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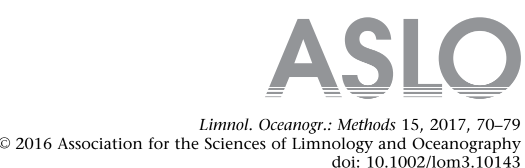

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A fast and direct liquid chromatography-mass spectrometry method to detect and quantify polyunsaturated aldehydes and polar oxylipins in diatoms

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Abstract

Polyunsaturated aldehydes (PUAs) are a group of microalgal metabolites that have attracted a lot of attention due to their biological activity. Determination of PUAs has become an important routine procedure in plankton and biofilm investigations, especially those that deal with chemically mediated interactions. Here we introduce a fast and direct derivatization free method that allows quantifying PUAs in the nanomolar range, sufficient to undertake the analysis from cultures and field samples. The sample preparation requires one simple filtration step and the initiation of PUA formation by cell disruption. After centrifugation the samples are ready for measurement without any further handling. Within one chromatographic run this method additionally allows us to monitor the formation of the polar oxylipins arising from the cleavage of precursor fatty acids. The robust method is based on analyte separation and detection using ultra high performance liquid chromatography-atmospheric pressure chemical ionization mass spectrometry (UHPLC-APCI MS) and enables high throughput investigations by employing an analysis time of only 5 min. Our protocol thus provides an alternative and extension to existing PUA determinations based on gas chromatography-mass spectrometry (GC-MS) with shorter run times and without any chemical derivatization. It also enables researchers with widely available LC-MS analytical platforms to monitor PUAs. Additionally, non-volatile oxylipins such as ω-oxo-acids and related compounds can be elucidated and monitored.

Polyunsaturated aldehydes (PUAs) are short-chained $\alpha,\beta,\gamma,\delta$-unsaturated aldehydes which came into focus of marine biologists due to their high bioactivity (Miralto et al. 1999). These fatty acid-derived metabolites are mainly produced by planktonic and benthic microalgae. As PUAs can play important roles in the interaction and regulation of algae-herbivore relationships, they have become a common parameter, determined along with other metadata in many studies. Being produced on cell damage of microalgae (Pohnert 2000; Pohnert 2002) PUAs have been made responsible for numerous effects on the grazer reproductive success such as the inhibition of copepod egg hatching or the action as causative agents for abnormal larval morphology (Miralto et al. 1999; Ianora et al. 2004; Poulet et al. 2007). However, the ecological relevance of these findings is still under discussion (Wichard et al. 2008). PUAs were not only found to be responsible for influencing diatom-copepod interactions, they also play a role in, e.g., intraspecific signaling and programmed cell death, allelopathy, and bacteria-phytoplankton interactions (Adolph et al. 2004; Vardi et al. 2006; Ribalet et al. 2008; Ribalet et al. 2014). Mainly diatoms are considered as important sources for PUAs but also other marine organisms, such as the brown alga *Dictyopteris* sp. and the green alga *Ulva* sp., as well as terrestrial plants and mosses produce $\alpha,\beta,\gamma,\delta$-unsaturated aldehydes (Noordermeer et al. 2001; Schnitzler et al. 2001; Stumpe et al. 2006; Alsufyani et al. 2014).

Biosynthetically, algal PUAs are derived from C_{16} and C_{20} polyunsaturated fatty acids (PUFAs) in a wound-activated process. These precursor fatty acids are enzymatically released within seconds after cell damage from phospholipids (Pohnert 2002) or glycolipids (d'Ippolito et al. 2004; Cutignano et al. 2006) and transformed to PUAs by a lipoxygenase (LOX)-hydroperoxide lyase (HPL) or a LOX-halolyase cascade (Pohnert 2000; d'Ippolito et al. 2004; Wichard and Pohnert 2006). Hexadecatrienoic acid (C16 : 3ω4) serves as precursor for 2*E*,4*Z*-octadienal (**3**), hexadecatetraenoic acid (C16 : 4ω1) for 2*E*,4*Z*,7-octatrienal (**5**), arachidonic acid (C20 : 4ω6) for 2*E*,4Z-decadienal (**4**), and eicosapentaenoic acid (C20 : 5ω3) for both 2*E*,4*Z*-heptadienal (**2**) and 2*E*,4*Z*,7Z-decatrienal (**6**) (Pohnert 2000; d'Ippolito et al. 2003; d'Ippolito et al. 2004; Pohnert et al. 2004). Besides PUAs, a multitude of other oxylipins are produced by diatoms such as hydroxy-, keto-, epoxyhydroxy-fatty acid derivatives (*see* Cutignano et al. 2011 and references

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therein), some of which are also biologically active (Ianora et al. 2011). Variability in the content of precursor molecules and enzyme activity leads to species-, strain- and even clone-specific oxylipin profiles that are further modulated by environmental factors (Wichard et al. 2005a; Ribalet et al. 2007; Taylor et al. 2009; Dittami et al. 2010; Gerecht et al. 2011). In addition, the physiological state of the cells determines their PUA profile (Vidoudez and Pohnert 2008).

To qualitatively or quantitatively analyze the oxylipin composition of diatoms from laboratory cultures and field samples several methods have been developed (Wendel and Jüttner 1996; Pohnert 2000; d'Ippolito et al. 2002a; Wichard et al. 2005b; Cutignano et al. 2011). Especially in field experiments, the low concentration and the inherent instability of PUAs requires elaborated sample preparation and sensitive detection methods for the direct determination of PUAs in filtered seawater samples (Vidoudez et al. 2011). Methods monitoring PUAs by gas chromatography-mass spectrometry (GC-MS) include the enrichment of the volatile PUA-containing oxylipin fraction by adsorption to resin and subsequent headspace GC-MS (Wendel and Jüttner 1996) or the more convenient solid phase micro extraction coupled to GC-MS (Pohnert 2000; Spiteller and Spiteller 2000). The rather labile aldehydes can be further stabilized by chemical derivatization (d'Ippolito et al. 2002a; Wichard et al. 2005b). Using pentafluorobenzylhydroxylamine (PFBHA), PUAs can be trapped in the aqueous phase without interfering with the enzymatic oxylipin production (Wichard et al. 2005b). This has been proven as a very robust and reliable method used by several laboratories (Taylor et al. 2007; Morillo-Garcia et al. 2014; Lavrentyev et al. 2015). Derivatization is followed by extraction using hexane giving highly reproducible results in GC-MS. Alternatively, high performance liquid chromatography (HPLC)-separation of PUA-derived dinitrophenylhydrazones has been introduced on liquid–liquid extracts of PUAs (Edwards et al. 2015). Recent studies focusing on oxylipin profiles of diatoms not only analyzed the volatile oxylipins using GC-MS but also screened for non-volatile oxylipins such as fatty acid hydroperoxides, hydroxy-, epoxyhydroxy fatty acids or ω-oxo-acids using liquid chromatography-mass spectrometry (LC-MS) (Barreiro et al. 2011; Ianora et al. 2015).

Our goal was to overcome the time-consuming derivatization processes by elaborating a method for the direct detection and quantification of PUAs and non-volatile oxylipins by ultra high performance liquid chromatography (UHPLC)-HRMS in a fast procedure not requiring extraction or derivatization steps, suitable for high sample capacities.

Materials and procedures

Reagents

2*E*,4*E*-hexadienal (**1**, 95%), 2*E*,4*E*-heptadienal (**2**, $\geq$97%), 2*E*,4*E*-octadienal (**3**, $\geq$95%), 2*E*,4*E*-decadienal (**4**, 85%), methanol (CHROMASOLV® Plus, for HPLC), and water (CHROMASOLV® Plus, for HPLC) were purchased from Sigma-Aldrich (Germany). Vanillin (VEB Laborchemie Apolda, Germany) was used as internal standard (IS).

Algal cultivation and enumeration

The marine diatom *Chaetoceros didymus* Na20B4 was obtained from W. Kooistra (Stazione Zoologica Anton Dohrn, Naples, Italy). The strains *Skeletonema costatum* RCC75 and *Thalassiosira rotula* RCC776 were obtained from the Roscoff Culture Collection (RCC, Roscoff, France). Stationary cultures were inoculated in artificial seawater (Maier and Calenberg 1994) in polystyrene culture flasks (Sarstedt, Germany) and grown without agitation at 11.3 ± 0.42°C under an illumination of 15 μmol photons m^{-2} s^{-1} on a 14 : 10 light : dark cycle (OSRAM Lumilux Cool White L36W/840). After 2 weeks the cultures reached 1–4 $\times$ 10^5 cells mL^{-1} (Table 1) and were aliquoted into four replicates with 40 mL each. For cell enumeration 1.5 mL of each culture were fixed with 10 μL mL^{-1} acid Lugol's solution (Rodhe et al. 1958).

Cell abundance of *C. didymus* was determined with a Nanoplankton Chamber (PhycoTech Inc., St. Joseph, MI) and of *S. costatum* and *T. rotula* with a Fuchs-Rosenthal hemocytometer (Laboroptik, Friedrichsdorf, Germany) using a Leica DM2000 microscope (Heerbrugg, Switzerland). At least 400 cells or 16 mm^2 were counted in four replicates.

Sample preparation

For cell harvesting, 40 mL of each replicate were concentrated by filtration on Whatman® GF/C filters (ø 47 mm) under reduced pressure (600 mbar). Cells were rinsed off the filter with 0.5 mL artificial seawater. The suspensions were transferred to 1.5 mL Eppendorf-tubes and 5 μL vanillin (700 μmol L^{-1} in MeOH : H_2O; 1 : 1; *v* : *v*) were added as IS. After 10 s vortexing, PUA formation was initiated by 1 min pulsed (50%) ultrasound treatment in an ice-cold water bath using a Bandelin Sonopuls HD 2070 (Berlin, Germany). Tubes were closed and incubated for 10 min in a water bath at room temperature to complete PUA formation (Pohnert 2000). Samples were cooled for 2 min in an ice-bath before adding 270 μL methanol. These cell lysates were centrifuged with an Eppendorf centrifuge 5415 D (9,300 rcf; 3 min), supernatants transferred to 1.5 mL glass vials, sealed air tight with a Teflon septum, and subsequently measured by UHPLC-HRMS. Aliquots of 40 mL artificial seawater were processed as described above as blank samples.

UHPLC-HRMS Orbitrap measurements

UHPLC was carried out using a Thermo Scientific (Bremen, Germany) UltiMate HPG-3400 RS binary pump and a WPS-3000 auto sampler which was set to 10°C and which was equipped with a 25 μL injection syringe and a 100 μL sample loop. The injection volume was set to 10 μL. The chromatography column Phenomenex (Aschaffenburg, Germany) Kinetex® C-18 RP (50 $\times$ 2.1 mm; 1.7 μm) was kept at 25°C within

Table 1. Cell abundance of harvested diatom cultures as mean cell abundance ± SD (cells mL^{-1}; $n = 4$).

Algal species	Strain	Mean cell abundance ± SD (cells mL^{-1})
Chaetoceros didymus	Na20B4	$1.9 \times 10^5 \pm 1.9 \times 10^4$
Skeletonema costatum	RCC75	$4.0 \times 10^5 \pm 3.5 \times 10^4$
Thalassiosira rotula	RCC776	$9.7 \times 10^4 \pm 8.6 \times 10^3$

the column compartment TCC-3200 and elution was carried out using a gradient (Table 2). Eluent A was water with 2% acetonitrile and 0.1% formic acid (*v* : *v*). Eluent B was acetonitrile with 0.1% formic acid (*v* : *v*). The UHPLC was coupled to a Thermo Scientific™ Q Exactive plus™ hybrid quadrupole-Orbitrap mass spectrometer equipped with an atmospheric pressure chemical ionization (APCI) source. To minimize source contamination a solvent delay was set at the beginning (0.0–0.2 min) and end (3.5–5.0 min) of the chromatographic run. For monitoring of the PUAs **1**–**4** a targeted selective ion monitoring (tSIM) in the positive ionization mode was used with the following parameters: $[M+H]^+$; m/z = x (Table 3) ± 0.2; resolution = 70,000; AGC target = 5×10^4; maximum IT = 254 ms. For untargeted monitoring of other relevant oxylipins such as **5**–**9** a simultaneous full scan was set to: m/z = 70–500; resolution = 70,000; AGC target = 5×10^6; maximum IT = 254 ms. Further settings were: sheath gas flow rate = 40 arbitraty units; aux gas flow rate = 15 arbitraty units; sweep gas flow rate = 0 arbitraty units; discharge current = 8.0 μA; capillary temperature = 350°C; S-lens RF level = 33; vaporizer temperature = 360°C; acquisition time frame = 0.5–3.5 min. MS^2 experiments were recorded with stepped normalized collision energy of 15, 30, and 45 selected by the calculated mass ± 0.2 m/z starting at 50 m/z.

Quantification

Peak detection and integration were carried out using the Thermo Scientific™ Xcalibur™ 3.0.63 Quan Browser software with the following settings: mass tolerance = 10 ppm; mass precision = 5 decimals; compounds = C_6H_8O (**1**), $C_7H_{10}O$ (**2**), $C_8H_{12}O$ (**3**), $C_{10}H_{16}O$ (**4**), $C_8H_{10}O$ (**5**), $C_{10}H_{14}O$ (**6**); adduct = $[M+H]^+$; retention time window = 3 s; signal = XIC from tSIM experiment for the dienals (**1**–**4**) and XIC from full scan experiment for dienals (**1**–**4**) and trienals (**5**–**6**); peak detection algorithm = ICIS (Smoothing = 1); peak detection method = highest peak. Area ratios of each analyte relative to the IS were determined and the analyte concentration per volume or cell calculated via calibration curves and cell abundances. After blank subtraction all replicates were averaged.

Table 2. Gradient for UHPLC-HRMS measurement.

Time (min)	Flow (mL min^{-1})	Solvent B (%)	Curve*
0.0	0.400	35	5
0.5	0.400	35	5
3.0	0.675	100	8
4.0	0.675	100	8
4.1	0.675	35	5
5.0	0.400	35	5

Eluent A was water with 2% acetonitrile and 0.1% formic acid. Eluent B (= Solvent B) was acetonitrile with 0.1% formic acid. Curve 1–9 with 5 = linear gradient and 6–9 concave upward (Fig. 1a).
*Instrument parameter setting linear or non-linear solvent gradients.

Calibration, limit of detection (LOD), and limit of quantification (LOQ)

A PUA stock solution of 2*E*,4*E*-dienals (**1**–**4**; all 100 μmol L^{-1} in MeOH) was generated from commercial standards (*see* "Reagents").

Seven calibration solutions were freshly prepared independently for an all-in-one-quantification in the range from 1×10^{-8}–5×10^{-5} mol L^{-1} (MeOH : H_2O; 35 : 65; *v* : *v*) using vanillin as IS (5 μL of a 700 μmol L^{-1} solution in MeOH : H_2O; 1 : 1; *v* : *v*), and subsequently analyzed by UHPLC-HRMS in six technical replicates. The injection volume was set to 10 μL. The average peak area ratio analyte/IS was plotted against the nominal concentration of each analyte for the working range of 1×10^{-8}–1×10^{-5} mol L^{-1}. Each calibration in the data set was tested to be normally distributed, free of outliers and homoscedasticity was proven for the whole concentration range. A Mandel test was applied to test the linear model against the quadratic model. No statistically significant differences demonstrated linearity.

The LOD and LOQ were estimated by the lowest analyzed concentration that gave a signal-to-noise (S/N) ratio equal to or greater than 3 (LOD) and 10 (LOQ). The noise range directly before the eluting peak was evaluated. Whenever the analyzed concentrations did not match a S/N of 10 the LOQ was interpolated by linear regression of the three lowest calibration concentrations.

Precision and sample stability

The precision of the instrument was determined by re-injection of a quality control sample (QCS) at the beginning ($n = 6$, QCS_{start}) and end ($n = 6$, QCS_{end}) of the entire measurement regime. As QCS acted a freshly prepared sample of *S. costatum* as described (*see* above). Homoscedasticity of the peak area ratios of IS and selected PUAs (**2**–**4**) was proven for the QCS_{start} and QCS_{end} samples using the *F*-test ($\alpha = 5\%$).

Sample stability of the cell free samples was determined by re-capping the vials after injection and re-measurement after 7 days of storage at 5°C in darkness. This was exemplary conducted for *S. costatum* and *T. rotula* for all quantifiable

Table 3. PUA structures and diagnostic UHPLC-HRMS data as m/z $[M+H]^+$.

Chemical species	Structure	m/z $[M+H]^+$	LOD (mol L^{-1})	LOQ (mol L^{-1})
2*E*,4*Z*-hexadienal (**1**)		97.06479	1.0×10^{-8}	5.0×10^{-8}
2*E*,4*Z*-heptadienal (**2**)		111.08044	$<1.0 \times 10^{-8}$*	2.3×10^{-8}
2*E*,4*Z*-octadienal (**3**)		125.09609	5.0×10^{-8}	1.4×10^{-7}
2*E*,4*Z*,7-octatrienal (**5**)		123.08044	n.d.	n.d.
2*E*,4*Z*-decadienal (**4**)		153.12739	$<1.0 \times 10^{-8}$*	1.9×10^{-8}
2*E*,4*Z*,7*Z*-decatrienal (**6**)		151.11174	n.d.	n.d.

Limit of detection (LOD; in mol L^{-1}) and quantification (LOQ; in mol L^{-1}) as determined for the corresponding 2*E*,4*E*-isomers. n.d. = not determined. *S/N at the lowest analyzed concentration of 1×10^{-8} M was 5 (**2**) and 6 (**4**).

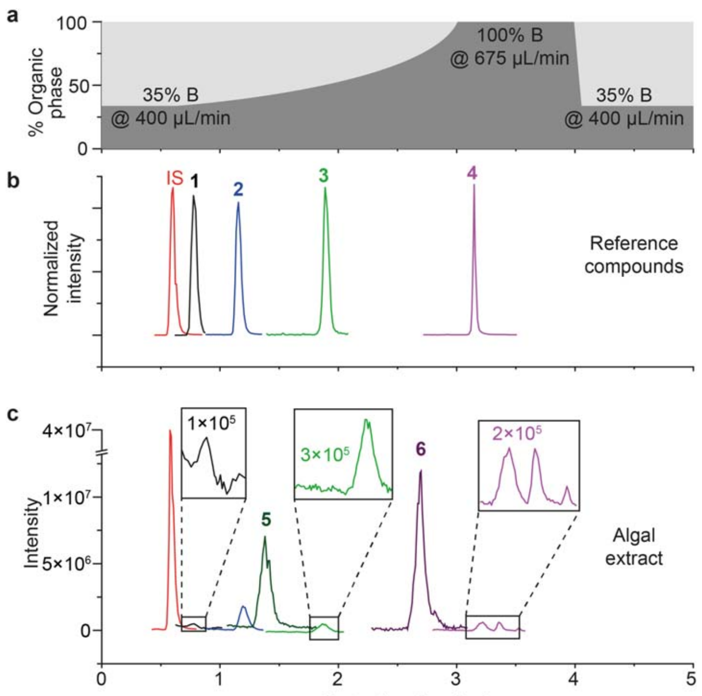

Fig. 1. UHPLC-HRMS analysis of polyunsaturated aldehydes including the gradient used for chromatography. (**a**) Gradient program for liquid chromatography (dark grey: organic phase (B); bright grey: aqueous phase). (**b**) Extracted ion chromatograms of the internal standard vanillin (IS, in red), and the authentic standards 2*E*,4*E*-hexadienal (**1**, in black), 2*E*,4*E*-heptadienal (**2**, in blue), 2*E*,4*E*-octadienal (**3**, in green), and 2*E*,4*E*-decadienal (**4**, in magenta) measured within one single run. Intensities are normalized to equal signal response. (**c**) Extracted ion chromatograms of a cell free extract of *Thalassiosira rotula* RCC776 harboring dienals (**1–4**) and the trienals octatrienal (**5**) and decatrienal (**6**) (color code see (**b**), trienals are in a darker color shade than their corresponding dienals). Note: In cell free extracts *E*/*Z*-isomers of PUAs are detected, resulting in more than one peak per aldehyde.

analytes. Sample stability over prolonged time was estimated by re-measurement of *T. rotula* extracts after 113 days storage at −20°C.

Assessment

Sample preparation

The sample preparation was modified according to former protocols (Wichard et al. 2005b) to achieve a fast analytical workflow allowing cell enrichment, cell wounding, incubation for wound-activated PUA production, and removal of suspended cell material within 20 min before UHPLC-HRMS measurement. The novel analytical approach allows determining PUAs within the complex cell-free algal lysate matrix without work- and time-intensive derivatization procedures as introduced by d'Ippolito et al. (2002a), Wichard et al. (2005b), or Edwards et al. (2015).

UHPLC-HRMS Orbitrap measurements

For quantification, a fast reversed-phase ultra-high performance liquid chromatography (RP-UHPLC) method was developed to separate PUAs. An elaborate curve-shaped 2.5 min gradient followed by a column wash with 100% organic eluent and re-equilibration of one minute was performed to achieve an optimal separation of the analytes. All analyzed compounds were baseline separated (Fig. 1b). The very short overall measurement time compared to former approaches (d'Ippolito et al. 2002a; Wichard et al. 2005b) now provides the opportunity of high sample throughput analyses, e.g., for monitoring experiments covering PUAs as well as other PUFA breakdown products. APCI proved to be superior compared to heated electro spray ionization (HESI). Sensitivity for the rather nonpolar short-chain aldehydes using HESI was two to three times lower compared to APCI (data not shown). For the dienals (**1–4**) targeted SIM analyses were used to enable maximum performance. For the analysis of trienals (**5–6**) and non-volatile oxylipins (**7–9**) a parallel analysis in full scan mode was executed. Hereby also potential unknown compounds may be identified. All method parameters were tested and verified with purchased dienal standards to ensure the adequate performance within all measurements.

Table 4. Mean concentration ± SD [fmol cell^{-1}] of the sums of the isomeric hexa- (**1**), hepta- (**2**), octa- (**3**), and decadienal (**4**), and of octa- (**5**) and decatrienal (**6**) in the exponentially growing marine diatom strains *Chaetoceros didymus* Na20B4 ($n = 4$), *Skeletonema costatum* RCC75 ($n = 4$), and *Thalassiosira rotula* RCC776 ($n = 3$).

	PUA concentration (fmol cell^{-1})					
Algae	**1**	**2**	**3**	**4**	**5**[a]	**6**[a]
C. didymus	n.d.	0.01 ± 0.001	n.d.	*	n.d.	n.d.
S. costatum	n.d.	0.48 ± 0.13	0.25 ± 0.09	*	0.08 ± 0.03	0.024 ± 0.005
T. rotula	*	0.18 ± 0.04	0.17 ± 0.03	0.03 ± 0.01	0.42 ± 0.08	1.08 ± 0.41

* = detected. n.d. = not detected.
[a]Trienal concentration as estimated if similar response factor as for the corresponding dienal is assumed.

Calibration, LOD, and LOQ

The statistical tests of normal distribution and trends passed for all calibration standards. As data showed no homoscedasticity over the working range of 1×10^{-8} mol L^{-1} to 1×10^{-5} mol L^{-1} a weighted linear regression was applied (1/y, Miller and Miller 2005). No significant difference was determined between the weighted linear and quadratic regression. According to this result the weighted linear model was accepted and used for quantification.

For each dienal LOD and LOQ were estimated based on the lowest analyzed concentration that on average gave S/N > 3 or 10, respectively (Table 3). The LOD ranged from 1 to 5×10^{-8} mol L^{-1} for the different analytes with the highest instrumental sensitivity for 2*E*,4*E*-decadienal.

Precision and sample stability

The precision of the instrument was successfully verified by homoscedasticity of re-measured QCS at the beginning (QCS_{start}) and end (QCS_{end}) of the entire measurement regime.

Re-measurement of samples after storage of seven days at reduced temperatures (5°C) and darkness resulted in a recovery rate of > 91% for all quantified compounds. Thus sample stability for 1 week was demonstrated. After additonal prolonged sample storage of 113 days at −20°C a recovery of > 84% for the dienals and of 50–77% for the trienals was determined (Supporting Information **S8**).

Dienal analysis in diatom cultures

Laboratory cultures of marine microalgae in exponential growth phase were investigated to evaluate the applicability of the new UHPLC-HRMS method for the detection and quantification of PUAs with two conjugated double bonds (dienals). Cultures of the bloom forming diatoms *C. didymus*, *S. costatum*, and *T. rotula* were analyzed. For the latter two species, PUA production was already quantified using a GC-MS method following derivatization (Wichard et al. 2005a). Concentrations determined in Wichard et al. (2005a) served as reference. *C. didymus* was investigated for the first time with regard to PUA production. Besides **2**, **3**, and **4** that are regularly recorded in diatoms also hexadienal (**1**) was detected.

Dienals were detected and quantified in all three diatom strains (Table 4). Heptadienal (**2**) was present in highest amounts in *S. costatum* (0.48 ± 0.13 fmol cell^{-1}) followed by octadienal (**3**) with 0.25 ± 0.09 fmol cell^{-1}. Decadienal (**4**) was present in quantifiable amounts only in *T. rotula* (0.03 ± 0.01 fmol cell^{-1}). Values for *S. costatum* can be compared to those from Wichard et al. (2005b) who investigated the same strain using PFBHA-derivatization. As in our study heptadienal (**2**) was the most dominant PUA with ca. 0.1 fmol cell^{-1} followed by octadienal (**3**) thus indicating the validity of the approach. In general, all results presented here are in accordance with earlier investigations and are well within the range of already observed species-, strain-, and culture dependent variability (Pohnert et al. 2002; Wichard et al. 2005a). Hexadienal (**1**) was detected in traces in *T. rotula* which is to our knowledge the first record of **1** in marine diatoms. Co-injection with an authentic standard confirmed the first evidence of the formation of hexadienal (**1**) by *T. rotula*. This detection of a low abundant, previously unknown metabolite supports the power of the direct PUA determination introduced here. Decadienal (**4**) was for the first time detected in traces in *S. costatum*.

Decadienal (**4**) was present in three of the four possible isomeric forms. The peak corresponding to the reference standard (2*E*,4*E*-isomer, $t_R = 3.14$ min) was followed by two peaks with identical mass ($t_R = 3.30$ min, $t_R = 3.45$ min) presumably corresponding to the 2*E*,4*Z*- and 2*Z*,4*E*-isomers since the fourth possible isomer with 2*Z*,4*Z* geometry has not been found in diatoms. We could not observe baseline separation of the isomers of **2** and **3**, which might be caused by their lower polarity and thereby shorter retention times. We therefore have to consider both isomers contributing to the integration for quantification. The origin of the different isomers is not fully understood; it was assigned to the release of isomeric mixtures by diatoms (Miralto et al. 1999) but also was discussed to be caused by isomerization occurring during sample processing (d'Ippolito et al. 2002a,b). Because our method induces minimum stress during sampling and the pure standards show only one signal without contribution of isomerization during the experimental procedure (Fig. 1b), we conclude that the production of isomeric mixtures by diatoms is more likely the explanation for the phenomenon.

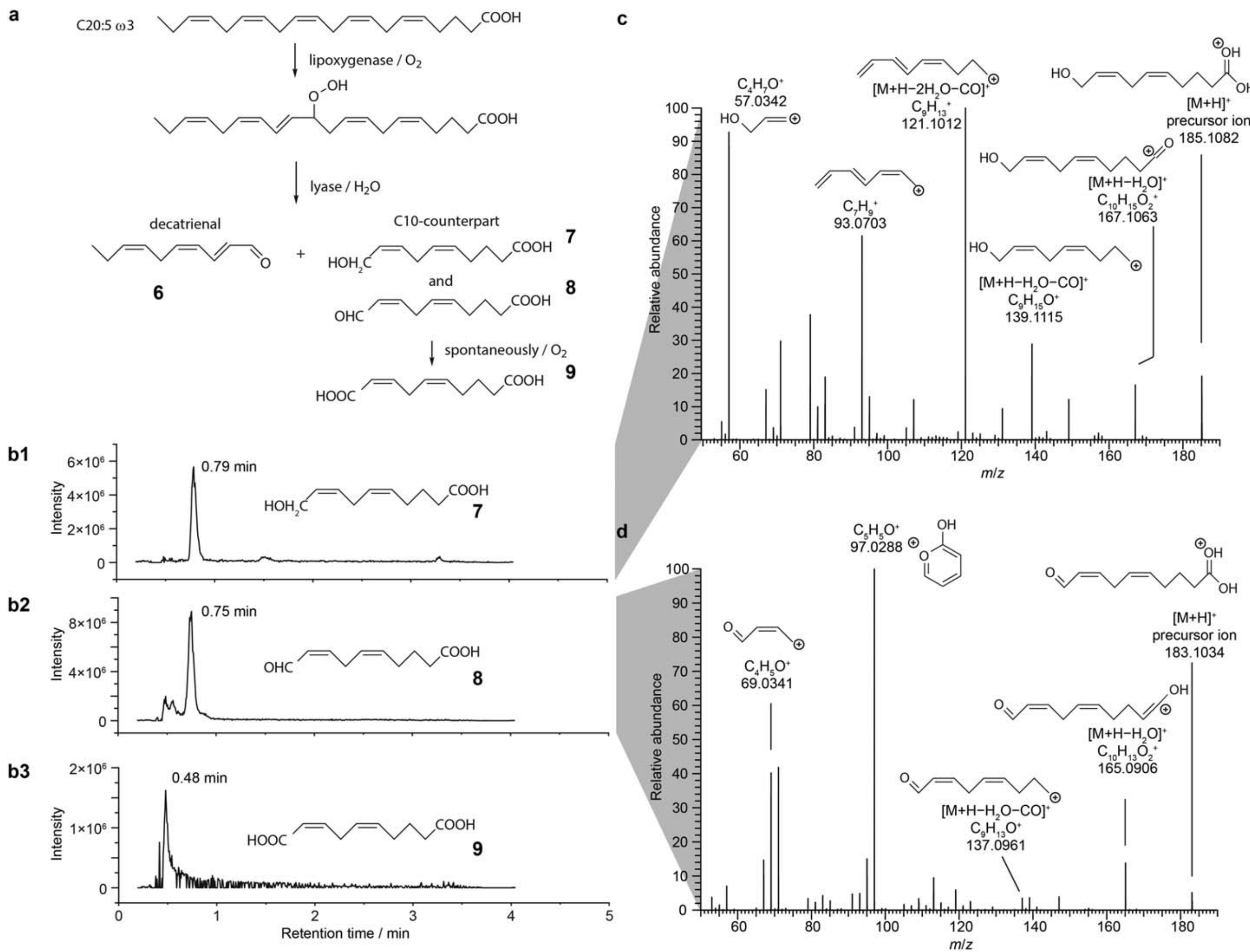

Fig. 2. UHPLC-HRMS analysis of polar eicosapentaenoic acid-derived products. (**a**) Lipoxygenase-mediated oxygenation and hydroperoxide lyase cleavage of eicosapentaenoic acid. All known (Barofsky and Pohnert 2007) and in this context newly observed products are listed. (**b**) Representative extracted ion chromatograms from a culture of *Thalassiosira rotula* RCC776 from the products **7** (**b1**), **8** (**b2**), and **9** (**b3**). (**c**) MS^2 spectrum of **7** obtained from **b1** including the main fragments. (**d**) MS^2 spectrum of **8** obtained from **b2** including the main fragments. Detailed fragmentation trees including the assignment to the spectra can be found in the Supporting Information (***S4–S7***).

Trienal analysis in diatom cultures

Trienals, PUAs with three double bonds, such as 2*E*,4*Z*,7-octatrienal (**5**) and 2*E*,4*Z*,7*Z* -decatrienal (**6**) are also released by marine algae and show comparable or even higher activity compared to dienals (Miralto et al. 1999; Jüttner 2005; Romano et al. 2010). For **5** and **6** no commercial standards are available and thus chromatograms were screened in full scan mode. Chromatographic runs were evaluated by monitoring the calculated accurate masses of the trienals (Table 3; for extracted mass spectra *see* Supporting Information **S1**). Both trienals were assigned to intense peaks eluting ca. 0.5 min earlier compared to the dienals of identical chain length (Fig. 1c). Decatrienal (**6**) was confirmed by analysis of its MS^2 spectrum (Supporting Information **S2–S3**). Peak shapes indicate the occurrence of isomers that are not baseline separated. The trienals **5** and **6** were thus determined as sum of all isomers and detected in quantifiable amounts in *T. rotula* and *S. costatum* but not in *C. didymus*. The trienal concentration can be estimated by comparison of the peak areas in full scan mode and assuming similar response factors as for the corresponding dienals (Table 4). Due to the fact that these values are not supported by referencing to synthetic standards different response factors in the MS might lead to a slight overestimation of one of the groups. The relative proportion of trienals compared to the dienals was as low as 12% in *S. costatum* and as high as 80% in *T. rotula*. *S. costatum* contained more octatrienal (**5**) compared to decatrienal (**6**), while **6** was the major PUA in *T. rotula*. The relative

proportions of these metabolites are in accordance with previous investigations using derivatization-based methods (d'Ippolito et al. 2002a; Wichard et al. 2005a,b).

Non-volatile oxylipins in diatom cultures

During the formation of PUAs from fatty acids a second product is released in diatoms namely short-chain hydroxylated fatty acids: 6*E*-8-hydroxyoct-6-enoic acid accompanying **3** and **5**, and 5*Z*,8*Z*-10-hydroxydeca-5,8-dienoic acid (**7**) accompanying **4** and **6** (Fig. 2a; Barofsky and Pohnert 2007). Analogous to these metabolites, formation of heptadienal (**2**) from eicosapentaenoic acid would result in 5*Z*,8*Z*,11*Z*-13-hydroxytrideca-5,8,11-trienoic acid, a metabolite that has not been detected so far in diatom lysates. As no commercial standards are available for these oxylipins the chromatograms were screened in full scan mode and monitored for the calculated accurate masses of these oxylipins. An intense peak eluting at around 0.79 min (Fig. 2, b1) could be assigned to **7** and was fully confirmed by its high resolution mass and by the evaluation of the according MS^2 spectrum (Fig. 2c). The presence in quantifiable amounts in *T. rotula* but not in *C. didymus* and *S. costatum* is in accordance with the high amounts of decatrienal (**6**) released by *T. rotula* (Table 4). Additionally, a concomitantly eluting peak at 0.75 min (Fig. 2, b2) was tentatively assigned to the corresponding ω-oxo-acid 5*Z*,8*Z*-10-oxodeca-5,8-dienoic acid (**8**) based on evaluation of the accurate mass and the MS^2 spectrum (Fig. 2d). The ω-oxo-acid may be formed as a shunt product of the lyase which seems to produce similarly to known P450 enzymes the corresponding oxo analogue (Noordermeer et al. 2001; Grechkin and Hamberg 2004). Supporting this hypothesis is the fact that evidence for the oxidation product, namely the dicarboxylic acid (**9**) was found (Fig. 2, **b3**) which easily can be obtained as oxidation product from **8** on air contact.

Discussion

The developed method provides a sensitive technique to measure and identify oxylipins including dienals, trienals, as well as ω-oxidized-acids within complex biological matrices. In contrast to previously published methods it can monitor the wide array of diatom-derived oxylipins within one single chromatographic run. This will enable mechanistic studies of fatty acid metabolism in diatoms and other oxylipin-producing organisms. The method will also facilitate investigations on the activity of the hitherto poorly investigated polar oxylipins derived from LOX-HPL reactions, thereby opening up new fields of research. Especially the fact that no bias is introduced due to the lack of the need for extraction procedures, the method provides direct access to the wound-activated metabolism of diatoms. The first identification of hexadienal (**1**) in diatom extracts shows that the untargeted measurement of previously unidentified compounds is possible in parallel to targeted PUA analysis. In addition it demonstrates that the newly introduced method allows for sensitive detection of novel products that were previously overseen, presumable due to their problematic extraction or derivatization. Small required sample volumes compared to existing methods (d'Ippolito et al. 2002a) and the short analysis time make this method ideal for higher throughput surveys of cellular PUA concentrations in naturally occurring phytoplankton assemblages as well as cultures. Due to the high plasticity of the parameter "PUA" such high throughput analyses will enable a close monitoring of PUA in diatoms under the influence of abiotic and biotic stressors. A quantitative comparison of the performance of the newly introduced protocol and previous approaches is hindered by the fact that neither, d'Ippolito et al. (2002a) nor Edwards et al. (2015) report limits of detection. Compared to Wichard et al. (2005b) our method is more sensitive (1.7×10^{-8} mol L^{-1} vs. 7.2×10^{-8} mol L^{-1} determined in GC-EI-MS mode). In case of sensitivity problems, the method would easily allow a lowering of the LOD by scaling up the volume of filtered samples to increase the analyte signal intensities. Compared to derivatization-based methods we avoid handling of potentially toxic metabolites and extraction protocols using organic solvents. Further, in contrast to PFBHA-derivatized PUAs where each isomer results in two peaks we only detect one signal per analyte resulting in less complex chromatograms. However, PFBHA treatment traps PUAs and allows the detection of even highly reactive metabolites that might be overlooked using our protocol. In conclusion, we provide a fast and direct determination of PUAs that is as sensitive as established protocols based on derivatization. We are able to pick up and quantify all PUAs previously detected in the phytoplankton species investigated here as well as other novel apolar and polar oxylipins. We also introduce the use of wide spread LC-MS analytical platforms and provide an alternative for labs that do not have access to GC-MS methods.

Comments and recommendations

In this study laboratory cultures of marine diatoms were investigated. To apply the method on field samples the UHPLC-HRMS measurements can be used without further adjustment.

The sensitivity is determined by the amount of PUA-producing cells on the GF/C filter and their PUA production. Sensitivity can thus easily be increased by filtration of more biomass. In case of samples containing larger particles or herbivores an additional filtration step removing those might be required. The method allows high throughput investigations and our monitoring of stability indicates that usage of a cooled (5°C) auto sampler would be sufficient for accurate measurements of larger sample sets. If prolonged storage time is required, as in the case of field experiments, sample instability could easily be compensated using standards that are processed and stored under identical conditions

as the field samples. In this study cell damage and the following PUA production were achieved by ultrasound treatment, which was adjusted to diatom cells. For other phytoplankton members with non-silicified cell walls the ultrasound treatment can be modulated accordingly to achieve sufficient cell damage; alternative methods like repeated freezing and thawing cycles might be utilized for cell disruption. Mass spectrometers that provide accurate mass measurements are recommended to filter out the background noise of the complex sample matrix to obtain high analyte sensitivities. However, even nominal mass resolution together with the added selectivity by chromatographic retention time is sufficient for the determination of PUAs. In these cases, additional specificity might be brought in using MS^2 protocols. When analyzing high salinity matrices a routine cleaning of the corona discharge needle is recommended as salt precipitation influences the analyte ionization.

References

Adolph, S., and others. 2004. Cytotoxicity of diatom-derived oxylipins in organisms belonging to different phyla. J. Exp. Biol. **207**: 2935–2946. doi:10.1242/jeb.01105

Alsufyani, T., A. H. Engelen, O. E. Diekmann, S. Kuegler, and T. Wichard. 2014. Prevalence and mechanism of polyunsaturated aldehydes production in the green tide forming macroalgal genus *Ulva* (Ulvales, Chlorophyta). Chem. Phys. Lipids **183**: 100–109. doi:10.1016/j.chemphyslip.2014.05.008

Barofsky, A., and G. Pohnert. 2007. Biosynthesis of polyunsaturated short chain aldehydes in the diatom *Thalassiosira rotula*. Org. Lett. **9**: 1017–1020. doi:10.1021/ol063051v

Barreiro, A., and others. 2011. Diatom induction of reproductive failure in copepods: The effect of PUAs versus non volatile oxylipins. J. Exp. Mar. Biol. Ecol. **401**: 13–19. doi:10.1016/j.jembe.2011.03.007

Cutignano, A., and others. 2006. Chloroplastic glycolipids fuel aldehyde biosynthesis in the marine diatom *Thalassiosira rotula*. ChemBioChem. **7**: 450–456. doi:10.1002/cbic.200500343

Cutignano, A., N. Lamari, G. d'Ippolito, E. Manzo, G. Cimino, and A. Fontana. 2011. Lipoxygenase products in marine diatoms: A concise analytical method to explore the functional potential of oxylipins. J. Phycol. **47**: 233–243. doi:10.1111/j.1529-8817.2011.00972.x

d'Ippolito, G., I. Iadicicco, G. Romano, and A. Fontana. 2002a. Detection of short-chain aldehydes in marine organisms: The diatom *Thalassiosira rotula*. Tetrahedron Lett. **43**: 6137–6140. doi:10.1016/S0040-4039(02)01283-2

d'Ippolito, G., and others. 2002b. New birth-control aldehydes from the marine diatom *Skeletonema costatum*: Characterization and biogenesis. Tetrahedron Lett. **43**: 6133–6136. doi:10.1016/s0040-4039(02)01285-6

d'Ippolito, G., G. Romano, T. Caruso, A. Spinella, G. Cimino, and A. Fontana. 2003. Production of octadienal in the marine diatom *Skeletonema costatum*. Org. Lett. **5**: 885–887. doi:10.1021/ol034057c

d'Ippolito, G., and others. 2004. The role of complex lipids in the synthesis of bioactive aldehydes of the marine diatom *Skeletonema costatum*. Biochim. Biophys. Acta Mol. Cell Biol. Lipids **1686**: 100–107. doi:10.1016/j.bbalip.2004.09.002

Dittami, S. M., T. Wichard, A. M. Malzahn, G. Pohnert, M. Boersma, and K. H. Wiltshire. 2010. Culture conditions affect fatty acid content along with wound-activated production of polyunsaturated aldehydes in *Thalassiosira rotula* (Coscinodiscophyceae). Nova Hedwigia **136**: 231–248. doi:10.1127/1438-9134/2010/0136-0231

Edwards, B. R., K. D. Bidle, and B. A. S. van Mooy. 2015. Dose-dependent regulation of microbial activity on sinking particles by polyunsaturated aldehydes: Implications for the carbon cycle. Proc. Natl. Acad. Sci. USA **112**: 5909–5914. doi:10.1073/pnas.1422664112

Gerecht, A., G. Romano, A. Ianora, G. d'Ippolito, A. Cutignano, and A. Fontana. 2011. Plasticity of oxylipin metabolism among clones of the marine diatom *Skeletonema marinoi* (Bacillariophyceae). J. Phycol. **47**: 1050–1056. doi:10.1111/j.1529-8817.2011.01030.x

Grechkin, A. N., and M. Hamberg. 2004. The "heterolytic hydroperoxide lyase" is an isomerase producing a short-lived fatty acid hemiacetal. Biochim. Biophys. Acta Mol. Cell Biol. Lipids **1636**: 47–58. doi:10.1016/j.bbalip.2003.12.003

Ianora, A., and others. 2004. Aldehyde suppression of copepod recruitment in blooms of a ubiquitous planktonic diatom. Nature **429**: 403–407. doi:10.1038/nature02526

Ianora, A., and others. 2011. Impact of the diatom oxylipin 15*S*-HEPE on the reproductive success of the copepod *Temora stylifera*. Hydrobiologia **666**: 265–275. doi:10.1007/s10750-010-0420-7

Ianora, A., and others. 2015. Non-volatile oxylipins can render some diatom blooms more toxic for copepod reproduction. Harmful Algae **44**: 1–7. doi:10.1016/j.hal.2015.02.003

Jüttner, F. 2005. Evidence that polyunsaturated aldehydes of diatoms are repellents for pelagic crustacean grazers. Aquat. Ecol. **39**: 271–282. doi:10.1007/s10452-005-3419-9

Lavrentyev, P. J., G. Franze, J. J. Pierson, and D. K. Stoecker. 2015. The effect of dissolved polyunsaturated aldehydes on microzooplankton growth rates in the Chesapeake Bay and Atlantic coastal waters. Mar. Drugs **13**: 2834–2856. doi:10.3390/md13052834

Maier, I., and M. Calenberg. 1994. Effect of extracellular Ca^{2+} and Ca^{2+}-antagonists on the movement and chemoorientation of male gametes of *Ectocarpus siliculosus* (Phaeophyceae). Bot. Acta **107**: 451–460. doi:10.1111/j.1438-8677.1994.tb00820.x

Miller, J. N., and J. C. Miller. 2005. Statistics and chemometrics for analytical chemistry. Pearson Education.

Miralto, A., and others. 1999. The insidious effect of diatoms on copepod reproduction. Nature **402**: 173–176. doi: 10.1038/46023

Morillo-Garcia, S., and others. 2014. Potential polyunsaturated aldehydes in the Strait of Gibraltar under two tidal regimes. Mar. Drugs **12**: 1438–1459. doi:10.3390/md12031438

Noordermeer, M. A., G. A. Veldink, and J. F. G. Vliegenthart. 2001. Fatty acid hydroperoxide lyase: A plant cytochrome P450 enzyme involved in wound healing and pest resistance. ChemBioChem **2**: 494–504. doi:10.1002/1439-7633(20010803)2:7/8 < 494::AID-CBIC494 > 3.0.CO;2-1

Pohnert, G. 2000. Wound-activated chemical defense in unicellular planktonic algae. Angew. Chem. Int. Ed. **39**: 4352–4354. doi:10.1002/1521-3773(20001201)39:23 < 4352::AID-ANIE4352 > 3.0.CO;2-U

Pohnert, G. 2002. Phospholipase A_2 activity triggers the wound-activated chemical defense in the diatom *Thalassiosira rotula*. Plant Physiol. **129**: 103–111. doi:10.1104/pp.010974

Pohnert, G., and others. 2002. Are volatile unsaturated aldehydes from diatoms the main line of chemical defence against copepods?. Mar. Ecol.: Prog. Ser. **245**: 33–45. doi: 10.3354/meps245033

Pohnert, G., S. Adolph, and T. Wichard. 2004. Short synthesis of labeled and unlabeled 6*Z*,9*Z*,12*Z*,15-hexadecatetraenoic acid as metabolic probes for biosynthetic studies on diatoms. Chem. Phys. Lipids **131**: 159–166. doi:10.1016/j.chemphyslip.2004.04.011

Poulet, S. A., A. Cueff, T. Wichard, J. Marchetti, C. Dancie, and G. Pohnert. 2007. Influence of diatoms on copepod reproduction. III. Consequences of abnormal oocyte maturation on reproductive factors in *Calanus helgolandicus*. Mar. Biol. **152**: 415–428. doi:10.1007/s00227-007-0701-5

Ribalet, F., T. Wichard, G. Pohnert, A. Ianora, A. Miralto, and R. Casotti. 2007. Age and nutrient limitation enhance polyunsaturated aldehyde production in marine diatoms. Phytochemistry **68**: 2059–2067. doi:10.1016/j.phytochem.2007.05.012

Ribalet, F., L. Intertaglia, P. Lebaron, and R. Casotti. 2008. Differential effect of three polyunsaturated aldehydes on marine bacterial isolates. Aquat. Toxicol. **86**: 249–255. doi: 10.1016/j.aquatox.2007.11.005

Ribalet, F., and others. 2014. Phytoplankton cell lysis associated with polyunsaturated aldehyde release in the Northern Adriatic Sea. PLoS One **9**: e85947. doi:10.1371/journal.pone.0085947

Rodhe, W., R. A. Vollenweider, and A. Nauwerck. 1958. The primary production and standing crop of phytoplankton, p. 299–322. *In* A. A. Traverso [ed.], Perspectives in marine biology. Univ. of California Press.

Romano, G., A. Miralto, and A. Ianora. 2010. Teratogenic effects of diatom metabolites on sea urchin *Paracentrotus lividus* embryos. Mar. Drugs **8**: 950–967. doi:10.3390/md8040950

Schnitzler, I., G. Pohnert, M. E. Hay, and W. Boland. 2001. Chemical defense of brown algae (*Dictyopteris* spp.) against the herbivorous amphipod *Ampithoe longimana*. Oecologia. **126**: 515–521. doi:10.1007/s004420000546

Spiteller, D., and G. Spiteller. 2000. Identification of toxic 2,4-decadienal in oxidized, low-density lipoprotein by solid-phase microextraction. Angew. Chem. Int. Ed. **39**: 583–585. doi:10.1002/(SICI)1521-3773(20000204)39:3 < 583::AID-ANIE583 > 3.0.CO;2-O

Stumpe, M., and others. 2006. Biosynthesis of C_9-aldehydes in the moss *Physcomitrella patens*. Biochim. Biophys. Acta. **1761**: 301–312. doi:10.1016/j.bbalip.2006.03.008

Taylor, R. L., G. S. Caldwell, H. J. Dunstan, and M. G. Bentley. 2007. Short-term impacts of polyunsaturated aldehyde-producing diatoms on the harpacticoid copepod *Tisbe holothuriae*. J. Exp. Mar. Biol. Ecol. **341**: 60–69. doi: 10.1016/j.jembe.2006.10.028

Taylor, R. L., K. Abrahamsson, A. Godhe, and S. A. Wangberg. 2009. Seasonal variability in polyunsaturated aldehyde production potential among strains of *Skeletonema marinoi* (Bacillariophyceae). J. Phycol. **45**: 46–53. doi:10.1111/j.1529-8817.2008.00625.x

Vardi, A., and others. 2006. A stress surveillance system based on calcium and nitric oxide in marine diatoms. PLoS Biol. **4**: 411–419. doi:10.1371/journal.pbio.0040060

Vidoudez, C., and G. Pohnert. 2008. Growth phase-specific release of polyunsaturated aldehydes by the diatom *Skeletonema marinoi*. J. Plankton Res. **30**: 1305–1313. doi: 10.1093/plankt/fbn085

Vidoudez, C., R. Casotti, M. Bastianini, and G. Pohnert. 2011. Quantification of dissolved and particulate polyunsaturated aldehydes in the Adriatic Sea. Mar. Drugs **9**: 500–513. doi:10.3390/md9040500

Wendel, T., and F. Jüttner. 1996. Lipoxygenase-mediated formation of hydrocarbons and unsaturated aldehydes in freshwater diatoms. Phytochemistry **41**: 1445–1449. doi: 10.1016/0031-9422(95)00828-4

Wichard, T., and others. 2005a. Survey of the chemical defence potential of diatoms: Screening of fifty one species for $\alpha,\beta,\gamma,\delta$-unsaturated aldehydes. J. Chem. Ecol. **31**: 949–958. doi:10.1007/s10886-005-3615-z

Wichard, T., S. A. Poulet, and G. Pohnert. 2005b. Determination and quantification of $\alpha,\beta,\gamma,\delta$-unsaturated aldehydes as pentafluorobenzyl-oxime derivates in diatom cultures and natural phytoplankton populations: Application in marine field studies. J. Chromatogr. B: Anal. Technol. Biomed. Life Sci. **814**: 155–161. doi:10.1007/s10886-005-3615-z

Wichard, T., and G. Pohnert. 2006. Formation of halogenated medium chain hydrocarbons by a lipoxygenase/hydroperoxide halolyase-mediated transformation in planktonic

microalgae. J. Am. Chem. Soc. **128**: 7114–7115. doi: 10.1021/ja057942u

Wichard, T., and others. 2008. Influence of diatoms on copepod reproduction. II. Uncorrelated effects of diatom-derived α,β,γ,δ-unsaturated aldehydes and polyunsaturated fatty acids on *Calanus helgolandicus* in the field. Prog. Oceanogr. **77**: 30–44. doi:10.1016/j.pocean.2008.03.002

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Conflict of Interest

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