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Evidence that polyunsaturated aldehydes of diatoms are repellents for pelagic crustacean grazers

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Abstract

Evidence is given that odour compounds of diatoms serve as potential repellents for crustacean grazers. Novel repellent-test and odour-test apparatus allowed the determination of repellent activity of diatom derived compounds, activated by freezing and thawing or mechanical disintegration, and pure compounds, respectively. Epilithic diatom biofilms when activated, produced odour compounds that were determined by GC–MS to be polyunsaturated aldehydes (PUA). 2(*E*),4(*Z*),7(*Z*)-Decatrienal and 2(*E*),4(*Z*)-octadienal were the major compounds, and 2(*E*),4(*Z*)-heptadienal was a minor compound. These PUA were each accompanied by small amounts of the *E*,*E*-isomers in positions 2 and 4. 2(*E*),4(*E*),7(*Z*)-Decatrienal was the most active repellent tested and exhibited a RC_{50} value (indicating the concentration of a compound necessary for a 50% reduction of swimming crustaceans in the assay vial) of 3.5 μM in a defined water column. Quantitative analyses showed that upon activation diatom biofilms produced large amounts of eicosapentaenoic acid (EPA) of which only a minor part was degraded to PUA. The major part of EPA was retained in the cells whilst the major part of PUA was released into the surrounding water. The data are consistent with the hypothesis that diatoms damaged by grazers develop free EPA in the cells that is toxic to grazers, and release PUA into the water that serve as warning signals to grazers. Diatoms and other phytoplankton species, that have the capacity to form these compounds, might benefit from such a reaction because the producers live in colonies or assemblages and the death of one cell liberates a cloud of repellent compounds into the water which reduces the grazing pressure on the remaining cells. Such activated defence reactions may help explain food selection and avoidance in freshwater and marine ecosystems.

Introduction

In any successful survival strategy, the perception of suitable food is an indispensable requirement for grazers. Many studies in terrestrial ecosystems have shown that chemical cues to detect and select food particles are given high priority by consumers (Paré and Tumlinson 1999). Such infochemicals allow communication by both remote signals and those that need close or direct contact between organisms. The long-range cues are odour compounds that are active as repellents or attractants and need extremely low concentrations for their perception, while the short-range cues are stimulants or deterrents that primarily act via the taste system of animals (Lindstedt 1971). The rationale

behind both strategies is the avoidance of toxic and indigestible food.

Feeding deterrents are defined as compounds which prevent continuation of feeding or force termination of feeding. Several compounds fitting this property have been detected in marine macroalgae and benthic cyanobacteria. A general property of these compounds is that they are effective against particular grazers rather than being active against all grazers. Ypoamide was isolated in a bioassay-guided procedure from cyanobacterial assemblages as a broadly acting deterrent against generalist fishes (Nagle and Paul 1998). Mixtures of several bioactive compounds isolated from monospecific tufts of cyanobacteria off Guam behaved as deterrents against generalist fishes, but were stimulants to specialist fishes (Nagle and Paul 1999). Activated defence caused by physical damage, as observed by eating or simulated by crushing, was reported for *Halimeda* sp. In this green alga, halimedatetraacetate is bioconverted upon activation to halimedatrialdehyde which is a greater deterrent to herbivorous fish than the educt (Paul and van Alstyne 1992). Another example is dimethylsulfoniopropionate which is widely distributed in marine macroalgae. Upon activation it is split into dimethylsulfide and acrylate. The latter was shown to deter feeding by sea urchins (van Alstyne et al. 2001).

Experiments have shown that pelagic organisms also utilize chemical defences. Apo-fucoxanthinoids derived from carotenoids in the marine diatom *Phaeodactylum tricornutum* are efficient deterrents against the harpacticoid copepod, *Tigriopus californicus* (Shaw et al. 1995). Dimethylsulfoniopropionate, a constitutive component of *Emiliania huxleyi*, reduced grazing to a variable degree, depending on grazer taxa (Strom et al. 2003). For freshwater phytoplankton, defined compounds have not yet been described, but experiments clearly indicate the existence of deterrents. Calanoid copepods (*Diaptomus birgei*) discriminated strongly against some cyanobacteria when they were administered in low concentrations in the presence of a high-quality chlorophyte (DeMott and Moxter 1991). The food selection and discrimination of individual particles can best be explained by the presence of deterrents in rejected cyanobacteria, but the compounds responsible are not known. Experiments with fragmented filaments of *Planktothrix rubescens* from which the microcystins were removed by extraction were still rejected by *Eudiaptomus* and it appears therefore that compounds other than the toxic microcystins are responsible for the deterrent reaction (Kurmayer and Jüttner 1999). A similar behaviour was also demonstrated for *Daphnia galeata* which did not ingest a mutant of *Microcystis* deficient in microcystin synthesis (Rohrlack et al. 1999).

Much less is known in aquatic ecosystems on repellents and attractants that are long-range cues to control the uptake of food organisms by grazers. It is generally accepted that aquatic invertebrates are able to perceive odours (Larsson and Dodson 1993) and have a potential for remote chemoreception (Moore et al. 1999). The large number of odour receptors detected in the nematode *Caenorhabditis elegans* and the fruit fly *Drosophila melanogaster* support this view (Krieger and Breer 1999; Firestein 2001). The few studies with freshwater grazers to detect chemical cues for remote chemoreception were contradictory. Experiments with *Daphnia* in a Y-tube olfactometer demonstrated positive chemotaxis to water from cultures of an edible green alga (*Scenedesmus acuminatus*) and cyanobacterium (van Gool and Ringelberg 1996). In later experiments in which water of a culture of a different chlorophyte (*Scenedesmus obliquus*) was applied, positive chemotaxis could not be detected (Roozen and Lürling 2001). In both experiments analyses of odour compounds were not performed. The absence of positive chemotaxis of the latter experiment can be twofold. Either the grazers were unable to detect the odour compounds or the algal strains used did not produce odour compounds.

In the present investigation, I demonstrate that algal odour compounds can be perceived by crustacean grazers and can cause repellent reactions. Components of the experiments were benthic diatoms, which are efficient producers of odour compounds upon activation, and pelagic grazers of the genera *Daphnia*, *Cyclops* and *Eudiaptomus*. The distribution of compounds produced by activation by freezing and thawing or mechanical disintegration, between diatom biomass and water phase, showed that the physical behaviour of polyunsaturated aldehydes (PUA) would enable the induction of remote sensing. It is hypothesized that these compounds are warning signals for the toxic eicosapentenoic acid that is formed in toxic concentrations and retained in the cells.

Materials and methods

Crustaceans

Planktonic crustaceans were collected from Lake Zürich between March and July 2002. The animals were concentrated by several vertical tows between 25 and 0 m depth with a net of 250 μm mesh size in the middle of the lake near to Kilchberg. To remove the larger phytoplankton species (primarily the filamentous cyanobacterium *Planktothrix*), the collected crustaceans were suspended in artificial freshwater (moderately hard, for composition see Jüttner 2001) and subjected to another filtration. When predators (*Chaoborus*, *Leptodora* and *Bythotrephes)* were present in high numbers, these were removed by filtration through a coarser net (400 μm mesh size). The crustaceans were suspended again in artificial freshwater and stored at 8 °C in the dark for 2–7 h. For the experiments, actively swimming crustaceans (*Eudiaptomus gracilis*, *Cyclops abyssorum*, *C. vicinus*, *C. bohater*, *Cyclops* sp., *Mesocyclops leuckarti*, *Bosmina* sp., *Daphnia hyalina* and *D. galeata*) were sampled with a wide orifice polyethylene pipette (4 mm i.d.) to separate them from dead matter (carapaces), and inactive crustaceans which assembled at the bottom of the bottle, and some *Daphnia* and *Bosmina* which had air bubbles and concentrated at the surface of the water. The species composition (around 300 individuals) was determined under a dissection microscope after addition of ethanol. The crustacean composition used for repellent assays against odour compounds varied in the range of 48–84% *Cyclops*, 10–37% *Bosmina* and *Daphnia*, and 1–15% *Eudiaptomus*. The temperature of the lake water at 20 m depth from which the major part of crustaceans was isolated increased from 4.6 to 7.8 °C during the experimental period.

Collection and activation of diatoms

Diatom biofilms of the littoral zone of Lake Zürich were collected and activated by a freeze–thaw cycle as described recently (Jüttner 2001). Briefly, only the loose part of the epilithic diatom biofilms growing on cobbles was wiped off by hand under lake water in a basket. The whirled up diatom suspension was poured off several times to remove the mineral fraction. The diatoms were concentrated by settling for a few minutes. The supernatant lake water was removed and the remaining dense diatom suspension was frozen at −196 °C in liquid nitrogen and stored at −20 °C. Before conducting bioassays, the frozen diatom biomass was thawed. By freezing and thawing, activation takes place leading to the formation of toxins and repellents in the diatom biomass.

Repellent assay of activated diatoms

Repellent assays of activated diatoms against crustaceans were conducted in a newly designed repellent-test apparatus, which was constructed like a pipe organ from glass tubes (Figure 1). First, the repellent test-apparatus was filled with 40 ml of artificial freshwater containing the crustaceans until the water reached the level of the constrictions on the vertical tubes. The number of the crustaceans (67% *Cyclops*, 7% *Bosmina* and *Daphnia* and 26% *Eudiaptomus*) was adjusted so that a total of about 400 individuals was present in the apparatus. At this density, approximately 15 animals were swimming in each vertical tube. Into each vertical glass tube, an insert was introduced. These inserts were glass tubes (40 mm length, 7 mm o.d.) which fitted tightly inside the vertical tubes of the apparatus and were closed at the base with a 30×30 mm hydrophilic polyester net (1-μm mesh size, SEFAR PETEX 07-1/2^{TM}, Sefar AG, Heiden, Switzerland) allowing exchange of molecules but no diatom cells. The apparatus with inserts was cooled to 8 °C in a fridge, before 1 ml activated diatom suspension was added to the inserts. Every second insert received 1 ml of artificial lake water as a control. After incubation at 8 °C in the dark for 2–3 h, the crustaceans swimming below the net in the vertical tubes were counted. Repellent reactions were quantified by comparing the number of swimming animals in each vertical tube beneath the inserts filled with diatoms, with that in the control tubes filled with artificial freshwater. In experiment A, 15 inserts were filled with activated diatoms and 10 with artificial lake water as controls. Five apparatus were used in parallel. In experiment B the numbers were 16 and 12, respectively, in four apparatus. The chlorophyll *a* concentration of the activated diatom suspension was 380 nmol g^{-1} wet biomass in

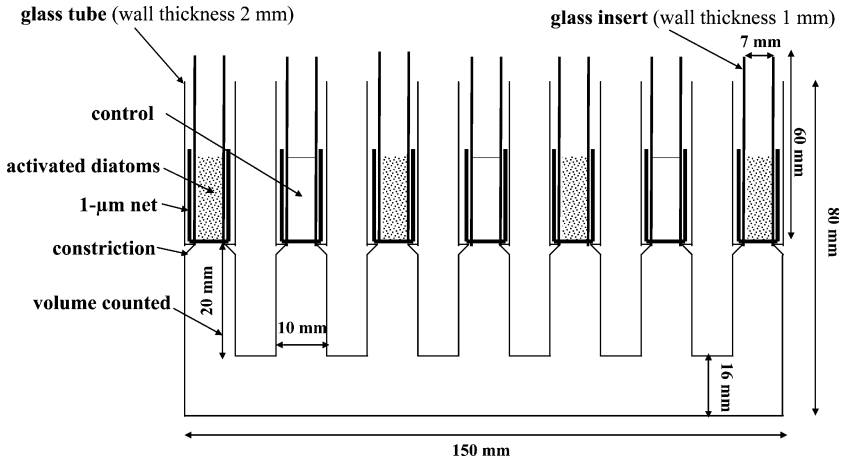

Figure 1. Design of the repellent-test apparatus. Inserts covered with nets on their bases were introduced into the vertical tubes and charged with activated diatoms or freshwater as the control. See text for explanations.

experiment A and 230 nmol g^{-1} biomass in experiment B. The dominant diatoms were *Diatoma ehrenbergii* and *D. vulgaris* which were accompanied by minor amounts of *Cymbella lanceolata*, *Navicula* sp., *Nitzschia* sp., *Gomphonema* sp., *Achnanthes minutissima*, *Stephanodiscus* sp. and *Synedra* sp.

Repellent assay of chemicals

A similar but smaller apparatus (odour-test apparatus) than that described above was used to conduct repellent assays of PUA and fatty acids. The glass tubes had an inner diameter of 3 mm (Figure 2). Dye experiments with methylene blue have shown that the smaller tube width is superior because mixing between the overlaying test water and freshwater did not occur in the observation time. A density gradient was achieved by adding an overlay of ambient temperature (25 °C) onto the cold water (8 °C) of the odour-test apparatus. The procedures were as follows: the odour-test apparatus was filled with artificial freshwater and crustaceans (about 150 animals) and cooled down to 8 °C in a fridge in about 15 min. Overlays of artificial freshwater (100 µl aliquots) containing different concentrations of PUA or fatty acids and not more than 1% ethanol were added to 5 tubes. Two tubes that got an overlay with 100 µl artificial freshwater containing 1% ethanol served as controls. Different concentrations of a compound

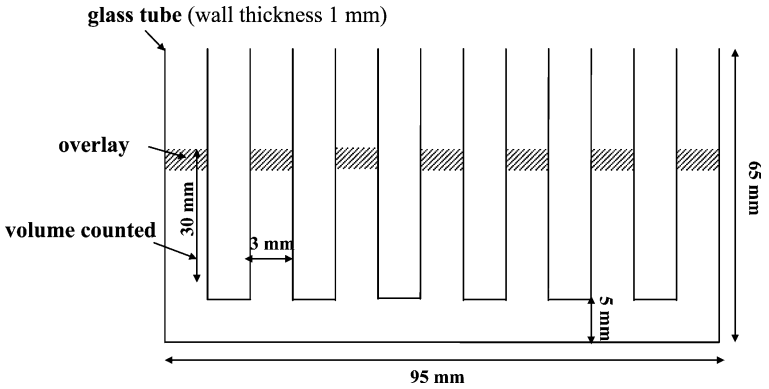

Figure 2. Design of the odour-test apparatus. A 25 °C warm artificial lake water containing different concentrations of the odour compounds was layered on 8 °C cold lake water that contained the crustacean grazers. See text for explanations.

(5 concentrations and two controls) were assayed in the same apparatus. After 15 min storage in the dark at 8 °C, the reaction of the crustaceans was measured by counting the swimming animals in the vertical part of the apparatus. RC_{50} values allowed the comparison of the repellent efficiency of different chemicals. They were defined as the concentration of an odour compound causing a 50% reduction in the number of swimming animals in the vertical water column after application of the overlay as compared with control overlays of artificial freshwater containing 1% ethanol. For each compound, at least seven different concentrations were assayed in five parallel apparatus. Four replicates were performed for EPA and at least eight for the PUA. Medians were calculated for each concentration and used for the generation of RC_{50} values. The RC_{50} values were calculated according to LC_{50} values applying the program GraphPad, Prism, San Diego. StatView, SAS Institute, Inc., was used for the statistical analysis.

Toxicity assay

The fairy shrimp *Thamnocephalus platyurus* was used to determine the LC_{50} acute grazer toxicity of PUA. The assay introduced by Centeno et al. (1995) was performed as described by Jüttner (2001). The use of closed vessels for the assay was essential to avoid any loss of the volatile aldehydes during incubation.

Distribution of aldehydes and EPA between the liquid and solid phase

The loose part of epilithic biofilms was harvested with a spatula and left standing in a basket to allow the cells to settle. The supernatant lake water was poured off to obtain a dense diatom suspension. An aliquot of 25 g (wet mass) of the suspension was diluted in 125 ml of water and frozen, initially at −196 °C with liquid nitrogen before storage at −20 °C. The cells activated by this procedure were thawed and left standing at ambient temperature for 2.5 h to complete the enzymatic reactions. The enzymes were denatured by adding 5 ml of 5% (v/v) H_2SO_4 to give a suspension at pH 1.5. After 10 min the suspension was neutralized with 10 M NaOH. The suspension was separated by passage through a glass fibre filter (GF 92, Schleicher and Schuell, Dassel, Germany) that has been purified with dichloro methane to analyse the filtrate and the retained diatom cake for PUA.

Analysis of volatile organic compounds (VOC)

The procedure of the stripping analysis of VOC was recently described (Durrer et al. 1999). The filtrate (25 ml) or 1.0 g of the diatom filter cake was diluted with water to 250 ml and, as an internal standard, 35 μl of a solution (102 μg ml^{-1}) of deuterated geosmin ((±)-*trans*-1α,10β-[$^2H_3$]dimethyl-9α-decalol) were added with a Hamilton syringe introducing the orifice below the water level. Fifty millilitres of this solution were placed in a 250-ml round-bottomed flask and 10 g of purified NaCl were added. The flask was connected to a closed loop stripping device and stripped for 30 min. The VOC were adsorbed onto a cartridge containing 150 mg Tenax TA (60–80 mesh). The cartridge was removed, the VOC thermally desorbed, cryofocussed on a capillary column at 0 °C and chromatographed. The GC column was a 30 m DB-1301 (0.32 mm i.d., 0.25 μm film thickness, chemically bound), the temperature program: 4 min isotherm at 0 °C, followed by an increase of the oven temperature to 170 °C at a rate of 5 °C min^{-1}. The head pressure of the carrier gas helium was 50 kPa, the split ratio 1:15. The retention times of the PUA were: 2(*E*),4(*Z*)-heptadienal (20.0 min), 2(*E*),4(*E*)-heptadienal (20.5 min), 2(*E*),4(*Z*)-octadienal (23.3 min), 2(*E*),4(*E*)-octadienal (23.7 min), 2(*E*), 4(*Z*),7(*Z*)-decatrienal (29.2 min) and 2(*E*), 4(*E*), 7(*Z*)-decatrienal (29.8 min). Full EI spectra in the range from m/z 29 to m/z 269 (4 scans s^{-1}) were recorded. Quantitative analyses were performed using authentic aldehydes (for the sources see Jüttner 1984; Jüttner et al. 1986; Wendel and Jüttner 1996). The aldehydes were stripped, chromatographed and the response factors determined (n = 12) for the fragment ions at m/z 81. The same response factors as determined for the *E*-isomers were used for the *Z*-isomers which were not available as reference compounds in sufficient amounts and purity. The fragment ion at m/z 115 served as a

calibration signal for the deuterated geosmin used as the internal standard.

Analysis of free eicosapentaenoic acid (EPA)

The *tert*-butyldimethylsilyl derivative was used to quantify the free EPA according to the procedure published recently (Jüttner 2001). EPA was analysed in 25 ml of the filtrate and 675 mg of the diatom filter cake suspended in 25 ml of water. The solutions/suspensions were supplemented with 50 μl deuterated stearic acid-d_{35} (98 atom% D, 1.125 μg μl^{-1} CH_2Cl_2) as an internal standard and extracted 5 times with 25 ml dichloromethane. The combined extracts were dried over 5 g of anhydrous Na_2SO_4 and filtered through a filter covered with 5 g of anhydrous Na_2SO_4. After evaporation of the solvent, the residue was dissolved in 1 ml of dichloromethane in an ultrasonic bath. Forty microlitres of the extract from the diatom cake or 80 μl of the extract from the filtrate was transferred into 1.5-ml glass vials with a Teflon lined septum. Twenty microlitres of *N*-(*tert*-butyldimethylsilyl)-*N*-methyltrifluoroacetamide (MTBSTFA) and 40 μl dichloromethane were added through the septum to the extract from the diatom cake, but only MTBSTFA was added to the extract from the filtrate. The reaction time at ambient temperature was between 40 min and 3 h. One microlitre of the diatom fraction and 5 μl of the filtrate fraction were injected into a combined gas chromatograph–mass spectrometer. The fragment ions used for calibration of EPA were m/z 359 $[M\text{-}57]^+$ and m/z 376 for deuterated stearic acid.

Analysis and sources

Chlorophyll *a* (Chl *a*) was quantitatively determined in 90% acetone extracts on a spectrophotometer (Cary 3) using the equation given by Jeffrey and Humphrey (1975). MTBSTFA and EPA were obtained from Fluka/Buchs, Switzerland, and deuterated stearic acid was from Aldrich.

Results

Repellent activity of activated diatoms

Repellent activities of natural diatom biofilms, mainly comprised of the genus *Diatoma*, were determined after activation of the diatoms in a newly constructed repellent-test apparatus. Two experiments conducted closely to each other in time showed strong repellent activities of the activated diatoms against crustaceans (Figure 3). The overall significance of repellent activities in the first experiment (A, 26 March 2002) was $p < 0.0001$ ($n = 10$) and in the second (B, on 3 April 2002) $p = 0.004$ ($n = 12$, ANOVA unpaired *t*-test).

Determination of VOC

To elucidate the structures of the compounds responsible for the repellent reaction of the crustaceans, GC–MS analyses of the VOC in the activated diatom biofilms were performed. The compounds were determined by comparison of the mass fragmentation patterns and retention times with those of known compounds. A typical gas chromatogram of VOC is presented in Figure 4 and shows the presence of six different PUA. 2(*E*),4(*Z*),7(*Z*)-Decatrienal was dominant and

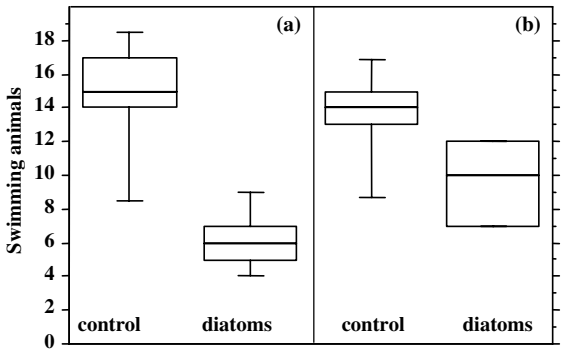

Figure 3. Repellent assay of activated diatoms with the repellent-test apparatus. The number of swimming crustaceans beneath inserts filled with artificial lake water (control) and activated diatom biofilms harvested from the littoral zone of Lake Zürich were counted. The central horizontal line in the boxes indicates the median, the top the 75th percentile and the bottom the 25th percentile. The upper and lower cross-line indicate the 90th and 10th percentile, respectively. Analysis of activated diatoms of experiment A on 26 March 2002 [380 nmol Chl *a* (g wet biomass)$^{-1}$; 8 nmol of 2,4-heptadienal (μmol Chl *a*)$^{-1}$; 34 nmol of 2,4-octadienal (μmol Chl *a*)$^{-1}$; 121 nmol of 2,4,7-decatrienal (μmol Chl *a*)$^{-1}$] and of experiment B on 3 April 2002 [227 nmol Chl *a* (g biomass)$^{-1}$; 7 nmol of 2,4-heptadienal (μmol Chl *a*)$^{-1}$; 119 nmol of 2,4-octadienal (μmol Chl *a*)$^{-1}$; 111 nmol of 2,4,7-decatrienal (μmol Chl *a*)$^{-1}$]. For each experiment the sum of the *E*/*Z* isomers is given.

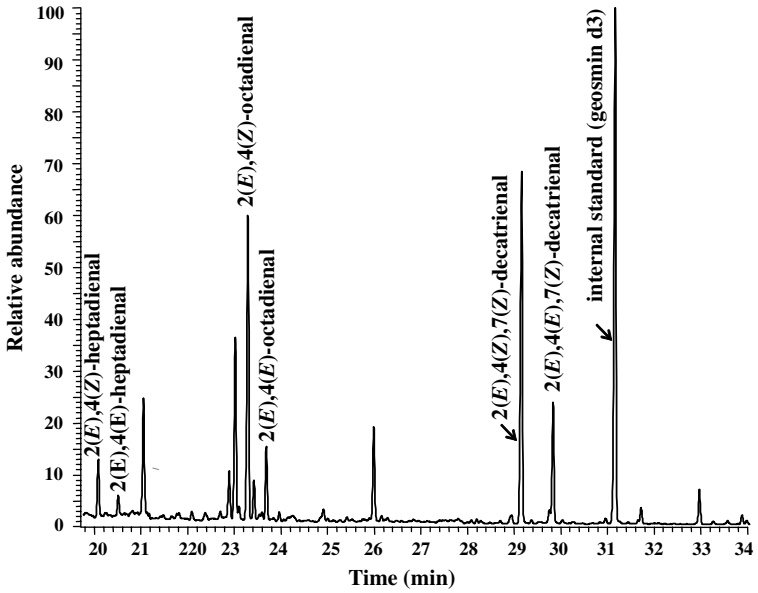

Figure 4. Gas liquid chromatogram of the VOC of an activated diatom biofilm.

exhibited concentrations of up to 112 pmol per nmol Chl *a* and was accompanied by its *EEZ*-isomer. In addition, isomers of 2,4-heptadienal and 2,4-octadienal were present. The *E*,*Z*- and *E*,*Z*,*Z*-configurations rather than the *E*,*E*- and *E*,*E*,*Z*-configurations were dominant for all PUA.

Determination of repellent efficiency

The contribution of different PUA to the repellent activity of activated diatoms against grazers was studied with pure compounds in an odour-test apparatus. Only the isomers that had an *E*-configuration in position 4 were available in purities and amounts high enough to conduct bioassays. The analyses clearly showed that grazers can perceive these compounds but at different concentrations. 2(*E*),4(*E*),7(*Z*)-Decatrienal was the most effective odour component of the diatom biofilms causing repellent reactions in the crustaceans. In Figure 5, box plots are shown displaying percentiles of swimming crustaceans beneath different concentrations of 2(*E*),4(*E*),7(*Z*)-decatrienal. The repellent reactions at 83 μM (p-value < 0.001, $n = 22$) and at 8 μM (p-value 0.02, $n = 22$) were significant and a trend was observed at 0.8 μM (p-value 0.44, $n = 22$) and 0.08 μM (p-value 0.56, $n = 22$, ANOVA unpaired t-test). To compare the repellent efficiency of PUA and EPA, RC_{50} values were determined (Table 1). 2(*E*),4(*E*),7(*Z*)-Decatrienal was by far the most efficient repellent exhibiting a RC_{50} value as low as 3.5 μM. Some activity was also found for 2(*E*),4(*E*)-decadienal, but the lower members of the PUA and EPA showed only weak repellent activities.

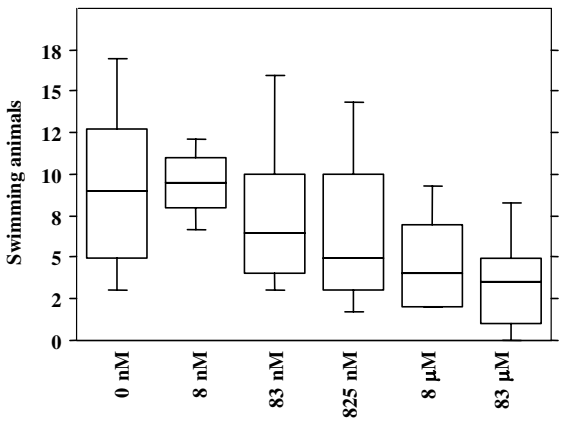

Figure 5. Repellent activity of 2(*E*),4(*E*),7(*Z*)-decatrienal against crustaceans determined in the odour-test apparatus. The number of swimming crustaceans in the vertical tubes are given for overlays containing concentrations between 0 and 83 μM (control $n = 35$; 8 nM $n = 14$; 83 nM to 83 μM $n = 22$). See text for further explanations.

Table 1. RC_{50} values (repellent concentration causing 50% loss of swimming crustaceans in the assay vial) of compounds found in activated diatom biofilms.

Compound	RC_{50} (μM)
2(*E*),4(*E*),7(*Z*)-Decatrienal	3.5
2(*E*),4(*E*)-Decadienal	42
2(*E*),4(*E*)-Octadienal	390
2(*E*),4(*E*)-Heptadienal	350
Eicosapentaenoic acid	230

Distribution of PUA and EPA

To examine the distribution of PUA and EPA between lake water and the diatom biomass, quantitative analyses were performed for both matrices. In a typical experiment after activation of the diatoms by freezing and thawing, the volume ratio of lake water to biomass was adjusted to approximately 40:1 (v:v). Table 2 presents the concentrations of the different PUA in lake water and the diatom biomass, and gives the mol percentage of an odour compound found in lake water. When the analysis was extended to EPA, striking differences in the distribution of both compound classes were observed (Table 3). While the major part of aldehydes was found in the lake water phase, the opposite was true for EPA.

Acute toxicity

The *Thamnocephalus platyurus* acute toxicity assay was applied to determine the 24-h LC_{50} values for the PUA found in diatom biofilms. The highest toxicity was found for 2(*E*),4(*E*),7(*Z*)-decatrienal exhibiting a 24-h LC_{50} of 13 μM (mean of $n = 3$). 2(*E*),4(*E*)-Heptadienal and 2(*E*),4(*E*)-octadienal were less toxic (45 and 72 μM, respectively).

Seasonal occurrence

The experiments with natural diatom biofilms were conducted over a period of 2 years. Although the abundance of species varied slightly, the PUA were found in similar concentrations in the diatom biofilms (Table 4) allowing comparison of experiments conducted with diatoms taken at different times. Experiments conducted on 27 February 2002 showed that disintegration of diatom cells by mechanical forces (for 5 min in a mixer) resulted in similar concentrations of aldehydes as those obtained after freezing at -196 °C and thawing (Table 4). Although disintegration in a mixer more closely resembled the disintegration of diatom cells caused by grazer attack freezing and thawing was preferred because of its ease in performance.

Discussion

In the experiments described here, benthic diatoms were used as repellent sources and pelagic crustaceans of Lake Zürich as target organisms. By means of two differently constructed assay systems, diatoms and pure compounds could be tested for their capacity to act as repellents. The design of the repellent-test apparatus was extremely efficient and allowed the measurement of repellent molecules released from activated diatom biofilms. The reduction in swimming crustaceans in the zone adjacent to the repellent source served as a measure of repellent activity. The cause of the

Table 2. Concentration of PUA in diatom biofilms activated by a freeze–thaw cycle.

PUA released	PUA nmol (μmol Chl *a*)$^{-1}$		PUA in lake water (mol%)
	Lake water	Biomass	
2(*E*),4(*Z*)-Heptadienal	6.9 ± 1.7	1.2 ± 0.6	81
2(*E*),4(*E*)-Heptadienal	2.5 ± 0.7	0.6 ± 0.2	81
2(*E*),4(*Z*)-Octadienal	52.5 ± 1.7	1.2 ± 0.5	98
2(*E*),4(*E*)-Octadienal	12.1 ± 2.6	1.4 ± 0.5	89
2(*E*),4(*Z*),7(*Z*)-Decatrienal	49.4 ± 19.3	4.8 ± 1.9	91
2(*E*),4(*E*),7(*Z*)-Decatrienal	15.6 ± 8.3	2.3 ± 1.5	87

The concentrations in the aqueous phase (140 ml) and biomass (3.6 ml biovolume = 6.7 μmol Chl *a*) were normalized to Chl *a* (SD $n = 5$). The mol percentages of the aldehydes in the lake water are also given.

Table 3. Distribution of PUA and EPA between the aqueous phase (140 ml) and biomass (3.6 ml biovolume) of diatom biofilms activated by a freeze–thaw cycle (SD for $n = 5$).

Matrix	nmol		μM		mol%	
	PUA	EPA	PUA	EPA	PUA	EPA
Lake water	939	274 ± 19	7	2	92	4
Biovolume	81	6623 ± 1125	23	1845	8	96

The absolute amounts and the calculated concentrations of PUA and EPA in each matrix, as measured under the experimental conditions, are given. In addition, the mol percentage distribution (mol%) of a compound between both matrices is presented.

Table 4. Concentration of PUA per algal biomass observed in epilithic diatom biofilms of the littoral zone of Lake Zürich in the cold season (SD for $n = 3$ of the March and April samples).

Sampling date	Activation		Concentration of PUA (pmol (nmol Chl a)$^{-1}$)					
	−196 °C	Mix	*EZ*H[a]	*EE*H	*EZ*O	*EE*O	*EZZ*D	*EEZ*D
27 Feb 2001	x		3.0	0.67	11	1.5	66	15
27 Feb 2001		x	5.2	1.3	14	1.8	51	15
19 Mar 2001	x		2.5 ± 0.3	0.56 ± 0.16	8.4 ± 0.5	0.80 ± 0.55	52 ± 8.7	12 ± 3.0
26 Mar 2002	x		6.9 ± 1.7	0.97 ± 0.36	31 ± 2.6	2.9 ± 0.8	112 ± 18	9.3 ± 1.5
2 Apr 2002	x		6.3 ± 1.4	1.0 ± 0.3	112 ± 23	6.4 ± 2.0	106 ± 24	4.5 ± 1.5

The collected diatom biofilms were activated by freezing to −196 °C or mechanical disintegration for 5 min in a mixer (mix) before analysis.

[a]*EZ*H – 2(*E*),4(*Z*)-Heptadienal; *EE*H – 2(*E*),4(*E*)-heptadienal; *EZ*O – 2(*E*),4(*Z*)-octadienal; *EE*O – 2(*E*),4(*E*)-octadienal; *EZZ*D – 2(*E*),4(*Z*),7(*Z*)-decatrienal; *EEZ*D – 2(*E*),4(*E*),7(*Z*)-decatrienal.

changed swimming behaviour responsible for the decrease in grazers in the water beneath the overlay was not studied further, but may be a reduction in swimming activity as recently shown for kairomones (Seely and Lutnesky 1998). Because the diatoms were only separated from the swimming field of the grazers by a net, liberated grazer repellents could easily pass this barrier. Unlike membranes frequently used, unspecific sorption of compounds to the net material applied here can be assumed to be very low.

Different odour concentrations were tested in the odour-test apparatus for their ability to repel grazers. A stable gradient between both phases preventing mixing in the experimental time, was achieved using different water temperatures. The structures of the assay systems outlined above turned out to be superior to other experimental designs described in the literature. Particularly wide spread is the use of U-tubes (Pennak 1973) and Y-tubes (van Gool and Ringelberg 1996; Baumgärtner et al. 2002), which allow the measurement of the preference for one arm over the other. Five-chambered flow-through systems have also been used (Laurén-Määttä et al. 1997). The apparatus described here permitted the simultaneous assay of 7 different samples. By multiplication of the number of apparatus, which could be loaded very rapidly, data sets can be obtained which allow the determination of RC_{50} values. The effectiveness of different biochemicals as causative agents for the repellent reaction can be quantified and structure related data can be obtained.

Benthic epilithic diatoms proved to be a rich and permanent source of repellents. *Diatoma* was dominant in all of the biofilms in this study, but determining which diatom is responsible for the repellent activity can only be obtained using pure cultures. The diatoms activated by a freeze–thaw cycle liberated two different groups of molecules from the precursor compounds, polyunsaturated fatty acids of which EPA was the major component, and PUA. In a previous study, EPA has been determined as the most toxic compound for grazers in activated diatoms (Jüttner 2001). It behaves like many vitamins that are toxic in high concentrations. Gas chromatographic and mass spectrometric analyses allowed the determination of the structures of the PUA. 2(*E*),4(*Z*),7(*Z*)-Decatrienal was found to be present in the

highest concentration on most sampling dates. Other important compounds were 2(*E*),4(*Z*)-octadienal and 2(*E*),4(*Z*)-heptadienal. They were accompanied by minor concentrations of the *E*-isomers of the double bond between carbon 4 and 5.

Quantitative analyses were conducted to trace those compounds, which might be responsible for the repellent activity and to allow the comparison of their actual concentrations in diatom biofilms with the determined RC_{50} values. After activation by freezing and thawing, the concentrations of both EPA and PUA were analysed in the diatom biomass and the lake water. The volume of the lake water was chosen arbitrarily, to allow general observations on the different distributions of the compounds in both matrices. The concentration of EPA was extremely low in lake water as expected from the low solubility of this compound in water, and the high concentrations found in the biomass support the hypothesis of EPA as a toxin (Jüttner 2001). The opposite distribution was observed with the PUA which were primarily dissolved in the lake water. The concentration of 7 μM was sufficient to explain a significant repellent reaction as based on the RC_{50} of 2(*E*),4(*E*),7(*Z*)-decatrienal of 3.5 μM.

The concentrations of compounds shown here form only one of many possible scenarios. Both EPA (Jüttner 2001) and PUA (Pohnert 2000) are formed during disintegration in the diatom cell in a very fast reaction. The maximum concentration of EPA and PUA in the cellular biomass can be calculated to be 1847 and 30 μM, respectively. Whilst EPA is retained within the cellular biomass, the PUA are dissolved in the surrounding lake water and may quickly form a robust microzone around the cell (Moore et al. 1999). A grazer causing the disintegration of one diatom cell will experience a cloud of repellents when a new attack is performed. The microzones are thought to persist in the boundary layer of small particles for some time (Wolfe 2000) and the existence of robust microzones, stable for about 10 min, around point sources has been shown (Blackburn et al. 1998). The concentrations would be sufficiently high to establish an odour concentration reaching RC_{50} values of 2(*E*),4(*E*),7(*Z*)-decatrienal, while the concentrations of EPA in the lake water would be insufficient to reach the rather high RC_{50} of 235 μM.

2(*E*),4(*E*),7(*Z*)-Decatrienal was the most active repellent found in this study. Reduction of chain length and number of double bonds of the molecule reduced the repellent activity. A major part of the PUA was present in the *Z*-configuration of the double bond positions at carbon 4 and 7 which are the first biosynthetically formed compounds after cleavage of the hydroperoxy fatty acid. Isomerization into the more stable *E*-double bond proceeds rapidly and special care is required to keep the isomerization reaction low. Only the *E*-isomers in the double bond position of carbon 4 were available for the bioassays in sufficient amounts.

The acute grazer toxicity of PUA against *Thamnocephalus platyurus* required concentrations about twice as high as the LC_{50}-values determined previously in the sea urchin embryo cleavage assay and the copepod egg hatching assay (Miralto et al. 1999).

PUA and EPA are liberated in very fast reactions upon activation of the diatom cells. EPA, which is a precursor compound in the formation of decatrienals in the marine diatom *Thalassiosira rotula* (Pohnert 2002) is not present in live cells but is liberated by hydrolysis of lipids. The biochemical reactions observed by mechanical disintegration may be similar to the wounding effect caused by grazers. Freezing and thawing the diatom biomass resulted in similar concentrations of lipoxygenase derived VOC as observed by mechanical forces. The quantitative analyses of activated biomass of diatoms revealed that only a small part of EPA is converted by lipoxygenases and hydroperoxide lyases to PUA leaving the major part as an insoluble toxin in the particulate. The accumulation of EPA indicates that the oxygenation reaction is limited.

The concomitant formation of EPA and PUA is an advanced repellent reaction. In a few enzymatic reactions a toxin and an infochemical can be produced from lipids, which are essential constituents of the cell, and major components of membranes. Such an induced repellent reaction would be of benefit if this reaction takes place in a multicellular organism or if the cells are organized to live in colonies. In both cases the repellent reaction induced by disintegration of a primary victim cell would be advantageous to the other cells in the assemblage preventing further attack and would aid the survival of the remaining cells. Such a cooperative defence can also be important in bio-

films exhibiting a patchy growth habit. In addition, one could imagine that unicellular flagellates living in swarms would benefit from such a reaction.

Numerous photoautotrophic freshwater microorganisms are known to exhibit such a reaction cascade after activation. PUA were first detected during a bloom of *Synura uvella* in a freshwater lake (Jüttner 1981). They have also been found using monoxenic cultures of diatoms (*Melosira varians* and *Fragilaria*, Wendel and Jüttner 1996, 1997) and chrysophytes (*Dinobryon cylindricum* and *Synura petersenii*, Rashash et al. 1995). A common feature of these producers is that they are colony-forming planktonic algae. The formation of PUA has also been observed in epilithic diatom biofilms of lotic waters (Jüttner and Dürst 1997). Recently PUA were detected in the marine diatom *Thalassiosira* and were made responsible for embryogenic toxicity in crustaceans (Miralto et al. 1999). Their wide spread occurrence in benthic and pelagic colony-forming photoautotrophes may be an indication of the great ecological importance of these compounds.

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