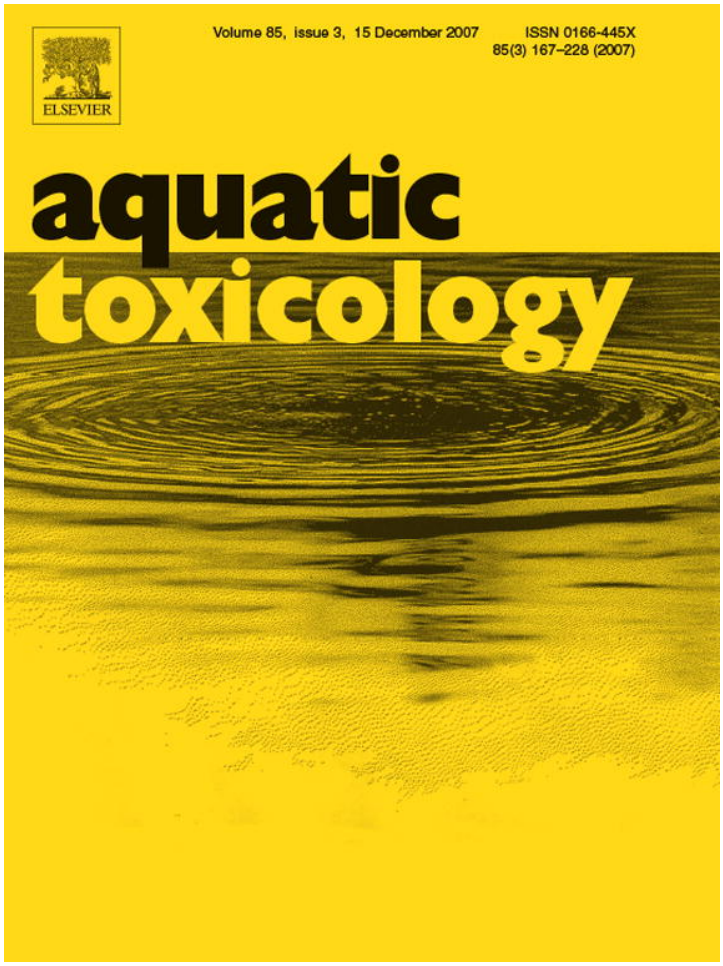

ELSEVIER

Aquatic Toxicology 85 (2007) 219–227

AQUATIC TOXICOLOGY

www.elsevier.com/locate/aquatox

Growth inhibition of cultured marine phytoplankton by toxic algal-derived polyunsaturated aldehydes

François Ribalet [a], John A. Berges [b], Adrianna Ianora [a], Raffaella Casotti [a,*]

[a] *Stazione Zoologica Anton Dohrn di Napoli, Villa Comunale, I80121 Napoli, Italy*
[b] *Department of Biological Sciences, University of Wisconsin-Milwaukee, Milwaukee, WI 53211, USA*

Received 16 July 2007; received in revised form 10 September 2007; accepted 11 September 2007

Abstract

Several marine diatoms produce polyunsaturated aldehydes (PUAs) that have been shown to be toxic to a wide variety of model organisms, from bacteria to invertebrates. However, very little information is available on their effect on phytoplankton. Here, we expand previous studies to six species of marine phytoplankton, belonging to different taxonomic groups that are well represented in marine plankton. The effect of three PUAs, 2*E*,4*E*-decadienal, 2*E*,4*E*-octadienal and 2*E*,4*E*-heptadienal, was assessed on growth, cell membrane permeability, flow cytometric properties and morphology. A concentration-dependent reduction in the growth rate was observed for all cultures exposed to PUAs with longer-chained aldehydes having stronger effects on growth than shorter-chained aldehydes. Clear differences were observed among the different species. The prymnesiophyte *Isochrysis galbana* was the most sensitive species to PUA exposure with a lower threshold for an observed effect triggered by mean concentrations of 0.10 μmol L^{-1} for 2*E*,4*E*-decadienal, 1.86 μmol L^{-1} for 2*E*,4*E*-octadienal and 3.06 μmol L^{-1} for 2*E*,4*E*-heptadienal, and a 50% growth inhibition (EC_{50}) with respect to the control at 0.99, 2.25 and 5.90 μmol L^{-1} for the three PUAs, respectively. Alternatively, the chlorophyte *Tetraselmis suecica* and the diatom *Skeletonema marinoi* (formerly *S. costatum*) were the most resistant species with 50% growth inhibition occurring at concentrations at least two to three times higher than *I. galbana*. In all species, the three PUAs caused changes in flow cytometric measures of cell size and cell granulosity and increased membrane permeability, assessed using the viability stain SYTOX Green. For example, after 48 h 51.6 ± 2.6% of *I. galbana* cells and 15.0 ± 1.8% of *S. marinoi* cells were not viable. Chromatin fragmentation was observed in the dinoflagellate *Amphidinium carterae* while clear DNA degradation was observed in the chlorophyte *Dunaliella tertiolecta*. Concentrations used are in a significant range for affecting growth and performance of phytoplankton living in close vicinity of PUA-producing algae. Thus, PUAs may act as allelochemicals by mediating interactions among planktonic organisms.

Keywords: Toxicity; Diatoms; Growth inhibition; Viability; Allelochemicals; EC_{50}

1. Introduction

In the last decade unicellular algae have been shown to produce a wide range of secondary metabolites which are often released in the environment (Hay, 1996). For most of these compounds, it remains unclear whether they are released as semiochemicals, i.e. information-conveying molecules, or if they are simply degradation products expelled by the producing organisms (Watson, 2003). However, it is becoming increasingly evident that a wide number of these compounds have biological functions, as, for example, pheromones (Pohnert and Boland, 2002), which mediate interactions between organisms of the same species, kairomones (Cembella, 2003) and allomones (Legrand et al., 2003), which mediate interactions between individuals of different species and that are beneficial to the producer (kairomones) or the receiver (allomones).

Polyunsaturated aldehydes (here abbreviated PUAs) are produced by the enzymatic degradation of polyunsaturated fatty acids which is activated soon after cell membrane disruption (d'Ippolito et al., 2002, 2004; Pohnert, 2002). PUAs produced include 2*E*,4*E*/*Z*-heptadienal, 2*E*,4*E*/*Z*-octadienal, 2*E*,4*E*/*Z*,7*Z*-octatrienal, 2*E*,4*E*/*Z*-decadienal and 2*E*,4*E*/*Z*,7*Z*-decatrienal, and these are described for both freshwater and marine phytoplankters, including chrysophytes, cryptophytes, cyanobacteria, synurophytes, prymnesiophytes and diatoms (Watson et al., 2001; Watson, 2003; Watson and Satchwill, 2003; Wichard et al., 2005b; Hansen et al., 2004).

* Corresponding author. Tel.: +39 0815833235; fax: +39 0817641355.
E-mail address: raffa@szn.it (R. Casotti).

0166-445X/$ – see front matter © 2007 Elsevier B.V. All rights reserved.
doi:10.1016/j.aquatox.2007.09.006

Among these PUAs, 2*E*,4*E*-decadienal has been widely used as a model aldehyde and has been shown to induce deleterious effects on the reproduction of several invertebrates, such as echinoderms, polychaetes, ascidians, crustaceans and molluscs (Caldwell et al., 2003). However, little information is available on other PUAs and on their effect on photosynthetic organisms that coexist in natural phytoplankton communities. Casotti et al. (2005) have investigated the effect of this aldehyde on the diatom *Thalassiosira weissflogii* in culture and shown that exposure to it induced reduction of the growth rate and triggered programmed cell death. It has also been suggested that decadienal may play a role to activate a stress-signalling mechanism mediated by Ca^{2+} and nitric oxide in the diatom *Phaeodactylum tricornutum* (Vardi et al., 2006). For these effects to occur in nature, however, it is necessary that PUAs are released in the surrounding medium following cell breakage. So far, only mastication by grazers has been suggested as possible trigger of PUA formation. However, cell lysis triggered by endogenous or exogenous factors such as senescence or viral attack, is also a possible and widespread phenomenon occurring at sea (Kirchman, 1999). Once released in the water, PUAs are expected to interact with organisms living in the vicinity of PUA-producing cells, by influencing their growth performance. Therefore, it is reasonable to propose a role as allelochemicals. A recent study on *Phaeocystis pouchetii* found no correlation between the presence of this species and diatom diversity (Hansen and Eilertsen, 2007), concluding that it is unlikely that PUA production can offer to the producer a competitive advantage against other diatom species. However, both *Phaeocystis* and diatoms produce PUAs and often coexist at sea, and the possibility that species other than diatoms are affected by PUAs released in the seawater cannot be ruled out.

The aim of the present study is to investigate the effect of three different PUAs on six species of algae belonging to taxonomic groups which are well represented in marine areas. Fluorescent stains detected by flow cytometry and epifluorescence were used to assess the effect of PUA exposures on growth rate, cell membrane integrity and morphology in culture.

2. Materials and methods

2.1. Algal cultures and experimental design

Axenic cultures of the diatom *Skeletonema marinoi* Sarno & Zingone (reallocated from *S. costatum*, strain CCMP2092; Sarno et al., 2005), the chlorophyte *Dunaliella tertiolecta* Butcher (strain CCMP1320), the prymnesiophyte *Isochrysis galbana* Parke (strain CCMP1323), the dinophyte *Amphidinium carterae* Hulburt (strain CCMP1314), the two prasinophytes *Tetraselmis suecica* (Kylin) Butcher (strain CCMP906) and *Micromonas pusilla* (Butcher) Manton & Parke (strain CCMP1646) were all obtained from the Provasoli-Guillard National Center for Culture of Marine Phytoplankton, Boothbay Harbor, USA. The cultures were grown semi-continuously at 15 °C (except for *M. pusilla* grown at 18 °C) with a 12–12 h light–dark cycle under a photon flux density of 150 μmol m^{-2} s^{-1} provided by white fluorescent tubes (Philips TLD 36W/950). Filtered seawater from the Gulf of Naples was amended with f/2 nutrients (Guillard, 1975) and used as medium. Cell growth was monitored by measuring cell numbers by flow cytometry. Growth rates were calculated as μ (day^{-1}) according to

$$\mu = \ln \frac{N_1/N_0}{t} \quad (1)$$

where N_0 and N_1 represent cell density at the start and the end of the growth period, and t is the time between measurements (in days).

For the toxicity tests 50 ml of each exponentially growing culture were inoculated with different concentrations (from 0.1 to 36 μmol L^{-1}) of each of the three PUAs. Initial cell density and growth rates are reported in Table 1. Experiments were replicated at least twice at different times, with three independent cultures for each PUA concentration in each replicate.

2.2. PUA preparation

The PUAs 2*E*,4*E*-heptadienal, 2*E*,4*E*-octadienal, and 2*E*,4*E*-decadienal were obtained from Sigma–Aldrich Inc. (Milano, Italy). Working solutions of PUAs were prepared by diluting the stock in absolute methanol (ROMIL, Cambridge, UK) at room temperature. The effective PUA concentration of the working solution was assessed spectrophotometrically before inoculation by using a 274 nm wavelength and a specific molar absorption coefficient of 31,000 (Pippen and Nonaka, 1958). The methanol had no effect on growth up to 7 μL 100% methanol mL^{-1} of culture (data not shown). The amount of aldehyde solution in each test was kept well below this threshold.

Table 1
Species used in this study, their taxonomic affiliation, average cell volume (μm^3) and growth rates at exponential phase (day^{-1})

Species	Taxonomic affiliation	Volume of control cells (μm^3)	Growth rate of control cells (day^{-1})
Micromonas pusilla (MP)	Prasinophyceae	4.7 ± 3.8	0.84 ± 0.05
Tetraselmis suecica (TS)	Prasinophyceae	1843 ± 362	0.96 ± 0.07
Isochrysis galbana (IG)	Prymnesiophyceae	26.3 ± 11.5	0.65 ± 0.03
Amphidinium carterae (AC)	Dinophyceae	2299 ± 635	0.43 ± 0.02
Dunaliella tertiolecta (DT)	Chlorophyceae	777 ± 276	0.69 ± 0.09
Skeletonema marinoi (SM)	Bacillariophyceae	63.4 ± 12.9	0.85 ± 0.08

Data are means of duplicate cultures from three independent experiments, with standard deviations ($n = 6$). Cultures were grown in f/2 medium with a 12–12 h light–dark illumination under a photon flux density of 150 μmol m^{-2} s^{-1}.

2.3. Flow cytometry, cellular parameters and cell viability

A Becton-Dickinson FACScalibur flow cytometer equipped with an air-cooled 488 nm argon-ion laser was used to estimate cell concentrations, percentage of viable cells and cell inherent optical parameters (scatter and fluorescence). The sheath fluid was natural seawater filtered onto 0.22 μm polycarbonate filters (Nuclepore, Pleasanton, USA). Optical properties of cells were assessed using forward angle light scatter (FALS) as a proxy of size and right angle light scatter (RALS), which is sensitive to the particle refractive index, as an indicator of changes in cell morphology that we term "granulosity". Red fluorescence was collected through a 650 long-pass filter and was used as a proxy for cell chlorophyll content. All values were expressed as a ratio between treated cells and non-treated cell units relative to the beads used as internal standards (1 μm Polysciences Fluorospheres, Warrington, USA, for *M. pusilla* and *I. galbana*, and 3.7 μm beads Coulter Flow-Set Fluorospheres, Beckman-Coulter, Fullerton, USA for *S. marinoi*, *D. tertiolecta*, *A. carterae*, and *T. suecica*). Data acquisition (10^4 cells on average for each sample) and analysis were performed using CellQuest software (Becton-Dickinson, San José, USA).

The percentage of viable cells was assessed in samples exposed to twice the EC_{50} concentration (determined for the individual PUAs; see below) 24 and 48 h after exposure. This concentration was chosen to compare species with different sensitivity to the PUAs at similar effect levels. Viability was assessed using the vital stain SYTOX Green (Molecular Probes, Leiden, The Nederlands) (Casotti et al., 2005). This stain does not penetrate live cells but only those with compromised plasma membranes. Optimal final concentration used was 500 nmol L^{-1} and time of incubation was 10 min. The green fluorescence of stained cells was collected through a 530/30 nm bandpass filter.

2.4. Cell and nuclear morphology

Morphological observations were obtained from at least 150 cells at 1000× magnification using an Axioskop 2 Microscope (Carl Zeiss GmbH, Jena, Germany) equipped with transmitted and epifluorescence light under blue excitation. Nucleus morphology observations were obtained from cells fixed with 1% paraformaldehyde and stained with the green-fluorescing DNA stain SYBR Green I (Molecular Probes, Leiden, The Netherlands) for 10 min at room temperature in the dark. For *D. tertiolecta* it was necessary to employ an antifading reagent (0.1% phenylenediamine, 50% glycerol and 50% PBS), made fresh daily, to prolong fluorescence (Noble and Fuhrman, 1998). Samples were taken from cultures exposed to twice the EC_{50} concentration (determined for the individual PUA; see below) at 24 and 48 h after exposure. This concentration was chosen to compare species with different sensitivity to the PUAs at similar effect levels.

Cell size was measured in living samples of control cultures (minimum 20 cells). Linear measurements were converted into cell volume using different geometric approximations: a sphere for *M. pusilla* and *I. galbana*, an ellipsoid for *A. carterae*, *D. tertiolecta* and *T. suecica*, and a cylinder for *S. marinoi*.

2.5. Statistical analyses

The acute toxicity of PUAs was expressed as EC_{50}, which is the concentration of PUA inducing a reduction of 50% in growth relative to the control, after 24 h exposure. EC_{50} values with 95% confidence interval were obtained from Probit analysis from a linear regression of percentage growth on logarithmic concentrations of PUA (Newman, 1995). Transformed EC_{50} values (natural logarithm of arcsinus of the square root of Probit values divided by 100) were used to obtain a normal distribution and equal variance to allow statistical comparison between treatments and species, using a post hoc Holm–Sidak multiple comparison test (SigmaStat 3.0, SPSS Inc., Chicago, USA). Data were fitted by a "dose–response model" using a non-linear regression with the following four-parameter logistic equation (Prism 4, GraphPad Software, San Diego, USA) which has been used before by Chèvre et al. (2002):

$$Y = \text{bottom} + \frac{\text{top} - \text{bottom}}{1 + (10^{\log \text{EC}_{50}}/10^{X})^{\text{slope}}} \quad (2)$$

where "bottom" indicates the background value in the absence of PUA, "top" the value representing the response produced by the highest PUA concentration, $\log EC_{50}$ the log of the PUA concentration that induces growth values situated halfway (50%) between bottom and top, and "slope" is the slope of the curve, used as a measure of the responsiveness of the algal growth to increments in PUA concentrations. The same equation was used to assess the value of the highest PUA concentration for which no effect is observed as compared to the control, which is where the model crosses the lower 95% confidence limit of the control. This value is defined as the statistical no effect concentration (SNEC). This parameter is preferred to the no observed effect concentration (NOEC), because it is less variable and less dependent on the experimental design (Chèvre et al., 2002).

3. Results

3.1. Effect of PUAs on growth rate

A concentration-dependent decrease of growth rate in cultures was observed for all cultures and for all PUAs, with 2*E*,4*E*-decadienal inducing a stronger effect than for the other polyunsaturated aldehydes (Holm–Sidak multiple comparison test, $P < 0.05$) (Fig. 1). When comparing SNEC values it is evident that the six species responded differently to the three PUAs (Table 2). The growth rate of *I. galbana* was significantly reduced with 2*E*,4*E*-decadienal concentrations above 0.10 μmol L^{-1} (95% CI: 0.08–0.13 μmol L^{-1}) while significant growth inhibition in *S. marinoi* started at concentrations only 20 times higher (2.20 μmol L^{-1} with 95% CI: 1.94–2.45 μmol L^{-1}) (Table 2). Except for *M. pusilla*, for which the SNEC for 2*E*,4*E*-octadienal was lower than for the other PUAs, the SNEC values for 2*E*,4*E*-octadienal and 2*E*,4*E*-heptadienal were higher than those for 2*E*,4*E*-decadienal. For *I. galbana*, these values were 19 and 30 times higher than those for

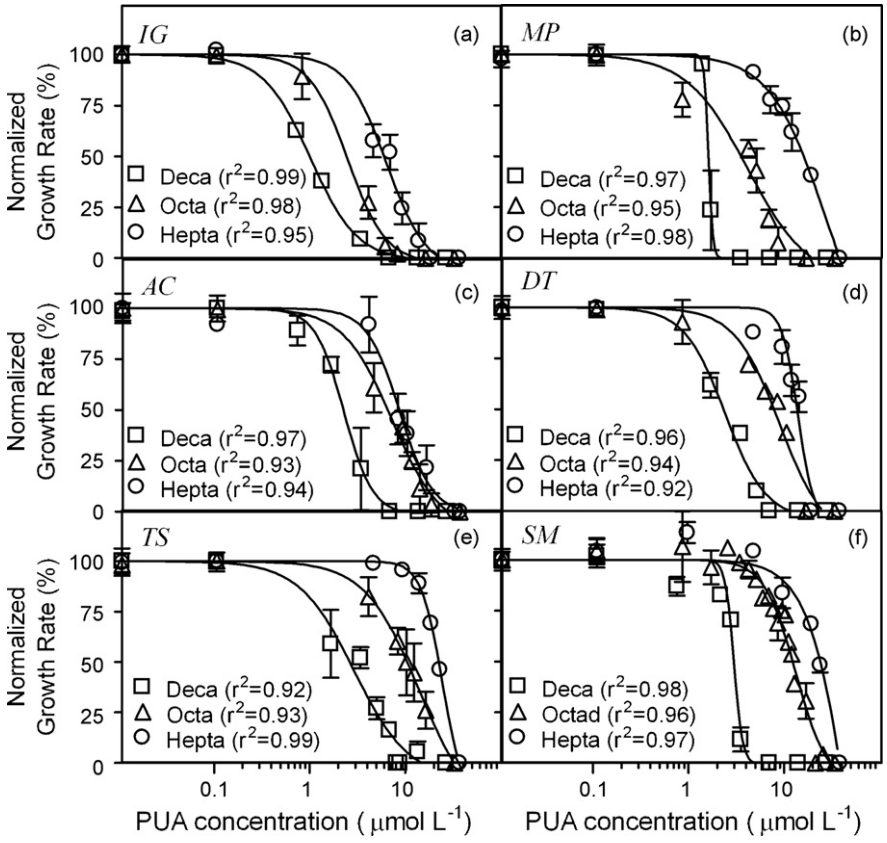

Fig. 1. Reduction in growth rates after 24 h as a function of PUA concentration ($\mu mol\ L^{-1}$) of 2*E*,4*E*-decadienal (deca), 2*E*,4*E*-octadienal (octa) and 2*E*,4*E*-heptadienal (hepta) for (a) *Isochrysis galbana* (IG); (b) *Micromonas pusilla* (MP); (c) *Amphidinium carterae* (AC); (d) *Dunaliella tertiolecta* (DT); (e) *Tetraselmis suecica* (TS) and (f) *Skeletonema marinoi* (SM). Data are normalized by the growth rate of the control cultures (untreated) and fitted using a non-linear regression model. The correlation coefficient (r^2) for each curve is indicated on their respective panels. Data are means of replicates with standard deviations ($n = 3$).

2*E*,4*E*-decadienal (1.86, 3.06 and 0.10 $\mu mol\ L^{-1}$, respectively; Table 2).

The acute toxicity of PUAs, estimated by the 24 h EC_{50} (which represents the PUA concentration inducing 50% growth inhibition), differed between species. *I. galbana* showed an EC_{50} of 0.99 $\mu mol\ L^{-1}$ for 2*E*,4*E*-decadienal, 2.25 $\mu mol\ L^{-1}$ for 2*E*,4*E*-octadienal and 5.90 $\mu mol\ L^{-1}$ for 2*E*,4*E*-heptadienal. Values were 2.02, 8.16 and 15.57 $\mu mol\ L^{-1}$ for *I. galbana* and *T. suecica*, while for *S. marinoi* the EC_{50} was 2.48, 8.94 and 18.17 $\mu mol\ L^{-1}$ for the three PUAs, respectively. No significant difference was observed between *S. marinoi* and *T. suecica*, and between *A. carterae* and *D. tertiolecta* when the EC_{50} values for each PUA were compared (Holm–Sidak multiple comparison test $P > 0.05$).

Aside from variation in the effective concentrations between species, changes were also noted in the growth rate with increasing PUA concentrations. This was reflected in the slope of the growth rate versus concentration relationship (see Fig. 1) which

Table 2
Values of the statistical no observed effect concentration ($\mu mol\ L^{-1}$), and of the 24 h EC_{50} ($\mu mol\ L^{-1}$) of six species of marine phytoplankton to 2*E*,4*E*-decadienal, 2*E*,4*E*-octadienal and 2*E*,4*E*-heptadienal

Species	Statistical no effect concentration ($\mu mol\ L^{-1}$)			24 h EC_{50} ($\mu mol\ L^{-1}$)		
	Decadienal	Octadienal	Heptadienal	Decadienal	Octadienal	Heptadienal
IG	0.10 (0.08–0.13)	1.86 (1.41–2.32)	3.06 (1.33–4.79)	0.99 (0.76–1.22)[a]	2.25 (1.68–2.91)[a]	5.90 (4.53–7.27)[a]
MP	1.42 (1.30–1.54)	0.41 (0.03–0.79)	4.03 (2.14–5.92)	1.19 (0.38–1.99)[a]	3.24 (2.39–4.08)[b]	11.32 (10.48–12.17)[b]
AC	1.27 (1.04–1.50)	4.75 (2.52–6.98)	4.50 (1.61–7.38)	1.67 (0.74–2.61)[b]	5.27 (3.15–7.39)[c]	8.95 (8.41–9.49)[b]
DT	1.20 (0.45–1.95)	4.03 (0.68–7.38)	12.11 (9.13–15.09)	2.17 (1.63–2.71)[b]	5.67 (5.22–6.11)[c]	10.71 (8.84–12.57)[b]
TS	1.50 (0.33–2.67)	5.30 (3.41–7.20)	12.79 (10.50–15.09)	2.02 (1.71–2.32)[b]	8.16 (5.69–10.60)[d]	15.57 (13.15–17.99)[c]
SM	2.20 (1.94–2.45)	3.23 (1.97–4.50)	3.61 (2.12–5.10)	2.48 (1.81–3.14)[b]	8.94 (7.28–10.60)[d]	18.17 (16.76–19.59)[c]

EC_{50} values were estimated by Probit analysis; the statistical no effect concentration was determined from the dose–response curve fitted by non-linear regression model at four parameters (see Section 2). Note that the lower the values, the more sensitive the species. Data are means of replicates with 95% confidence intervals ($n = 3$). IG is for *I. galbana*, MP for *M. pusilla*, AC for *A. carterae*, DT for *D. tertiolecta*, TS for *T. suecica* and SM for *S. marinoi*. EC_{50} values with the same superscript letters (a, b, c and d) indicate that the difference between two species for the same PUA is not significant ($P > 0.05$). A multiple comparison procedure was applied for comparing the values between the different species, for each aldehyde (Holm–Sidak method, SigmaStat 3.0).

Table 3
Values of the equation coefficients expressing the slope of the concentration–response curve, representing the responsiveness of the growth rate of a given species to an increment of PUA concentration, for the six phytoplankton species exposed to 2*E*,4*E*-decadienal, 2*E*,4*E*-octadienal and 2*E*,4*E*-heptadienal

Species	Slope		
	Decadienal	Octadienal	Heptadienal
IG	1.75 (1.58–1.91)	2.15 (1.64–2.67)	2.06 (1.30–2.81)
MP	16.28 (8.56–20.56)	1.35 (0.81–1.90)	1.50 (1.08–1.94)
AC	2.87 (3.78–1.96)	1.68 (1.01–2.35)	2.50 (1.46–3.54)
DT	2.01 (1.43–2.60)	1.81 (1.06–2.57)	5.19 (2.91–7.46)
TS	1.50 (0.92–2.07)	1.52 (0.77–2.28)	3.89 (3.30–4.48)
SM	9.54 (6.50–12.58)	2.61 (1.88–3.33)	1.92 (0.58–3.26)

Only absolute values are reported. Note that the higher the value, the more responsive the species to the PUA. Data are means of replicates with 95% confidence interval limits ($n = 3$). IG is for *I. galbana*, MP for *M. pusilla*, AC for *A. carterae*, DT for *D. tertiolecta*, TS for *T. suecica* and SM for *S. marinoi*.

we term "responsiveness" (Table 3). Values were very similar for most of the species and only slight differences were observed between the three PUAs. In the case of *S. marinoi* and *M. pusilla*, the growth rate was particularly responsive to a step-wise increase in 2*E*,4*E*-decadienal (slope coefficients of 9.54 and 16.28, respectively) as compared to 2*E*,4*E*-octadienal (slope coefficients of 2.61 and 1.92, respectively) and 2*E*,4*E*-heptadienal (slope coefficients of 1.35 and 1.50, respectively), while *T. suecica* and *D. tertiolecta* were slightly more responsive to 2*E*,4*E*-heptadienal (slope coefficients of 3.89 and 5.19, respectively) than the two other PUAs (slope coefficients of 1.50 and 2.01 for 2*E*,4*E*-decadienal, respectively; 1.52 and 1.81 for 2*E*,4*E*-octadienal, respectively).

3.2. Effect of PUAs on inherent optical parameters and morphology

FALS, RALS and RED were affected after 24 h PUA exposure in all species, but in varying ways (Fig. 2). After 24 h at twice the EC_{50} value, cell size, estimated by the FALS value, was slightly reduced in most of the species, except for *M. pusilla* for which a strong decrease was observed (39.9 ± 24.5%, 63.9 ± 4.6%, and 75.3 ± 12.0%, for 2*E*,4*E*-decadienal, 2*E*,4*E*-octadienal and 2*E*,4*E*-heptadienal, respectively); and in the case of *I. galbana*, a slight increase of FALS was observed (105.8 ± 4.6%, 112.7 ± 4.2%, and 111.4 ± 0.6%, for the three PUAs, respectively) (Fig. 2a). RALS, which is a parameter sensitive to changes in internal cell granulosity, increased in all species except *A. carterae* (Fig. 2b). The mean cell red fluorescence from chlorophyll decreased in all species. *A. carterae* fluorescence was the most affected, decreasing by 41.4 ± 16.3%, 59.6 ± 4.1% and 59.7 ± 0.2% of the control values for 2*E*,4*E*-decadienal, 2*E*,4*E*-octadienal and 2*E*,4*E*-heptadienal, respectively, indicating strong chlorophyll quenching as related to PUA toxicity (Fig. 2c). No effects on flow cytometric properties were observed between the three PUAs.

Vesicles and refractive bodies in the cytoplasm were observed in *D. tertiolecta*, *T. suecica* and *S. marinoi* (Fig. 3k, o and s), parallel to the observed increase in RALS, with respect to the

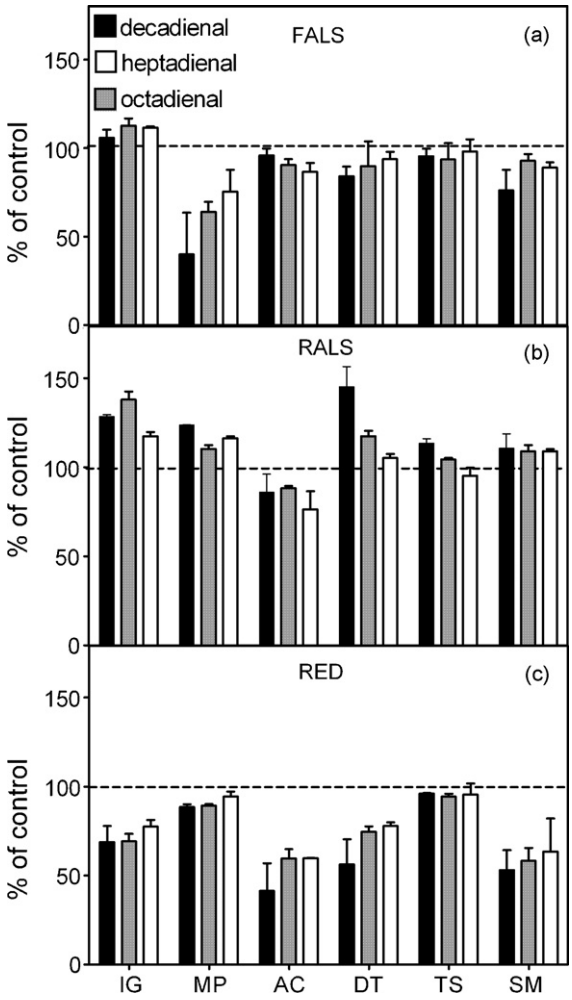

Fig. 2. Mean cellular values of (a) FALS, (b) RALS, and (c) RED fluorescence of *I. galbana* (IG), *M. pusilla* (MP), *A. carterae* (AC), *D. tertiolecta* (DT), *T. suecica* (TS) and *S. marinoi* (SM) after 24 h of incubation with 2*E*,4*E*-decadienal, 2*E*,4*E*-octadienal, or 2*E*,4*E*-heptadienal, at twice the concentration of the individual EC_{50} values each. The dotted line indicates the 100% which is the reference value (control). Data are means of replicates with standard deviations ($n = 3$).

control (Fig. 3i, m and q). In all species chromatin dispersal or complete DNA degradation (DT, Fig. 3i) as well as modifications of the shape of the nucleus were observed (Fig. 3). Such changes were observed in all cultures exposed to any PUA and were most evident after 48 h. No effect on the morphology was observed between the three PUAs (data not shown).

The percentage of SYTOX-positive cells (i.e. those judged to be non-viable) increased with time upon PUA exposure and varied among species (Fig. 4), largely reflecting the patterns shown in the growth rate data. After 24 h PUA exposure to 2*E*,4*E*-decadienal, 2*E*,4*E*-octadienal and 2*E*,4*E*-heptadienal, *I. galbana* had 15.2 ± 1.2%, 8.2 ± 0.9% and 4.9 ± 0.3% non-viable cells, respectively, and 51.6 ± 2.6%, 42.6 ± 2.8% and 48.3 ± 5.9% after 48 h exposure. In contrast, *T. suecica* had only 7.3 ± 1.8%, 8.0 ± 0.8% and 8.3 ± 1.5%, respectively, after 24 h exposure, and 15.9 ± 1.6%, 18.0 ± 0.4% and 21.9 ± 1.4%, respectively, after 48 h. The lowest percentage of dead cells was observed in *D. tertiolecta* culture with 2.0 ± 0.2%, 3.0 ± 1.8% and 4.3 ± 0.5%, respectively, at 24 h and 2.5 ± 2.4%, 12.0 ± 0.8%

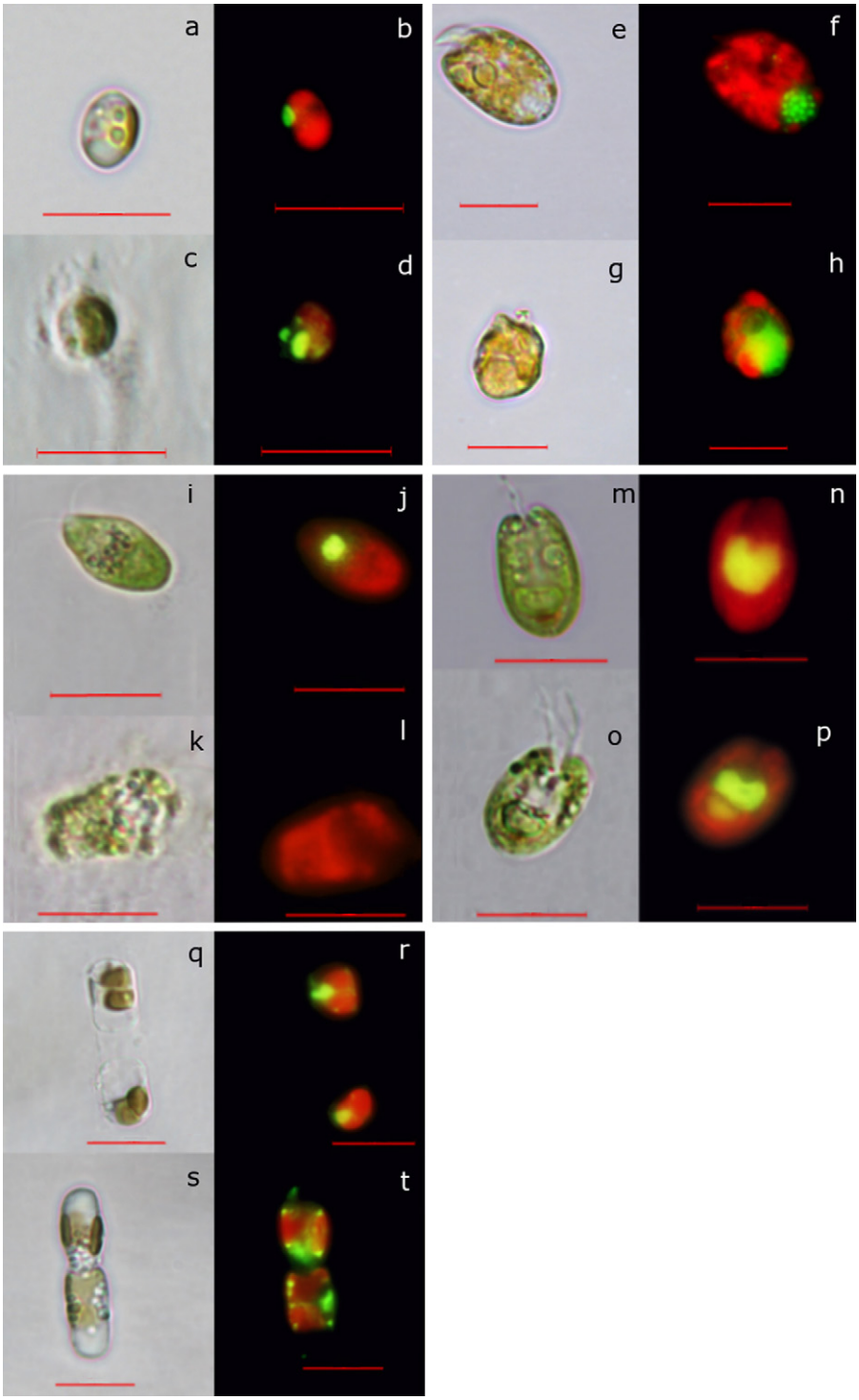

Fig. 3. Micrographs of *I. galbana* (a–d), *A. carterae* (e–h), *D. tertiolecta* (i–l), *T. suecica* (m–p) and *S. marinoi* (q–t). First two panels of each series are transmitted and epifluorescence pictures, respectively, of untreated cells fixed with paraformaldehyde 1% and stained with the DNA stain SYBR Green I (green fluorescence from the nucleus). The last two panels of each series are transmitted and epifluorescence pictures, respectively, of cells exposed to PUA for 48 h, fixed with paraformaldehyde 1% and stained with the DNA stain SYBR Green. Note the irregular shape of nuclei in treated cells and the presence of vesicles and refringent bodies. Scale bar is 10 μm.

and $11.6 \pm 3.8\%$, respectively, at 48 h. It is important to recognize that staining results may have been underestimated in *D. tertiolecta* because of the high level of chromatin degradation (Fig. 3o) which may have interfered with SYTOX staining.

4. Discussion

The three PUAs caused reduction in growth rate and cell membrane disruption, together with chlorophyll degradation, changes in cell size and cell granulosity in the six microalgae

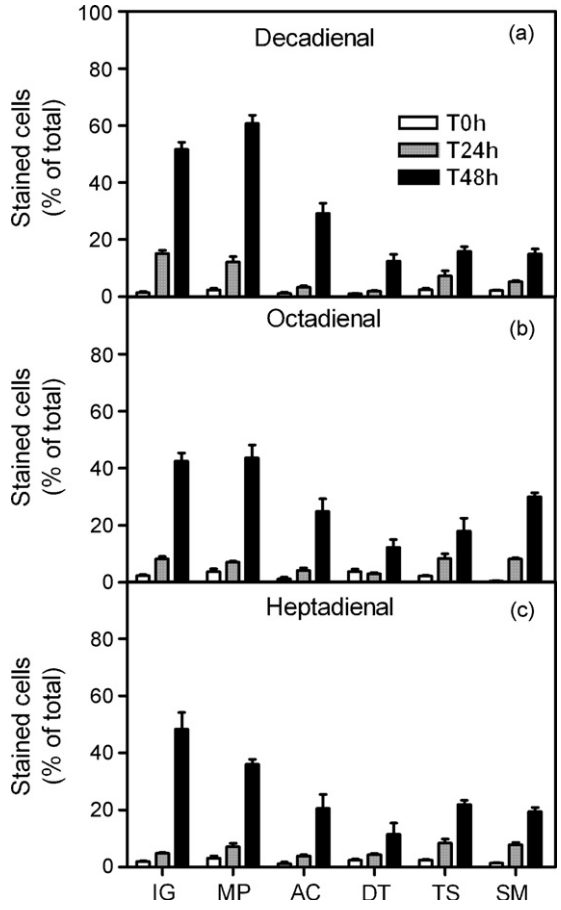

Fig. 4. Effect of the three PUAs on cell membrane permeability of *I. galbana* (IG), *M. pusilla* (MP), *A. carterae* (AC), *D. tertiolecta* (DT), *T. suecica* (TS) and *S. marinoi* (SM). Proportions of cells stained positively by SYTOX Green (non-viable) at time 0 h and after 24 and 48 h of incubation with (a) 2*E*,4*E*-decadienal, (b) 2*E*,4*E*-octadienal, (c) 2*E*,4*E*-heptadienal, at twice the concentration of the individual EC_{50}. Data are means of replicates with standard deviations ($n = 3$).

tested. These results confirm that PUAs trigger degeneration of a broad range of key physiological processes, as reported also by Spiteller et al. (2001). 2*E*,4*E*-Decadienal induced a stronger inhibition of growth as compared to 2*E*,4*E*-octadienal and 2*E*,4*E*-heptadienal, probably due to its longer alkyl chain that increases the reactivity of the molecule (Adolph et al., 2003).

The presence of vesicles and refractive bodies in the cytoplasm and the chromatin dispersal in some of the species tested are reminiscent of apoptosis, a mechanism of programmed cell death only recently discovered in algae (Franklin et al., 2006). Interestingly, exposure to 2*E*,4*E*-decadienal has been shown to induce programmed cell death in many different marine organisms, including diatoms (Casotti et al., 2005), copepods and sea urchin embryos (Romano et al., 2003), suggesting a common mode of action in both animals and plants. The range of concentrations of 2*E*,4*E*-decadienal (the most widely PUA used in toxicological studies so far) affecting animals varies widely, from 0.15 to 25.92 $\mu mol\,L^{-1}$ for crustaceans, and from 1.18 to 15.64 $\mu mol\,L^{-1}$ for echinoderms (see Caldwell et al., 2003). These concentrations appear to be higher than those identified for species in the present study, suggesting that algae may be more sensitive than animals. However, in the case of animals, sensitive stages such as eggs are used and exposure times are often shorter, making it difficult to compare.

Among species, in general, our data show a higher sensitivity to PUAs of smaller phytoplankton like *I. galbana* when compared to larger species like *T. suecica* and *S. marinoi*. This could be related to the smaller size of *I. galbana*, as the higher surface to volume ratio may underlie a higher potential for uptake of lipophilic compounds such as PUAs. Differing membrane characteristics may also play a role. The more sensitive species *I. galbana* and *M. pusilla* have no distinct or mineralized cell walls (Throndsen, 1997; Zhu et al., 1997) and so may be more vulnerable compared to other taxa with different cell wall properties (e.g. the diatom silicified cell wall or the highly structured cell wall of dinoflagellates). Prymnesiophytes (like *I. galbana*) are reported to have higher total lipid content as compared to prasinophytes, chlorophytes, dinophytes and diatoms (Reitan et al., 1994) and this may also be a factor to consider to explain its higher sensitivity as compared to the other species tested. It is, in fact, reported that toxicity of lipophilic compounds is related to the total lipid content of target cells, which increases their ability to penetrate the cell wall and to affect critical lipoprotein complexes within the cell membrane (Hutchinson et al., 1980). Detoxification mechanisms or presence of specific target sites for PUAs may vary among taxa, and altogether, the differential sensitivity of the tested species may result in differences in their ability to compete with other species in natural waters where diatoms or other PUA-producing algae are found.

Even within diatoms (putative producers of PUAs), there are species-specific differences in responsiveness to the same PUA. *P. tricornutum* shows an EC_{50} for growth of 7.1 $\mu mol\,L^{-1}$ for 2*E*,4*E*-decadienal (Vardi et al., 2006), which is much higher than what found for *T. weissflogii* (1.9 $\mu mol\,L^{-1}$; Casotti et al., 2005) and *S. marinoi* (2.4 $\mu mol\,L^{-1}$; this study). Also in this case, differences may be due to size and/or membrane mechanical or biochemical properties. But it should also be noted that none of these species are reported to produce 2*E*,4*E*-decadienal (Wichard et al., 2005a), and *P. tricornutum* does not produce PUAs at all, but other oxylipins (Pohnert et al., 2002). In our study *S. marinoi* was particularly tolerant to 2*E*,4*E*-octadienal and 2*E*,4*E*-heptadienal, which are the main PUAs produced by this species (Wichard et al., 2005a), whereas it was more sensitive to 2*E*,4*E*-decadienal. This suggests that *S. marinoi* has developed a moderate resistance against the compounds that it is most likely to encounter. This may also explain the Ca^{2+}–nitric oxide-mediated signalling system elicited by sublethal concentrations of 2*E*,4*E*-decadienal in *P. tricornutum* (Vardi et al., 2006), which could be interpreted as a general reaction to toxic compounds.

There are several important limitations in extrapolating the data of the present study to the natural realm. Concentrations tested appear to be high when compared to concentrations of PUAs that diatom species are reported to produce, ranging from 0.01 to 9.81 $fmol\,cell^{-1}$ (Wichard et al., 2005a). In the present study, single model compounds were used whereas in nature aldehydes are released from the same cell at the same time, and so it is likely that PUA effect will act additively, as predict by Faust et al. (2001). Moreover, due to the patchy nature

of phytoplankton at sea, it is reasonable to expect high local concentrations in the proximity of diatom cells.

Concentration of PUAs as a function of distance from the surface of the producing cell state can be estimated using a random-walk model based on simple molecular diffusion (Berg, 1983). This model uses the following formula to calculate the concentration (C) of a given compound at steady state as a function of distance from the cell (r):

$$C(r) = \frac{i}{4\pi Dr} \quad (3)$$

where i is the PUA production rate in fmol cell^{-1} s^{-1}, r the distance from the source in μm and D is the diffusion constant in μm^2 s^{-1}, which for PUAs has been conservatively calculated as ∼450 μm^2 s^{-1} based on its stereochemistry (Tucker and Nelken, 1982).

Thalassiosira rotula is reported to produce 6.35 fmol PUA cell^{-1} in culture (Wichard et al., 2005a) at a rate of 0.035 fmol cell^{-1} s^{-1} (Pohnert, 2000). We can therefore expect to find 6.24, 0.62 and 0.06 μmol PUA L^{-1} at 1, 10 and 100 μm in the surrounding of one single cell, respectively, soon after its lysis. This figure can be much higher at sea, if we base the calculations on values measured by Wichard et al. (2005b), which report concentrations as high as 47.7 fmol total PUAs cell^{-1} in phytoplankton samples dominated by diatoms in the English Channel. Based on this figure and using the same kinetic curve of Pohnert (2000) for PUA production, the rate was 0.265 fmol s^{-1}, and therefore the release of PUAs from each diatom cells can be estimated as 46.9, 4.7 and 0.5 μmol PUA L^{-1} at a distance of 1, 10 and 100 μm from the cell surface. Such concentrations are well within the significant range for affecting growth and performance of surrounding organisms. A similar approach has been used by Jackson (1980) to exclude the possibility that growth of phytoplankton in oligotrophic areas of the oceans could be supported by pulses of nutrients excreted from animals swimming near a phytoplankter, because of rapid dissipation. However, the D value we used is expected to be much lower than Jackson's, because of the high lipophilic nature of PUAs. Many studies have, indeed, reconsidered the estimates of the D parameter, showing that it can be much lower (Ploug and Passow, 2007). Unlike the situation in static cultures, PUA production is a continuous process, which is reinitiated when the product is removed from the enzyme (Fontana et al., 2007), and this may contribute to maintain high local concentrations at sea. In addition to this, our experiments probably underestimate the potential effects of PUAs because we used nutrient-replete cultures growing logarithmically. Ribalet et al. (2007) have shown that PUA production increases with age and nutrient limitation, suggesting that PUAs measured in cultures are potentially underestimating true production under natural conditions, for example at the end of a bloom when conditions are often far from optimal. Consequently, it is reasonable to expect that PUA concentrations at sea may affect the growth of algae surrounding PUA-producing cells, also considering that altered nutrient conditions often increase the sensitivity of target algae to toxicants (e.g. Fistarol et al., 2005).

Ultimately, to resolve such questions, *in situ* measurements of aldehydes are needed. Attempts to develop a sensitive method for the detection and quantification of dissolved PUAs in seawater are in progress (G. Pohnert, pers. comm.) and this is expected to represent a key step to understand the ecological role of these compounds in marine ecosystems and to stimulate new ideas for future research.

Acknowledgements

We thank Sabina Mazza for preliminary assays on *S. marinoi*. The project was carried out in the framework of the Research Mode Project ROSEMEB (Role of SEcondary Metabolites in driving ecosystem functionality and Maintaining Ecosystem Biodiversity) within the MarBEF Network of Excellence 'Marine Biodiversity and Ecosystem Functioning', which is funded by the Sustainable Development, Global Change and Ecosystems Programme of the European Community's Sixth Framework Programme (contract GOCE-CT-2003-505446). This publication is contribution MPS-07064 of MarBEF.

References

Adolph, S., Poulet, S.A., Pohnert, G., 2003. Synthesis and biological activity of α,β,γ,δ unsaturated aldehydes from diatoms. Tetrahedron Lett. 59, 3003–3008.

Berg, H.C., 1983. Random Walks in Biology. Princeton University Press, Princeton, NJ, USA.

Caldwell, G.S., Bentley, M.G., Olive, P.J.W., 2003. The use of a brine shrimp *Artemia salina* bioassay to assess the toxicity of diatom extracts and short chain aldehydes. Toxicon 42, 301–306.

Casotti, R., Mazza, S., Brunet, C., Vantrepotte, V., Ianora, A., Miralto, A., 2005. Growth inhibition and toxicity of the diatom aldehyde 2-*trans*,4-*trans* decadienal on *Thalassiosira weissflogii* (Bacillariophyceae). J. Phycol. 41, 7–20.

Cembella, A.D., 2003. Chemical ecology of eukaryotic microalgae in marine ecosystems. Phycologia 42, 420–447.

Chèvre, N., Slooten, K.B.-V., Tarradellas, J., Brazzale, A.R., Behra, R., Guettinger, H., 2002. Effects of dinoseb on the life cycle of *Daphnia magna*: modeling survival time and a proposal for an alternative to the no-observed-effect concentration. Environ. Toxicol. Chem. 21, 828–833.

d'Ippolito, G., Romano, G., Iadicicco, O., Miralto, A., Ianora, A., Cimino, G., Fontana, A., 2002. New birth-control aldehydes from the marine diatom *Skeletonema costatum*: characterization and biogenesis. Tetrahedron Lett. 43, 6133–6136.

d'Ippolito, G., Tucci, S., Cutignano, A., Romano, G., Cimino, G., Miralto, A., Fontana, A., 2004. The role of complex lipids in the synthesis of bioactive aldehydes of the marine diatom *Skeletonema costatum*. Biochim. Biophys. Acta 1686, 100–107.

Faust, M., Altenburger, R., Backhaus, T., Blanck, H., Boedeker, W., Gramatica, P., Hamer, V., Scholze, M., Vighi, M., Grimme, L.H., 2001. Predicting the joint algal toxicity of multi-component s-triazine mixtures at low-effect concentrations of individual toxicants. Aquat. Toxicol. 56, 13–32.

Fistarol, G., Legrand, C., Graneli, E., 2005. Allelopathic effect on a nutrient-limited phytoplankton species. Aquat. Microb. Ecol. 41, 153–161.

Fontana, A., d'Ippolito, G., Cutignano, A., Miralto, A., Ianora, A., Romano, G., Cimino, G., 2007. Chemistry of oxylipin pathways in marine diatoms. Pure Appl. Chem. 79, 475–484.

Franklin, D.J., Brussaard, C.P.D., Berges, J.A., 2006. What is the role and nature of programmed cell death in phytoplankton ecology? Eur. J. Phycol. 41, 1–14.

Guillard, R.R.L., 1975. Culture of phytoplankton for feeding marine invertebrates. In: Smith, W.L., Chanley, M.H. (Eds.), Culture of Marine Invertebrate Animals. Plenum Press, New York, pp. 29–60.

Hansen, E., Eilertsen, H.C., 2007. Do the polyunsaturated aldehydes produced by *Phaeocystis pouchetii* (Hariot) Lagerheim influence diatom growth during the spring bloom in Northern Norway? J. Plankton Res. 29, 87–96.

Hansen, E., Ernstsen, A., Eilertsen, H.C., 2004. Isolation and characterization of a cytotoxic polyunsaturated aldehyde from marine phytoplankter *Phaeocystis pouchetii* (Hariot) Lagerheim. Toxicology 199, 207–217.

Hay, M.E., 1996. Marine chemical ecology: what's known and what's next? J. Exp. Mar. Biol. Ecol. 200, 103–134.

Hutchinson, T.C., Hellebust, J., Tam, D., Mackay, D., Mascarenhas, R.A., Shiu, W.Y., 1980. The correlation of the toxicity to algae of hydrocarbons and halogenated hydrocarbons with their physical–chemical properties. In: Afghan, B.K., Mackay, D. (Eds.), Hydrocarbons and Halogenated Hydrocarbons in the Aquatic Environment. Plenum Press, New York, pp. 577–586.

Jackson, G.A., 1980. Phytoplankton growth and zooplankton grazing in oligotrophic oceans. Nature 284, 439–441.

Kirchman, D.L., 1999. Oceanography: phytoplankton death in the sea. Nature 398, 293–294.

Legrand, C., Rengefors, K., Fistarol, G.O., Graneli, E., 2003. Allelopathy in phytoplankton—biochemical, ecological and evolutionary aspects. Phycologia 42, 406–419.

Newman, M.C., 1995. Quantitative Methods in Aquatic Ecotoxicology. CRC Press, Boca Raton.

Noble, R.T., Fuhrman, J.A., 1998. Use of SYBR Green I for rapid epifluorescence counts of marine viruses and bacteria. Aquat. Microb. Ecol. 14, 113–118.

Pippen, E.L., Nonaka, M., 1958. A convenient method for synthesizing normal aliphatic 2,4-dienals. J. Org. Chem. 23, 1580–1582.

Ploug, H., Passow, U., 2007. Direct measurement of diffusivity within diatom aggregates containing transparent exopolymer particles. Limnol. Oceanogr. 52, 1–6.

Pohnert, G., 2000. Wound-activated chemical defense in unicellular planktonic algae. Angew. Chem. Int. Ed. 39, 4352–4354.

Pohnert, G., 2002. Phospholipase A2 activity triggers the wound-activated chemical defense in the diatom *Thalassiosira rotula*. Plant Physiol. (Rockv.) 129, 103–111.

Pohnert, G., Boland, W., 2002. The oxylipin chemistry of attraction and defense in brown algae and diatoms. Nat. Prod. Rep. 15, 108–122.

Pohnert, G., Lumineau, O., Cueff, A., Adolph, S., Cordevant, C., Lange, M., Poulet, S., 2002. Are volatile unsaturated aldehydes from diatoms the main line of chemical defence against copepods? Mar. Ecol. Prog. Ser. 245, 33–45.

Reitan, K.I., Rainuzzo, J.R., Olsen, Y., 1994. Effect of nutrient limitation on fatty acid and lipid content of marine microalgae. J. Phycol. 30, 972–979.

Ribalet, F., Wichard, T., Pohnert, G., Ianora, A., Miralto, A., Casotti, R., 2007. Age and nutrient limitation enhance polyunsaturated aldehyde production in marine diatoms. Phytochemistry 68, 2059–2067.

Romano, G., Russo, G.L., Buttino, I., Ianora, A., Miralto, A., 2003. A marine diatom-derived aldehyde induces apoptosis in copepod and sea urchin embryos. J. Exp. Biol. 206, 3487–3494.

Sarno, D., Kooistra, W.H.C.F., Medlin, L.K., Percopo, I., Zingone, A., 2005. Diversity in the genus *Skeletonema* (Bacillariophyceae). II. An assessment of the taxonomy of *S. costatum*-like species with the description of four new species. J. Phycol. 41, 151–176.

Spiteller, P., Kern, W., Reiner, J., Spiteller, G., 2001. Aldehydic lipid peroxidation products derived from linoleic acid. Biochim. Biophys. Acta 1531, 188–208.

Throndsen, J., 1997. The planktonic marine flagellates. In: Tomas, C.R. (Ed.), Identifying Marine Phytoplankton. Academic Press, San Diego, pp. 591–730.

Tucker, W.A., Nelken, L.H., 1982. Diffusion coefficients in air and water. In: Lyman, W.J., Reehl, W.F., Rosenblatt, D.H. (Eds.), Handbook of Chemical Property Estimation Methods. American Chemical Society, Washington, DC, pp. 17–25.

Vardi, A., Formiggini, F., Casotti, R., De Martino, A., Ribalet, F., Miralto, A., Bowler, C., 2006. A stress surveillance system based on calcium and nitric oxide in marine diatoms. PLoS Biol. 4, 411–419.

Watson, S.B., 2003. Cyanobacterial and eukaryotic algal odour compounds: signals or by-products? A review of their biological activity. Phycologia 42, 332–350.

Watson, S.B., Satchwill, T., 2003. Chrysophyte odour production: resource-mediated changes at the cell and population levels. Phycologia 42, 393–405.

Watson, S.B., Satchwill, T., Dixon, E., McCauley, E., 2001. Under-ice blooms and source-water odour in a nutrient-poor reservoir: biological, ecological and applied perspectives. Freshw. Biol. 46, 1–15.

Wichard, T., Poulet, S.A., Halsband-Lenk, C., Albaina, A., Harris, R., Liu, D., Pohnert, G., 2005a. Survey of the chemical defence potential of diatoms: screening of fifty one species for α,β,γ,δ-unsaturated aldehydes. J. Chem. Ecol. 31, 949–958.

Wichard, T., Poulet, S.A., Pohnert, G., 2005b. Determination and quantification of α,β,γ,δ-unsaturated aldehydes as pentafluorobenzyl-oxime derivates in diatom cultures and natural phytoplankton populations: application in marine field studies. J. Chromatogr. B 814, 155–161.

Zhu, C.J., Lee, Y.K., Chao, T.M., 1997. Effects of temperature and growth phase on lipid and biochemical composition of *Isochrysis galbana* TK1. J. Appl. Phycol. 9, 451–457.