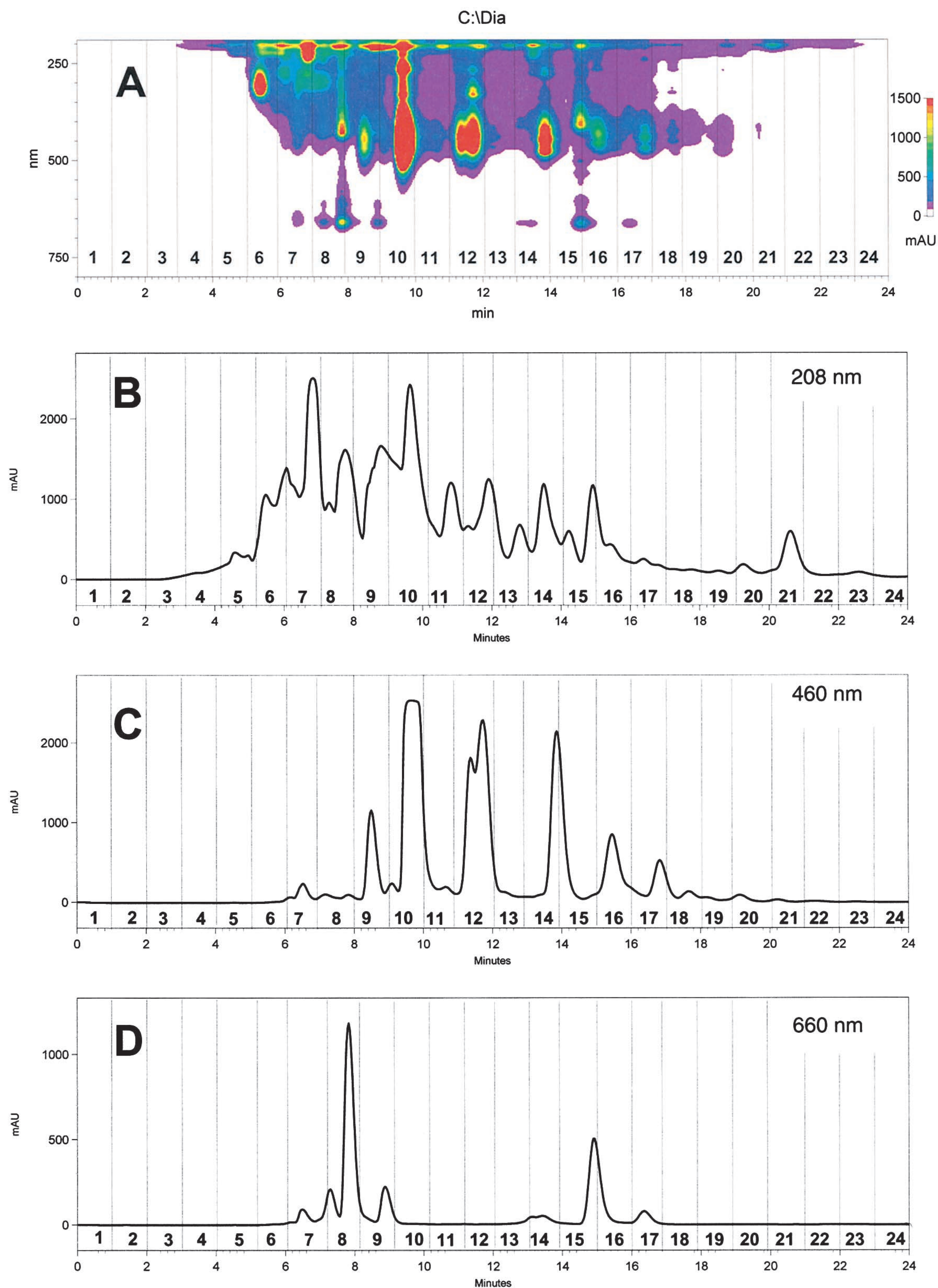



FIG. 1. HPLC separation of the 60% aq. methanolic extract of a natural diatom biofilm (8 March 1999) detected by a diode array system. Isoabsorbance plot (A) of wavelength versus time, and extracted chromatogram view for absorbance at 208 nm (B), 460 nm (C), and 660 nm (D). The latter two wavelengths were used for the detection of carotenoids and chls, respectively.

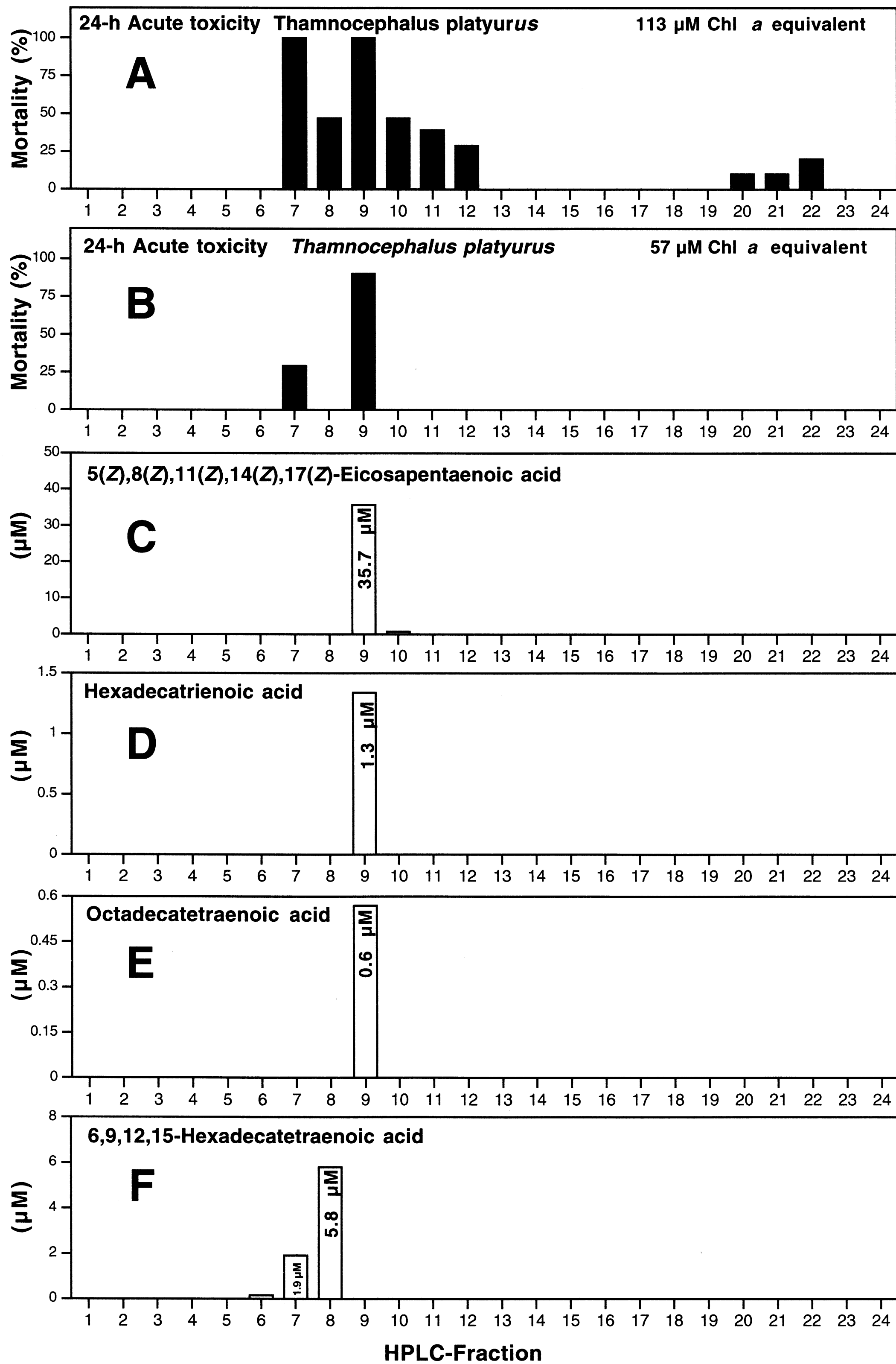



FIG. 2. Toxicity (24-h acute grazer toxicity assay) and concentration of free fatty acids (in the bioassays) of 24 fractions of the HPLC separation of Fig. 1. Toxicity was obtained for the fractions of an extract that corresponded to (A) 113 µM chl *a* and (B) 57 µM chl *a* in the diatom biofilm. The concentrations of the different free fatty acids (C–L) are given for the fractions of the more concentrated extract.

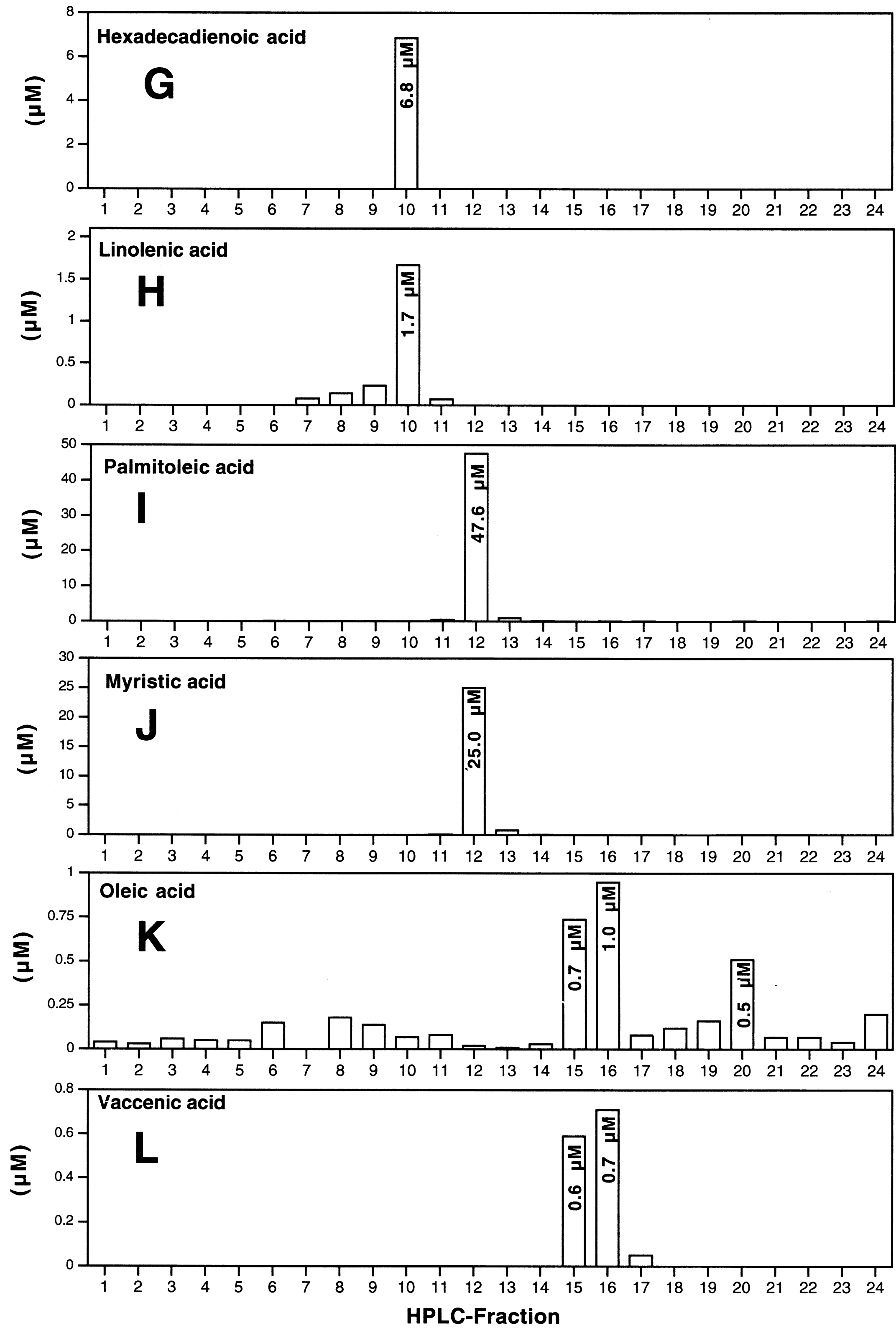



FIG. 2. Continued.

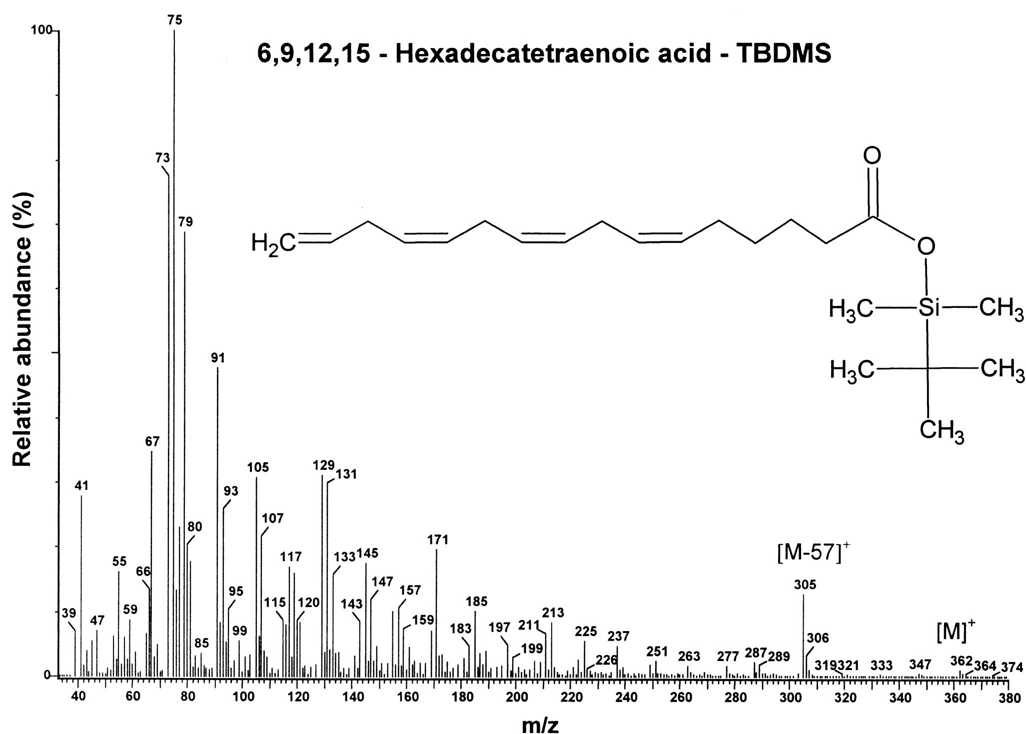
fraction of the more diluted series represented the extract of an equivalent of 57 μM chl *a* of the biofilm. As can be seen in Figure 2B, two distinct toxic fractions were observed; fraction 9 exhibited high 24-h acute grazer toxicity and a weaker toxicity was observed in fraction 7. At higher concentrations, representing 113 μM chl *a* of the extracted biofilm, the two former toxic fractions exhibited 100% mortality, and a number of other fractions showed moderate toxicity (Fig. 2A).

The toxic fractions were further studied by electrospray MS, and signals were observed that were indicative of unsaturated free fatty acids. The presence of these compounds was further supported by the absorption spectra that showed maxima at 203 nm as measured with a diode array detector of the HPLC. Therefore, each of the 24 fractions was analyzed for free fatty acids by GC-MS applying the MTBSTFA derivatization technique. The *tert.*butyldimethylsilyl derivatives turned out to be much more advantageous than the frequently used methyl esters. Their fragmentation behavior was very favorable and showed strong $[M\text{-}57]^+$ ions. These ions facilitated the quantitative determination of polyunsaturated fatty acids because acids with different molecular weight (chain lengths and number of double bonds) could easily be distinguished. As an example, the EI mass spectra of the *tert.*butyldimethylsilyl and methyl esters of 6,9,12,15-hexadecatetraenoic acid are presented in Figure 3.

The most toxic fraction (no. 9) that eluted in front of fucoxanthin contained high concentrations of all-*cis*-5,8,11,14,17-eicosapentaenoic acid (20:5Δ5,8,11,14,17) and small amounts of hexadecatrienoic acid and octadecatetraenoic acid (Fig. 2, C–E). There were no indications that other trace compounds were present. The second less toxic fraction (no. 7) coincided with the elution of 6,9,12,15-hexadecatetraenoic acid (16:4Δ6, 9,12,15) (Fig. 2F). This polyunsaturated fatty acid was also present in slightly higher concentration in fraction 8, which showed toxicity only in the more concentrated extract. The more concentrated extract (113 μM chl *a* equivalent) also exhibited toxicity in fractions 10 through 12, which contained hexadecadienoic acid, α-linolenic acid (18:3Δ9,12,15), palmitoleic acid (16:1Δ9), and myristic acid (14:0) (Fig. 2, G–J). Fractions 15 and 16, in which palmitic acid (16:0), oleic acid (18:1Δ9), and *cis*-vaccenic acid (18:1Δ11) eluted, did not show any toxicity under the applied conditions (Fig. 2, K and L).

Because the most toxic fraction coincided with the elution of 5,8,11,14,17-eicosapentaenoic acid, the next toxic fraction with 6,9,12,15-hexadecatetraenoic acid, and the fractions of weaker toxicity with α-linolenic acid and palmitoleic acid, these and other fatty acids were tested for toxicity as pure compounds. Most fatty acids were commercially available in high purity, with the exception of 6,9,12,15-hexadecatetraenoic acid, which was isolated from 1050 g wet weight of diatom biofilm by semipreparative HPLC. The identity of this compound and its purity was established by proton NMR spectrometry. The 24-h acute grazer toxicity for different free fatty acids was determined by the *T. platyurus* bioassay (Table 2). For the bioassay, fatty acids of high purity were used that were only slightly oxidized as shown by their low absorption at 236 nm in comparison with 203 nm. The $LC_{50}$ of 5,8,11,14,17-eicosapentaenoic acid was 34 μM, and this compound turned out to belong to the most toxic polyunsaturated fatty acid of this investigation. The concentration of this fatty acid in the toxic fraction 9 was sufficiently high to explain its toxicity. The next most toxic fatty acids were α-linolenic acid and γ-linolenic acid (18:3Δ 6,9,12), palmitoleic acid, and linoleic acid (18:2Δ9,12). Of these, only palmitoleic acid was present in sufficiently high concentrations to be regarded as the causative agent of the minor toxic fractions. Toxicity at 91 μM was observed for 6,9,12,15-hexadecatetraenoic acid. This concentration was too high to assume that this compound contributed significantly to the toxicity of fraction 7. Saturated fatty acids showed no toxicity.

Because the toxicity of diatom biofilms was markedly enhanced after freezing and thawing and the formation of free polyunsaturated fatty acids were responsible for this, the presence of free fatty acids was analyzed in live and once-frozen diatoms. When conventional extraction procedures were used, both samples exhibited nearly identical concentrations of free fatty acids (data not shown). In further experiments it was shown that the immediate introduction of live diatom cells into methanol at ambient temperature did not stop the hydrolysis of lipids, a process that is initiated very rapidly upon cell disintegration. Also, the application of the classical extraction solvent for lipids, methanol/dichloromethane, gave the same concentrations of free fatty acids both in live and frozen biofilms. Reduced concentrations of free fatty acids, particularly of the polyunsaturated species in live diatoms, were obtained by dipping the biofilm matter collected on glass-fiber filters into boiling methanol or boiling methanol/dichloromethane. The best results, however, were achieved with boiling acetone. Under this extraction procedure, unlike in frozen biomass, the concentrations of free fatty acids in live diatoms were very low or below the detection limit. The molar percentage of the free fatty acids obtained by different extraction procedures are presented in Table 3. In live diatoms the polyunsaturated fatty acids were below the detection limit, whereas saturated fatty acids were still present in low concentrations (Table 4). The total fatty acids in the harvested diatom biofilm over the experimental period and, for comparison, of a laboratory culture of *Gomphonema parvulum* are given in Table 5. The similar patterns suggest that diatoms are the dominant source of the major fatty acids. The even-numbered saturated fatty acids were also present as a further isomer and the odd-numbered in three to four isomers. These and several polyunsaturated fatty acids that could easily be detected by the M-57 cleavage of the *tert.*butyldimethyl-



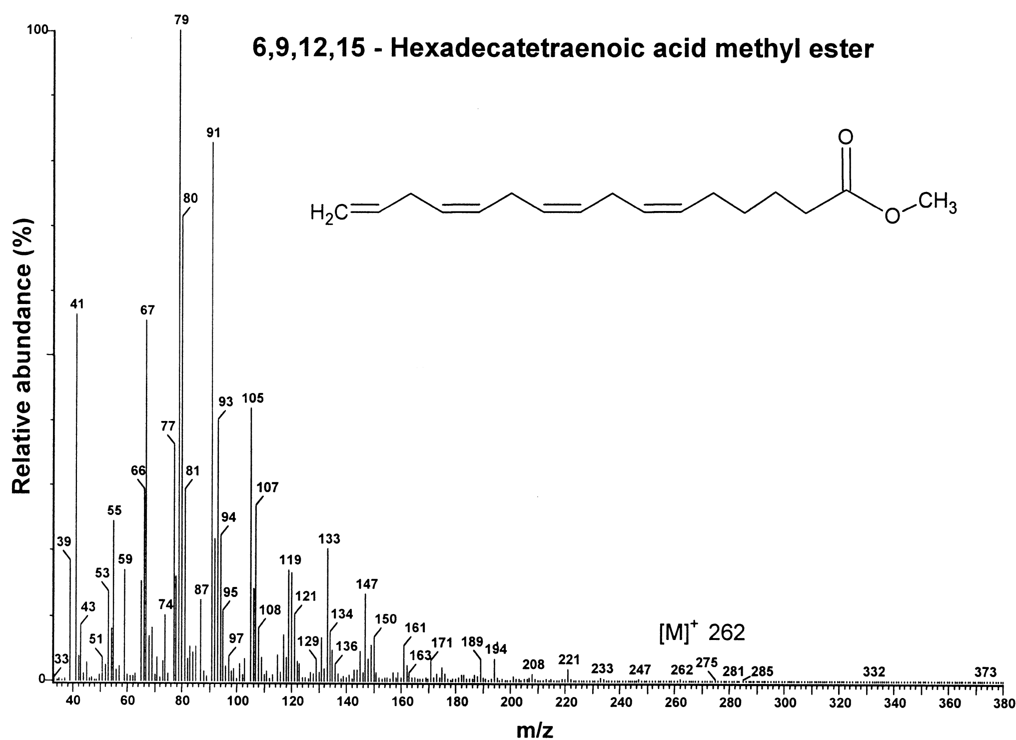




FIG. 3. EI mass spectrum of the *tert.*butyldimethylsilylester (A) and methylester of 6,9,12,15-hexadecatetraenoic acid isolated from diatom biofilms.

TABLE 2. Acute toxicity (24-h $LC_{50}$) and molecular mass ($M_r$) of saturated and unsaturated fatty acids against *Thamnocephalus platyurus.*

| Fatty acid | $M_r$ (g/mol) | 24-h $LC_{50}$ (μM) |
|---|---|---|
| 12:0 | 200 | 111 |
| 14:0 | 228 | > 405 |
| 16:0 | 256 | > 413 |
| 16:1Δ9 | 254 | 71 |
| 16:4Δ6,9,12,15 | 248 | 91 |
| 18:0 | 284 | > 317 |
| 18:1Δ6 | 282 | > 396 |
| 18:1Δ9 | 282 | > 500 |
| 18:1Δ11 | 282 | > 405 |
| 18:2Δ9,12 | 280 | 90 |
| 18:3Δ6,9,12 | 278 | 34 |
| 18:3Δ9,12,15 | 278 | 33 |
| 20:0 | 312 | > 370 |
| 20:5Δ5,8,11,14,17 | 302 | 34 |

TABLE 3. Effect of extraction conditions on the occurrence of free fatty acids (mol percentage of the total fatty acid) of diatom biofilms from the littoral zone of Lake Zürich and of a culture of *Gomphonema parvulum.*

| Source | Activation | Solvent | Temp-erature | Total free fatty acid (mol %) |
|---|---|---|---|---|
| Biofilm | −20° C | $H_3O^+/CH_2Cl_2$ | 24° C | 35 |
| Biofilm | −196° C | MeOH | Boiling | 53 |
| Biofilm | Grinding | Acetone | Boiling | 28 |
| Biofilm | 20% NaCl | Acetone | Boiling | 4 |
| Biofilm | No | MeOH | Boiling | 12 |
| Biofilm | No | $MeOH/CH_2Cl_2$ | Boiling | 16 |
| Biofilm | No | Acetone | Boiling | 0.4 |
| *Gomphonema* | No | Acetone | Boiling | 1 |

Activation was initiated by freezing at −20° C and −196° C (liquid nitrogen), acidification and extraction with $CH_2Cl_2$ ($H_3O^+/CH_2Cl_2$), addition of 20% NaCl (10 min), and grinding in a mortar. The extraction was performed with methanol (MeOH), methanol/dichloromethane, and acetone at ambient temperature and at boiling point. Activation was prevented (no activation) by dipping glass-fiber filters, on which the cells were concentrated, in the extraction solvents.

silyl derivatives were not investigated further because their low concentration made it rather unlikely that they contributed to the observed toxicity.

Previous experiments (Jüttner and Dürst 1997) showed the addition of high salt concentrations that cause osmotic stress initiate reactions of the lipoxygenase pathway that involves the liberation of fatty acids. This technique was used to analyze the liberation rate of free fatty acids upon stress. As shown in Figure 4, the hydrolysis of lipids and formation of free fatty acids proceeds very rapidly. High concentrations of free fatty acids were observed already after the shortest applied interval (1 min). Only the liberation of 5,8,11, 14,17-eicosapentaenoic acid continued for some minutes, whereas the liberation of the other fatty acids was already complete after this time period. Hydrolysis of lipids also took place when the cells were disintegrated mechanically (Table 3). The grinding of live diatoms in a mortar should imitate the action of grazers and should demonstrate the effect of mechanical disintegration of the cells.

## DISCUSSION

In this study, epilithic diatom biofilms, which frequently develop in the littoral zone of lakes and in running waters, were studied for chemical defense. The chemical defense of photoautotrophic biofilms is important to their persistence because these assemblages of organisms are heavily grazed by harpacticoid copepods (Fenchel 1998), ciliates, snails (Gregory 1983), nematodes (Farmer 1992), insect larvae (Peterson et al. 1998), water fowl, and other organisms. The organisms that graze on them have presumably developed physiological defense to overcome this defense mechanism of the food organism. To trace the molecules responsible for chemical defense in diatom biofilms, a bioassay organism was chosen that was not adapted to chemically protected cyanobacteria. The anostracan *T. platyurus,* which colonizes ephemeral puddles, was found in a previous study to lack resistance to cyanobacteria (Kurmayer and Jüttner 1999) and is also a suitable organism to trace the toxicity of diatoms (Jüttner 1999).

This study focused on polyunsaturated free fatty acids because all evidence pointed to the fact that 5,8,11,14,17-eicosapentaenoic acid is the major causative agent for the toxicity of diatom biofilms. One strong argument is that this acid was nearly pure in the HPLC fraction with the highest toxicity. The separation conditions were adjusted in such a way to achieve this result. As indicated by UV and visible light absorption measurements, GC-MS analysis of the MTBSTFA and TMSH derivatized fraction, and ES-MS, other compounds were present in this fraction only in very low amounts. This fact is fully supported by the toxicity experiments conducted with authentic compounds in high purity. The 24-h $LC_{50}$ of 5,8,11,14,17-eicosapentaenoic acid was sufficiently low to be consistent with the concentration of 5,8,11,14,17-eicosapentaenoic acid and activity of the most toxic fraction.

A different situation was observed with the second toxic peak of the HPLC eluate. Initially, 6,9,12,15-hexadecatetraenoic acid, present in this fraction and a typical fatty acid of cold-acclimated diatoms, was assumed to be responsible for the toxicity. However, 6,9,12,15-hexadecatetraenoic acid isolated in high purity exhibited only medium toxicity, and the concentrations in this fraction support the view that this compound contributed only a minor part to the overall toxicity. Other not yet identified compounds must be responsible for the observed toxicity of this fraction.

The toxicity of the diatom biofilm was not restricted to the occurrence of a particular diatom species. Seasonal analyses in which a species succession took place showed that the toxicity is a general phenomenon of many different epilithic diatoms (Jüttner in press).

Bioactivity caused by polyunsaturated fatty acids has been reported for several flagellates, and the pro-

TABLE 4. Percentage (mol%) of free fatty acids of the total fatty acid (free and bound) in live *Gomphonema parvulum* and diatom biofilms that were untreated or ground.

| | Percentage of free fatty acid (mol%) | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Sample | 14:0 | 16:0 | 16:1 | 16:2 | 16:3 | 16:4 | 18:0 | 18:1 | 18:2 | 18:3 | 20:5 |
| Live *Gomphonema* | 0.5 | 1.2 | 0.1 | 0.0 | 0.0 | 0.0 | 13 | 1.1 | 1.2 | 0.0 | 0.0 |
| Live biofilm | 0.4 | 1.0 | 0.2 | 0.1 | 0.0 | 0.0 | 4.1 | 1.6 | 0.8 | 0.9 | 0.0 |
| Ground biofilm | 38 | 21 | 32 | 32 | 17 | 22 | 19 | 23 | 20 | 32 | 43 |

Hydrolysis of lipids was stopped by dipping biomass-coated glass-fiber filters into boiling acetone.

duction of these compounds is responsible for the toxic effects of marine blooms of flagellates. The bloom forming *Chrysochromulina polylepis* Manton et Park (Yasumoto et al. 1990) and *Gymnodinium aureolum* excreted octadecapentaenoic acid, among other compounds. *Chrysochromulina* was also reported to cause death of the ciliate grazer *Favella ehrenbergii* Claparède et Lachmann (Carlsson et al. 1990), but direct proof of the responsible compound is not available. Toxic effects of polyunsaturated fatty acids produced by *Gymnodinium* cf. *mikimotoi* have been reported for embryogenesis of *Paracentrotus lividus* Lam. (Sellem et al. 2000) and *Asterina pectinifera* (Murakami et al. 1989).

The toxicity of polyunsaturated free fatty acids against various biotic processes is well established. This makes it rather difficult to explain the presence of a high pool of intracellular free fatty acids, as generally reported for different algae. In various marine diatoms, up to 26% of the total fatty acids were in the free form (Dunstan et al. 1994). *Pseudo-nitzschia multiseries* (Hasle) Hasle growing in the exponential phase exhibited 34% (Parrish et al. 1991) and *Gyrodinium aureolum* 7.5% of the total fatty acids in the free form (Parrish et al. 1993). The experiments of the present study show for the first time that, although often assumed to do so, the conventional extraction procedures do not stop the very rapidly proceeding hydrolysis of lipids and that free polyunsaturated fatty acids cannot be detected in live cells. Only saturated and monounsaturated fatty acids, which exhibit low or no toxicity, were present in low concentrations in live cells.

As in the wound reaction in higher plants (Rosahl 1996) and marine algae (Gerwick et al. 1999), a very rapid enzymatic hydrolysis of lipids is initiated as soon as the diatom cell looses its integrity. The integrity can be destroyed by freezing and thawing, due to solvents and salts, mechanical forces, or any other factor that leads to cell death. As has been shown for the induced reaction of DMSP and DMSP lyase, ingestion by grazers can initiate such reactions (Wolfe 2000). The ecological advantage of such a reaction is evident when the growth habit is colonial or patchy. The destruction of a single cell helps to protect the other cells from being grazed. The physical properties of polyunsaturated fatty acids are very suitable for a grazer toxin in an aquatic habitat. As a result of their low solubility in water, most fatty acids remain in the particles and render them poisonous. This defense strategy can be

TABLE 5. Total fatty acids in the diatom biofilms (*Diatoma*, *Gomphonema*) of the littoral zone of Lake Zürich over the experimental period in 2000 (date of sampling) and in a culture of *Gomphonema parvulum*.

| | Percentage of total fatty acids (mol%) | | | | | |
|---|---|---|---|---|---|---|
| | Biofilm | | | | | *Gomphonema parvulum* |
| Fatty acid | 11/1/00 | 1/2/00 | 8/2/00 | 1/3/00 | 29/3/00 | |
| 12:0 | 0.6 | 0.6 | 1.4 | 0.8 | 1.0 | 0.6 |
| 13:0 | 0.2 | 0.1 | 0.2 | 0.1 | 0.1 | 0.05 |
| 14:0 | 8.9 | 7.8 | 9.3 | 7.3 | 8.5 | 7.8 |
| 15:0 | 0.9 | 1.3 | 1.2 | 0.3 | 0.8 | 0.5 |
| 16:0 | 25.3 | 10.0 | 16.1 | 15.0 | 10.6 | 27.0 |
| 16:1Δ9 | 16.2 | 15.9 | 23.3 | 31.6 | 19.9 | 47.5 |
| 16:2 | 7.5 | 8.4 | 7.1 | 7.2 | 7.1 | 2.6 |
| 16:3 | 4.5 | 5.7 | 7.2 | 6.1 | 5.4 | 0.2 |
| 16:4 | 5.1 | 7.3 | 8.7 | 8.0 | 8.4 | 0.2 |
| 17:0 | 0.7 | 0.4 | 0.4 | 0.03 | 0.4 | 0.07 |
| 18:0 | 9.1 | 3.3 | 3.5 | 0.8 | 2.4 | 1.5 |
| 18:1Δ9 / 18:1Δ11 | 3.0 | 8.1 | 4.9 | 1.6 | 5.4 | 5.1 |
| 18:2Δ9,12 | 1.6 | 4.9 | 2.9 | 0.9 | 5.0 | 1.1 |
| 18:3Δ6,9,12 | 0.5 | 1.3 | 0.8 | 0.7 | 1.7 | 0.5 |
| 18:3Δ9,12,15 | 1.8 | 4.1 | 3.9 | 0.5 | 3.4 | — |
| 18:4 | 0.2 | 0.3 | 0.2 | 0.2 | 1.2 | 0.2 |
| 19:0 | 0.1 | 0.2 | 0.1 | 0.1 | 0.2 | 0.01 |
| 20:0 | 0.7 | 0.4 | 0.3 | 0.02 | 0.5 | 0.04 |
| 20:5Δ5,8,11,14,17 | 10.1 | 18.3 | 7.3 | 17.1 | 15.6 | 4.8 |
| 21:0 | 0.1 | 0.2 | 0.1 | 0.2 | 0.4 | 0.02 |
| 22:0 | 2.9 | 1.4 | 1.1 | 1.5 | 2.0 | 0.2 |

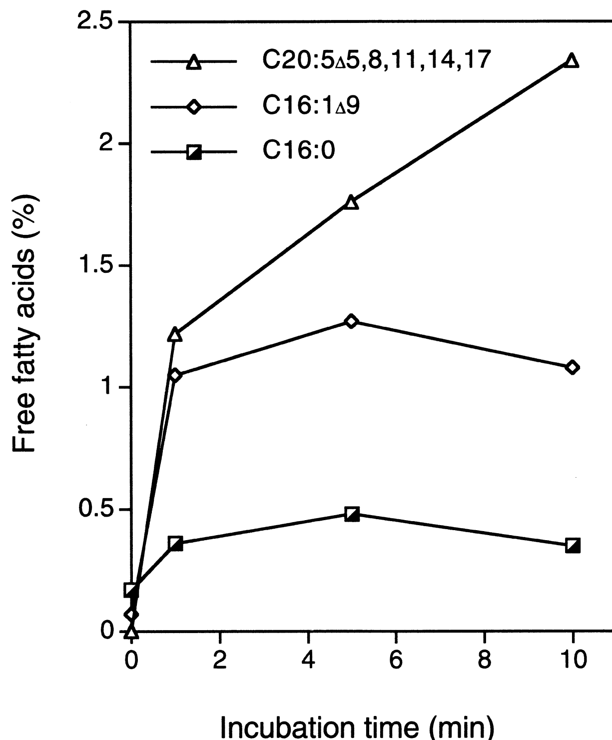



FIG. 4. Time course of the formation of free fatty acids (20:5Δ5,8,11,14,17; 16:0; 16:1Δ9) from lipids. The reaction was initiated by an osmotic shock (20% NaCl) and stopped with boiling acetone.

regarded as advanced from the standpoint of evolutionary biology because an essential cell constituent (phospholipid) is rapidly converted into a highly toxic grazer toxin and no extra cost for permanent defense molecules are required. This contrasts to the rather conservative strategy of cyanobacteria that have to spend an extra amount of energy for the synthesis of potent grazer toxins of complicated structures (Jüttner 1999).

The composition of fatty acids in freshwater diatoms have only rarely been investigated (Kates and Volcani 1966), but there is considerable knowledge of fatty acids available for brackish (Müller-Navarra 1995) and marine diatoms (Gillan et al. 1981, Parrish et al. 1991, Thompson et al. 1992, Dunstan et al. 1994). A diatom-dominated periphyton population consisting primarily of *Gomphonema*, *Nitzschia*, and *Navicula* was analyzed by Napolitano (1994). The composition of fatty acids in the diatom biofilms of the littoral zone of Lake Zürich was rather similar to the pattern found in Napolitano's study. Saturated fatty acids were dominated by palmitic acid, myristic acid, and stearic acid. The most important unsaturated fatty acid were palmitoleic acid and 5,8,11,14,17-eicosapentaenoic acid. Major amounts of α-linolenic acid and several mono- to tetraunsaturated fatty acids were also found. High concentrations of 6,9,12,15-hexadecatetraenoic acid, which was first isolated from marine phytoplankton with *Biddulphia sinensis* Grev. as the dominant organism (Klenk and Eberhagen 1962), were also present in the diatom biofilms. This polyunsaturated fatty acid, which is a typical constituent of diatom lipids, has so far only been found in cold-acclimated cells of other algal group (Thompson et al. 1992) and is assumed to be responsible for increase of membrane fluidity at low temperatures. It is interesting to note that in the present study the laboratory culture of *Gomphonema*, unlike the natural diatom biofilms, contained extremely low concentrations of this acid. This can be explained by the different temperatures to which the cultivated *Gomphonema* and the diatoms of the natural biofilms have been exposed. The water temperature in the lake was in the range of 4–6° C, whereas the growth temperature of *Gomphonema* was held at 20° C. Growth experiments with *Stauroneis amphioxys* Gregory, a sea ice diatom (Gillan et al. 1981), have shown an increase of this and other polyunsaturated fatty acids at a low temperature of 3° C. Inverse correlations of the concentration of this polyunsaturated fatty acid with temperature have also been demonstrated for several marine diatoms and flagellates (Thompson et al. 1992).

Fatty acid methyl esters, though widely used for the determination of fatty acids in diatoms (Gillan et al. 1981, Parrish et al. 1991, Dunstan et al. 1994), are not particularly suitable for quantitative analysis by MS. Applying electron impact ionization, the molecular ions are very small or absent and the low molecular fragment ions are not characteristic enough to distinguish easily between individual fatty acids of different molecular weight. Other derivatives like nicotinates (Keusgen et al. 1996) and other ionization techniques (Mansour et al. 1999, McLachlan et al. 1999) have been used to overcome this problem. In the present study, *tert.*butyldimethylsilyl derivatives of fatty acids were used (Mawhinney et al. 1986). These derivatives can be easily generated and exhibit sufficient strong fragment ions $(M-57)^+$ of high indicative value. The favored cleavage of the *tert.*butyl group generates a fragment ion that makes it easy to calculate the molecular weight and, hence, to differentiate fatty acids in their chain length and degree of unsaturation.

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