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*RAPID COMMUNICATION*

# SURVEY OF THE CHEMICAL DEFENCE POTENTIAL OF DIATOMS: SCREENING OF FIFTY ONE SPECIES FOR α,β,γ,δ–UNSATURATED ALDEHYDES

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**Abstract**—In recent years a negative influence of diatom-derived α,β,γ,δ-unsaturated aldehydes (PUA) on the reproductive success of copepods and invertebrates has been suggested. Since adverse chemical properties of diatoms would question the traditional view of the marine food web, this defense mechanism has been investigated in detail, but the PUA-release by test organisms has only been determined in a few cases. The observed effects were nevertheless frequently discussed from a general point of view often leading to contradictory conclusions. We have examined the PUA-production of 51 diatom species (71 isolates) in order to provide a basis for the interpretation of laboratory and field results on the influence of diatom food on the reproductive success of their consumers. PUA-production is species and strain dependent. Thirty-six percent of the investigated species (38% of the cultivated isolates) release α,β,γ,δ-unsaturated aldehydes upon cell disruption in concentrations from 0.01 to 9.8 fmol per cell. *Thalassiosira rotula* and *Thalassiosira pacifica*, major spring-bloom forming diatoms

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isolated from Roscoff (Bretagne, English Channel, France) and Puget Sound (Washington, USA) were among the PUA-producing strains.

**Key Words**—Alga/herbivore interactions, plankton, pentafluorobenzylhydroxylamin, copepod, reproductive success

## INTRODUCTION

Diatom-derived α,β,γ,δ-unsaturated aldehydes (polyunsaturated aldehydes, PUA) can interfere with the hatching success and larval development of copepods as well as with the embryonic development of invertebrates (see Ianora et al., 2003 and Pohnert, 2005 for reviews). Diatoms are unicellular algae that contribute substantially to the phytoplankton and are thus an important base of the marine food web. A generally negative impact of diatoms on herbivores would have major implications for the classical concept of plankton ecosystem functioning. Therefore, numerous studies, mainly based on laboratory experiments, have been carried out to clarify this predator-prey relationship. While some authors have proposed a deleterious effect of diatoms (Ianora et al., 2003), others have not found any adverse effects (e.g., Jonasdottir et al., 1998). Moreover, no general negative relationship between copepod egg hatching and diatom biomass was detected in the global field survey by Irigoien et al. (2002). Most of these studies correlated hatching parameters to the presence or absence of diatoms rather than monitoring PUA production of the diet, even if this is the postulated determining factor in these interactions. This is surprising, since only a few marine and fresh water diatoms have been investigated for PUA-production (Pohnert, 2005). Indeed, some of the controversially discussed results on reproductive parameters (Paffenhöfer et al., 2005) might be explained by variability of PUA production in different diatom species. To overcome this limitation and to provide a basis for further ecological and modelling studies, we conducted a survey of volatile PUA-production from representative diatoms of the major classes by using a protocol based on in situ trapping of the reactive metabolites (Wichard et al., 2005).

## METHODS AND MATERIALS

*Cultivation*. Seventy one diatom-isolates from different sources were investigated (Table 1). All isolates were grown in stationary cultures using 250 ml jars containing 100 ml artificial medium (Table 1). Sylvania (Germany) "Cool white deluxe (F36W 840, 4000K)" tubes provided illumination. A light regime of 16:8 (light/dark) with 30–40 $\mu E/m^2/sec$ light intensity was used. Generally, the growth temperature was 15.5°C, except for *Thalassiosira*

TABLE 1. OVERVIEW OF THE ISOLATES INVESTIGATED BY SOLID PAHSE MICROEXTRACTION AND PFBHA-DERIVATISATION

| Species | SPME/ PFBHA[a] | Detected PUA | Medium[b] | Source or culture no. |
|---|---|---|---|---|
| *Actinocyclus subtilis* | •/• | – | $f_2$ | CCAP 1000/1 |
| *Amphiphora paludosa* | –/• | +[c] | $f_2$ | SAG 15.83 |
| *Asterionella formosa* | •/• | –[k] | WC | SAG 8.95 |
| *Asterionellopsis glacialis* | •/• | + | $f_2$ | PLY 607 |
| *Chaetoceros calcitrans* | •/• | – | $f_2$ | CCAP 1010/11 |
| *Chaetoceros compressus* | •/• | + | $f_2$ | PLY 550 |
| *Chaetoceros muelleri* | •/• | – | $f_2$ | CCMP 1316 |
| *Coscinodiscus granii* | •/• | – | $f_2$ | Unknown |
| *Coscinodiscus* sp. | –/• | – | $f_2$ | RCC 773[d] |
| *Cyclotella meneghiniana* | •/• | – | WC | SAG 1020-1a |
| *Ditylum brightwellii* | •/• | – | $f_2$ | PLY 609 |
| *Ditylum brightwellii* | •/– | – | $f_2$ | Friday Harbor[e] |
| *Ditylum brightwellii* | –/• | – | $f_2$ | RCC 775[d] |
| *Fragilaria capucina* | •/• | – | WC | Lake Constance[f] |
| *Fragilaria* sp. | •/• | + | WC | Lake Constance[f] |
| *Gomphonema parvulum* | •/– | –[k] | WC | SAG 1032-1 |
| *Guinardia deliculata* | –/• | + | K | Roscoff[d] |
| *Guinardia striata* | –/• | – | K | Roscoff[d] |
| *Melosira nummuloides* | •/• | + | $f_2$ | CCAP 1048/6 |
| *Melosira sulcata* | •/• | + | $f_2$ | Jiaozhou Bay, China |
| *Navicula pelliculosa* | •/• | – | WC | SAG 1050-3 |
| *Navicula sallinicola* | –/• | – | $f_2$ | SAG 40.96 |
| *Navicula* sp. | •/• | – | $f_2$ | RCC 781[d] |
| *Navicula* sp. | •/– | – | K | RCC 457 |
| *Navicula transitans* | •/• | – | $f_2$ | RCC 80 |
| *Nitzschia* sp. | •/• | – | $f_2$ | RCC 782[d] |
| *Nitzschia closteridium* | –/• | – | $f_2$ | RCC 81 |
| *Nitzschia curvilineata* | –/• | – | $f_2$ | SAG 48.91 |
| *Nitzschia frustulum* | –/• | – | br | SAG 1052-2 |
| *Odontella regia* | •/• | +[c] | $f_2$ | RCC 772[d] |
| *Odontella sinensis* | •/– | – | $f_2$ | PLY 606 |
| *Paralia sulcata* | •/• | – | $f_2$ | DML[g] |
| *Phaeodactylum tricornutum* | •/• | – | br | UTEX 646 |
| *Phaeodactylum tricornutum* | •/• | – | br | SAG 1090-1a |
| *Phaeodactylum tricornutum* | –/• | – | br | SAG 1090-1b |
| *Phaeodactylum tricornutum* | •/• | – | $f_2$ | PLY 100 |
| *Pleurosigma normanii* | •/– | – | $f_2$ | MBA[g] |
| *Pseudonitzschia* sp. | •/• | – | $f_2$ | PLY 611 |
| *Rhizosolenia setigera* | –/• | – | K | Roscoff[d] |
| *Rhizosolenia setigera* | –/• | – | $f_2$ | CCMP 1820 |
| *Skeletonema costatum* | •/• | + | $f_2$ | RCC 75 |
| *Skeletonema costatum* | –/• | + | $f_2$ | SAG 19.99 |
| *Skeletonema costatum* | •/– | + | $f_2$ | CCMP 781 |
| *Skeletonema costatum* | •/– | + | $f_2$ | CCMP 784 |

TABLE 1. CONTINUED

| Species | SPME/ PFBHA[a] | Detected PUA | Medium[b] | Source or culture no. |
|---|---|---|---|---|
| *Skeletonema costatum* | •/– | + | $f_2$ | CCMP 2092 |
| *Skeletonema pseudocostatum* | •/• | + | $f_2$ | See reference[h] |
| *Skeletonema subsalsum* | –/• | + | WC | SAG 8.94 |
| *Stephanodiscus hantzschii* | •/• | – | WC | Lake Constance[f] |
| *Stephanodiscus minutulus* | •/• | – | WC | SAG 49.91 |
| *Stephanophyxis turris* | •/• | –[k] | $f_2$ | DML[g] |
| *Thalassionema nitzschioides* | –/• | – | K | RCC 785[d] |
| *Thalassiosira aestivalis* | •/• | + | $f_2$ | Dabob Bay (779)[e] |
| *Thalassiosira anguste-lineata* | •/• | + | $f_2$ | Dabob Bay (779)[e] |
| *Thalassiosira eccentrica* | •/• | – | $f_2$ | CCAP 1085/6 |
| *Thalassiosira minima* | •/• | + | $f_2$ | CCAP 1085/8 |
| *Thalassiosira nordenskioeldii* | •/• | + | $f_2$ | Dabob Bay (748)[e] |
| *Thalassiosira pacifica* | •/• | + | $f_2$ | Dabob Bay (779)[e] |
| *Thalassiosira pseudonana* | •/• | – | $f_2$ | CCAP 1085/12 |
| *Thalassiosira pseudonana* | •/• | – | $f_2$ | CCMP 1335 |
| *Thalassiosira pseudonana* | –/• | – | br | SAG 1020-1b |
| *Thalassiosira punctigera* | •/– | – | $f_2$ | Point Wells (748)[e] |
| *Thalassiosira rotula* | •/• | + | $f_2$ | CCMP 1018 |
| *Thalassiosira rotula* | •/• | + | $f_2$ | CCMP 1647 |
| *Thalassiosira rotula* | •/• | + | $f_2$ | CCMP 1812 |
| *Thalassiosira rotula* | –/• | + | $f_2$ | RCC 776[d] |
| *Thalassiosira rotula* | –/• | + | $f_2$ | Point Wells (805)[e] |
| *Thalassiosira rotula* | •/– | + | K | RCC 290 |
| *Thalassiosira* sp. | •/– | + | K | RCC 349 |
| *Thalassiosira weissflogii* | •/• | – | $f_2$ | Unknown |
| *Thalassiosira weissflogii* | –/• | – | br | SAG 122.79 |
| *Thalassiosira weissflogii* | –/• | – | $f_2$ | RCC 76 |
| *Prorocentrum micans*[i] | •/– | – | $f_2$ | Unknown |
| *Prorocentrum minimum*[i] | •/• | – | $f_2$ | RCC 291 |

*aestivalis* and *Skeletonema costatum* (CCMP 784), which were grown at 13°C and 23°C, respectively. Cultures reached the stationary phase after 2–3 wk, when 60–90 ml ($10^4$-$10^6$ cells/ml culture medium, depending on species) were harvested. The cell morphology of each culture was checked prior to harvest with light microscopy. Cells were counted with a Neubauer-improved haemocytometer in four replicates. The counting variance ranged from 10–35%.

*Quantitative PUA Analysis*. The cultures were harvested by filtration as described in Wichard et al. (2005). For a first rapid screening of the cultivated isolates, solid phase microextraction was performed with a polydimethylsiloxan fiber after wounding by sonication as described in Pohnert et al. (2002). To quantify PUA release upon cell damage, a protocol based on derivatization of PUA with O-(2,3,4,5,6-pentafluorobenzyl) hydroxylamin hydrochloride

(PFBHA·HCl) and subsequent GC/MS (EI) analysis was applied (Wichard et al., 2005). The limit of quantification for PUA in concentrated diatom cultures was 5 ng/ml. Each analysis was performed in triplicate.

*Quantitative Chlorophyll a + c analysis*. In cases where PUA were determined, the chlorophyll content of the diatom isolates was quantified as well. The extraction in 90% acetone and quantification was performed in triplicate according to the standardized method No 446.0 (U.S. Environmental protection agency: Microbiological and Chemical Exposure Research and references herein). Jeffrey and Humphrey's trichromatic equations were applied: Chlorophyll a (mg/l) = 11.85 $E_{664}$ – 1.54 $E_{647}$ – 0.08 $E_{630}$ and Chlorophylls $c_1 + c_2$ (mg/l) = −1.67 $E_{664}$ – 7.60 $E_{647}$ + 24.52 $E_{630}$. Each value was corrected by the absorbance at 750 nm. The ratio of PUA to Chl a + c of identical culture batches was calculated as PUA/Chl (ppm) = PUA (μg/ml)/Chl (μg/ml) × 1,000,000.

*Determination of Cell Volume*. Living cells were measured microscopically in planar view (minimum: 20 cells). Linear measurements were converted to cell volume using different geometric approximations: a cylinder for *Chaetoceros compressus*, *Guinardia deliculata*, *Melosira* spp., *Skeletonema subsala-*

*Footnotes to Table 1*

*Note*. A cross (+) identifies a diatom as PUA-producer. Among those, several strains were selected for quantification (see Table 2).

[a] Applied analytical method.

[b] $f_2$ = marine enriched medium (artificial seawater) and K = K medium (filtered seawater), see for references Pohnert et al., 2002; WC = fresh water medium; br = brackish water: $f_2$ medium diluted with Chu-12 medium (2 + 1), see for references Carotenuto and Lampert, 2004.

[c] Only traces of PUA were detected after PFBHA derivatisation.

[d] Strains were isolated from coastal waters off Roscoff in 2004.

[e] Strains were isolated (cruise number of research vessel "C.A. Barnes" in parenthesis) from Dabob Bay, Point Wells or Friday Harbour (Puget Sound and San Juan Island, respectively, Washington, USA) in 2001–2003.

[f] See Carotenuto and Lampert, 2004.

[g] No strain number is available.

[h] Genetically described in Pohnert et al., 2002.

[j] Dinoflagellates often used as a control diet for copepods.

[k] Acidic polyunsaturated aldehydes were detected: 12-oxo-dodeca-5,8,10-trienoic acid (*A. formosa*, *S. turris*) and 9-oxo-nona-5,7-dienoic acid (*G. parvulum*).

Abbreviations: CCAP (DML) Culture Collection of Algae and Protozoa (Dunstaffnage Marine Laboratory, Scotland); CCMP = Centre for Culture of Marine Phytoplankton Main, USA; PLY (MBA) = Marine Biological Association Plymouth, England; RCC = Roscoff Culture Collection, France; SAG = Culture Collection Göttingen, Germany; UTEX = The Culture Collection of Algae, University of Texas at Austin, TX, USA.

*tum*, and *Thalassiosira* spp.; a cylinder + 2 half spheres for *Skeletonema costatum* and *Skeletonema pseudocostatum*, and a cone for *Asterionellopsis glacialis*. The carbon content was determined by the carbon to volume relationship based on the equation C (pg/cell) = 0.288 × volume$^{0.811}$ (Menden-Deuer and Lessard, 2000). The PUA to carbon ratio was calculated as PUA/C (ppm) = PUA (fg/cell)/C (fg/cell) × 1,000,000.

## RESULTS AND DISCUSSION

Seventy one diatom-isolates were analyzed for PUA-formation upon cell damage by sonication. The diatoms were either obtained from algal collections or freshly isolated from coastal waters off Roscoff (48°45′ N and 3°58′ W, Bretagne, France) and during several cruises to Dabob Bay, Point Wells and Friday Harbour (47° 46.14′N and 122° 50.10′W/47° 44.63′ N and 122° 25.34′ W/48.535° N and 123.005° W, Washington, USA). A total of 50 different species was investigated, with an emphasis on the family Thalassiosiraceae and the species *Phaeodactylum tricornutum*, because these diatoms are widely used in bioassays on the reproductive success of copepods (Miralto et al., 1999; Pohnert et al., 2002; Paffenhöfer et al., 2005). Under defined culture conditions (see Method section), 27 PUA-producers were identified among the 71 isolates investigated (Table 1). Out of the PUA producers, two released the unsaturated aldehydes only in trace amounts. The PUA-production upon wounding of 20 selected isolates (18 marine and 2 freshwater) was quantified during the stationary growth phase. PUA-production ranged from 0.01 fmol PUA/cell (*Thalassiosira nordenskioeldii*) to 9.8 fmol PUA/cell (*Thalassiosira pacifica*) (Table 2). This wide range over four orders of magnitude, as well as the isolate-dependent variability of structurally different unsaturated aldehydes, reflects a high plasticity within the Bacillariophyceae.

Since the calculation of PUA per cell underestimates the aldehyde contribution of species with low cell volume but probably high cell abundance in a typical herbivore diet, the PUA to carbon (PUA/C) and the PUA to chlorophyll a + c (PUA/Chl) ratio were also calculated. With respect to the PUA/Chl and PUA/C ratios, other dominant producers, such as *Skeletonema pseudocostatum* (PUA/Chl = 40,650 ppm) or *Skeletonema costatum* (SAG 19.99, PUA/C = 488 ppm), come to the fore.

Within the Bacillariophyceae, more than half of the investigated species do not produce PUA upon wounding in the stationary growth phase. In the light of the ongoing discussion about the influence of diatoms on herbivores, this result stresses that a general PUA-mediated effect can not be assumed for any given phytoplankton bloom, but that a species and strain-specific analysis is required.

TABLE 2. QUANTIFICATION OF PUA PER CELL, PER CHLOROPHYLL a + c AND PER CARBON CONTENT (STATIONARY PHASE)

| Producer of polyunsaturated aldehydes | Analysis of PUA | | | | | | Analysis of Chl a + c | | Analysis of carbon | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | PUA | C7:2 | C8:2 | C8:3 | C10:2 | C10:3 | Chl a + c | PUA/Chl a + c | cell vol. | C/cell | PUA/C |
| | fmol/cell | % of total PUA | | | | | $\mu g/10^6$ cells | ppm | $\mu m^3$ | pg/cell | ppm |
| *Thalassiosira pacifica* | 9.81 ± 0.069 | 70 | 19 | 11 | 0 | 0[a] | 6.58 ± 0.20 | 113,368 | 1,256 | 94 | 11,859 |
| *Melosira nummuloides* | 8.68 ± 0.424 | 7 | 72 | 0 | 2 | 19 | 11.7 ± 1.52 | 95,584 | 5,011 | 288 | 3,717 |
| *Thalassiosira rotula* (CCMP 1647) | 6.35 ± 0.289 | 5 | 32 | 11 | 2 | 50 | 3.36 ± 0.23 | 255,331 | 1,574 | 113 | 7,364 |
| *Thalassiosira rotula* (origin: Roscoff) | 5.69 ± 0.472 | 7 | 16 | 18 | 0 | 58 | 36.4 ± 0.16 | 21,573 | 12,972 | 624 | 1,348 |
| *Chaetoceros compressus* | 2.82 ± 0.635 | 31 | 12 | 0 | 8 | 49 | 3.67 ± 0.17 | 103,668 | 1,704 | 120 | 2,395 |
| *Thalassiosira aestivalis* | 1.54 ± 0.266 | 78 | 16 | 6 | 0 | 0 | 70.6 ± 6.96 | 2,462 | 4,630 | 270 | 525 |
| *Thalassiosira anguste-lineata* | 1.53 ± 0.079 | 69 | 17 | 14 | 0 | 0 | 48.5 ± 7.38 | 3,597 | 2,245 | 150 | 913 |
| *Thalassiosira rotula* (origin: Point Wells) | 1.27 ± 0.108 | 24 | 8 | 41 | 0 | 27 | 46.6 ± 4.50 | 3,459 | 16,406 | 755 | 212 |
| *Thalassiosira rotula* (CCMP 1812) | 1.04 ± 0.030 | 24 | 28 | 23 | 0 | 24 | 7.69 ± 0.55 | 9,672 | 3,497 | 215 | 588 |
| *Skeletonema pseudocostatum* | 0.38 ± 0.041 | 50 | 49 | 1 | 0 | 0 | 1.10 ± 0.31 | 40,650 | 186 | 20 | 2,343 |
| *Thalassiosira rotula* (CCMP 1018)[c] | 0.22 ± 0.048 | 31 | 45 | 24 | 0 | 0 | 8.10 ± 0.28 | 3,179 | 1,593 | 114 | 278 |
| *Guinardia deliculata* | 0.18 ± 0.015 | 100 | 0 | 0 | 0 | 0 | 17.4 ± 1.01 | 1,122 | 3,712 | 226 | 94 |
| *Skeletonema costatum* (RCC 75) | 0.13 ± 0.016 | 58 | 38 | 3 | 0 | 0 | 2.21 ± 0.11 | 6,623 | 286 | 28 | 578 |
| *Fragilaria* sp. | 0.10 ± 0.010 | 65 | 32 | 0 | 3 | 0 | n.d. | n.d. | 452[b] | 41 | 252 |
| *Asterionellopsis glacialis* | 0.05 ± 0.005 | 68 | 28 | 4 | 0 | 0 | 6.09 ± 0.03 | 960 | 176 | 19 | 343 |
| *Thalassiosira minima* | 0.05 ± 0.002 | 23 | 10 | 0 | 0 | 61 | 1.16 ± 0.18 | 5,960 | 172 | 19 | 393 |
| *Skeletonema subsalsum* | 0.04 ± 0.014 | 0[a] | 80 | 0 | 20 | 0 | n.d. | n.d. | 227 | 23 | 303 |

TABLE 2. CONTINUED

| Producer of polyunsaturated aldehydes | Analysis of PUA | | | | | | Analysis of Chl a + c | | Analysis of carbon | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | PUA | C7:2 | C8:2 | C8:3 | C10:2 | C10:3 | Chl a + c | PUA/Chl a + c | cell vol. | C/cell | PUA/C |
| | fmol/cell | % of total PUA | | | | | µg/$10^6$ cells | ppm | µm$^3$ | pg/cell | ppm |
| *Thalassiosira nordenskioeldii* | 0.01 ± 0.004 | 0[a] | 100 | 0 | 0 | 0 | 35.2 ± 2.19 | 121 | 3,776 | 229 | 14 |
| *Melosira sulcata* | 0.01 ± 0.001 | 0 | 100 | 0 | 0 | 0 | n.d. | n.d. | 369 | 35 | 36 |
| *Skeletonema costatum* (SAG 19.99) | 0.01 ± 0.001 | 81 | 19 | 0 | 0 | 0 | 3.45 ± 0.13 | 260 | 11 | 2 | 488 |

*Note*. Species sorted by descending total amount of PUA released (fmol/cell). Valuse: mean ± SD.

[a] Traces were detected;

[b] See reference (Carotenuto and Lampert, 2004);

[c] While Pohnert et al. (2002) did not detect any PUA in this strain using solid phase microextraction, the more sensitive PFBHA-derivatisation reveals that this species has to be considered as a weak PUA-producer. Isomeric mixtures of C7:2 = 2,4-heptadienal, C8:2 = 2,4-octadienal, C8:3 = 2,4,7-octatrienal, C10:2 = 2,4-decadienal and C10:3 = 2,4,7-decatrienal were detected.

Recently, the hypothesis that PUA-production could be the reason for poor copepod reproductive success during spring blooms of diatoms was proposed (Ianora et al., 2004). In this context, it is interesting to note that some of the most abundant spring-bloom forming species like *Thalassiosira* spp. (e.g. *Th. rotula* and *Th. pacifica*) release high amounts of PUA. These species were isolated from different habitats, such as the Adriatic Sea (Miralto et al., 1999), the coastal waters off Roscoff (NE Atlantic) and Dabob Bay (NE Pacific).
Because the ability to produce PUA is distributed heterogeneously in the major classes of Bacillariophyceae, one cannot predict the defensive potential of certain species. Moreover, PUA-production within different isolates of one species ranges widely, and thus case-specific chemical investigations accompanying bioassays are required. For example, the different *Thalassiosira rotula* isolates investigated release PUA in a wide range of concentrations from 0.15 to 6.34 fmol/cell. In this study, only cultures in the stationary growth phase were investigated. This culture condition was selected since it is also used in most laboratory investigations. Additional variation of PUA-production during different phases of diatom blooms or growth phases of cultures might have to be taken into account as well.

Based on this survey, we not only recommend performing future bioassays along with chemical analyses, but also urge for a reconsideration of the general conclusions drawn in the past. It is likely, that the observed reduction of hatching success in several studies/regions may not be due to the formation of deleterious PUA, but may have other causes. On the other hand, in regions where major PUA producers are the main constituents of blooms, there might be effects on the reproduction of herbivorous grazers and their population dynamics (Ianora et al. 2004; Halsband-Lenk et al. unpublished). Whether secondary production can be significantly affected by this chemically mediated interaction in such ecosystems requires further investigation.

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