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Age and nutrient limitation enhance polyunsaturated aldehyde production in marine diatoms

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Abstract

Skeletonema marinoi produces 2,4-heptadienal, 2,4-octadienal, and 2,4,7-octatrienal, the latter only in traces. In nutrient-replete cultures, the production of potentially defensive polyunsaturated aldehydes (PUA) increases from the exponential to the stationary phase of growth from 1.2 fmol cell^{-1} ($\pm$0.4 fmol cell^{-1} SD) to 4.2 fmol cell^{-1} ($\pm$1.0 fmol cell^{-1} SD), with 2,4-heptadienal as the dominant aldehyde. The plasticity of PUA production with age of the culture supports the hypothesis of a direct link between toxin production and cell physiological state. N- and P-limited cells in stationary phase produced 1.4 and 1.8 fold higher amounts of PUA than control cultures and 10.7 and 4.6 times higher PUAs when compared to their own exponential growth phase, respectively. The increase in PUA production in the nutrient-limited cultures was not paralleled by an increase in the total amount of precursor fatty acids indicating that physiological stress might trigger an enhanced expression or activity of the enzymes responsible for PUA production, i.e. chemical defense increase in aged and nutrient-stressed diatoms. If this holds true during blooms, grazers feeding at the end of a bloom would be more affected than early-bloom grazers.

Keywords: *Skeletonema*; Harmful algal blooms; Chemical defense; Polyunsaturated fatty acids; Growth phase; Nutrient limitation

1. Introduction

Diatoms are eukaryotic unicellular algae responsible for more than 50% of marine primary production (Nelson et al., 1995). They are major representatives of the seasonal blooms that occur in the ocean and have traditionally been considered the main source of food for predatory zooplankton, such as copepods. Recent evidence indicates that certain diatom species induce a drastic reduction in the reproductive response of copepods (Poulet et al., 1994) due to blockage of mitotic divisions during embryogenesis or induction of apoptosis in developing embryos (Miralto et al., 1999b, Ianora et al., 2004). At the metabolic level, hatching failure has been related to the presence of a family of polyunsaturated short-chain aldehydes (here abbreviated as PUAs). PUA production for chemical defense was first attributed to the marine planktonic diatom *Thalassiosira rotula* (Miralto et al., 1999b). Several different PUAs such as 2,4-heptadienal, 2,4-octadienal, 2,4,7-octatrienal, 2,4-decadienal and 2,4,7-decatrienal, and oxo-acids bearing a similar unsaturated aldehyde structure element have been described since in several marine and freshwater diatoms in culture (reviewed in Pohnert, 2005; Wichard and Pohnert, 2006) and in natural phytoplankton samples (Wichard et al., 2005b).

PUAs are not present in intact cells and are synthesized mainly by wounded cells through enzymatic transformation of free polyunsaturated fatty acids (here abbreviated as PUFAs) (Pohnert, 2000). This defensive reaction is

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under the control of a phospholipase A_2-galactolipase/lipoxygenase/hydroperoxide lyase enzyme cascade (Pohnert, 2002; d'Ippolito et al., 2004) which is activated within seconds after crushing of the cell. As the transformation occurs immediately after cell disruption, the regulation through transcription and *de novo* protein biosynthesis of the enzymes is highly unlikely. A strict correlation has been found between the PUA and the PUFA composition of phospholipids and chloroplast-derived glycolipids in diatoms (Pohnert, 2002; d'Ippolito et al., 2004). Since virtually no free PUFAs have been found in intact cells, and addition of PUFAs to wounded diatoms leads to increased formation of PUAs, availability of free fatty acids appears to be a limiting factor for aldehyde production (Pohnert, 2002).

The question arises as to how the PUA production varies depending on the physiological conditions before cell disruption occurs.

A certain variability of the lipidic pool has been observed in response to changes in physiological conditions or environmental factors, including age of culture, nutrients or temperature (for a review, Groth-Nard and Robert, 1993). For many phytoplankton species, production of toxins and other secondary metabolites is strongly modulated by physiological conditions and age (reviewed in Legrand et al., 2003; Ianora et al., 2006). Diatom PUAs are rather dependant on the available lipid resources and a few key enzymes, in contrast to these toxins, which require elaborate enzymatic activity for their production. We show here that PUA production of diatoms is also strongly dependant on the physiological and environmental conditions during growth.

The bloom-forming diatom *Skeletonema marinoi* (separated from *S. costatum*, Sarno et al., 2005) produces the C_7 aldehyde 2*E*-4*Z*-heptadienal (in the following heptadienal), as well as the C_8 aldehydes 2*E*-4*Z*-octadienal (octadienal) and 2*E*-4*Z*,7*Z*-octatrienal (octatrienal), which are derived from eicosapentaenoic (EPA), hexadecatrienoic (HDTRI), and hexadecatetraenoic (HDTERTA) acid, respectively (Pohnert, 2005). *S. marinoi* forms dense, almost monospecific blooms in late-winter in the Northern Adriatic Sea (Mediterranean Sea) that strongly effect copepod reproduction and recruitment (Miralto et al., 1999a; Ianora et al., 2004). During the bloom, PUA production per cell correlates positively with *S. marinoi* cell numbers and, in turn, with phytoplankton lysis rates which are higher in the final stages of the bloom (Casotti, R., unpubl.), suggesting a potential release of PUA into the seawater without grazing.

The present study aimed at quantifying potential production of PUAs per cell in cultures of *S. marinoi* during the different growth phases, from exponential to declining. PUA production has been measured in cultures grown under nitrate and phosphate limitation. The data obtained show that the wound-activated production of PUAs per *S. marinoi* cell was higher when cultures reached the stationary growth phase. The same was true for N- and P-limited cultures.

2. Results

2.1. Growth under standard conditions

The average growth rate of *S. marinoi* in exponential phase under standard conditions was $0.96\ d^{-1} \pm 0.05\ d^{-1}$ SD. The cultures attained cell concentrations of 1.05×10^6 cell ml^{-1} at the stationary phase, and these remained constant for 6 days before the declining phase (Fig. 1a). We

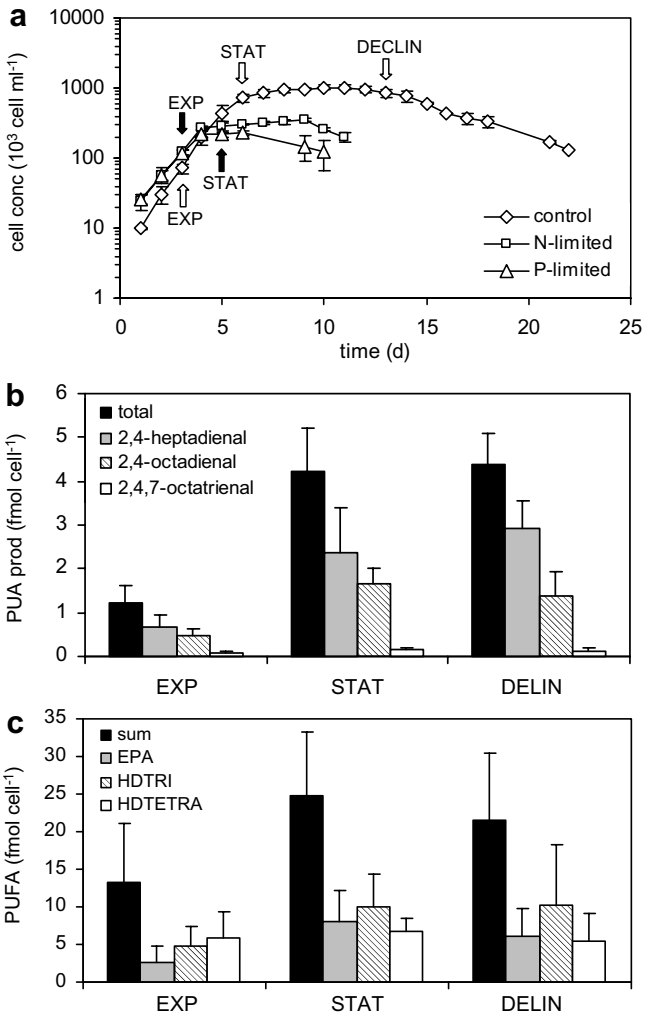

Fig. 1. (a) Average cell concentrations of *S. marinoi* during growth in batch cultures under nutrient-replete (open diamonds) and under N- (open squares) and P- limitation (open triangles). Arrows indicate sampling points for analyses of polyunsaturated aldehydes (PUAs) and their precursor polyunsaturated fatty acids (PUFAs). White arrows for nutrient-replete and black arrows for nutrient-limited cultures. (b) PUA production in nutrient-replete cultures at the three growth phases indicated in panel (a). Different PUAs are indicated in the legend. (c) PUFAs at the three growth phases indicated in panels (a) and (b). Different PUFAs are indicated in the legend. Sum indicates the sum of the three PUA-precursor fatty acids. EPA is eicosapentaenoic acid, HDTRI is hexadecatrienoic acid, and HDTETRA is hexadecatetraenoic acid. For plots of nutrient-replete cultures, data are means of replicates from 9 independent experiments, while for nutrient-limited cultures, $n = 3$. EXP, STAT, and DECLIN indicate the exponential, stationary, and declining phase of growth, respectively. Error bars are standard deviations.

tested the hypothesis that nitrate and phosphate could be the factors limiting growth in the control cultures causing their entrance in the stationary phase of growth, by adding these nutrients separately to triplicate control cultures in the same concentrations as in the f/2 medium (883 and 36 μmol l^{-1}, respectively) at the onset of the stationary phase of growth. The cultures showed no increase in cell concentration and therefore both nutrients could be excluded to be limiting growth in the stationary phase.

No significant change in forward angle light scatter (FALS, used as a proxy of size) was observed (Student's *t*-test, $p > 0.05$), while Right Angle Light Scatter (RALS, indicator of internal granular structure) increased progressively (Table 1) with time. The increase in RALS was reflected in an increase in granularity of the diatom cytoplasm, as observed by microscopy. Red fluorescence from chlorophyll increased by 32.0% from the exponential to stationary phase and then decreased again during the declining phase (Table 1). Chlorophyll *a* (Chl *a*) from HPLC followed the same trend (Table 1). The proportion of non-viable cells, assessed using the SYTOX Green nucleic acid stain, was very low in the exponential phase (SYTOX Green-positive cells $0.7 \pm 0.1\%$ SD), while it increased with culture age ($3.6 \pm 0.8\%$ SD in the stationary and $9.3 \pm 1.5\%$ SD in the declining phase – Table 1). The onset of stationary growth led to an increase in cellular C, while particular organic nitrogen (PON) remained constant (Table 2).

Total PUA content per cell produced in response to wounding in the exponential phase of growth was 1.23 ± 0.37 fmol $cell^{-1}$ SD and increased significantly in the early stationary phase (4.21 ± 0.99 fmol $cell^{-1}$ SD, Student *t*-test, $p < 0.001$), to remain stable until the early declining phase (4.39 ± 0.69 fmol $cell^{-1}$ SD, Fig. 1b). The relative increase followed the same trend when PUAs were normalized by Chl *a* or particulate organic carbon (POC, not shown). Among the PUAs, heptadienal was always the most abundant, accounting for 53.7% of total PUAs in exponential, 56.8% in stationary, and 68.8% in declining phases of growth. Octadienal contributed to 39.5% of total PUAs in exponential, 39.6% in stationary and 32.3% in declining phases (Fig. 1b). Octatrienal accounted for a lower proportion of total PUAs, decreasing from 6.8% (exponential) to 3.7% (stationary) to 2.6% (declining). Absolute values were significantly different only for exponential vs stationary phase for all three aldehydes (Student's *t*-test, $p < 0.01$).

Average content per cell of the three PUFAs which serve as precursors of the unsaturated aldehydes followed the same pattern than that observed for PUAs. These PUFAs were found in concentrations of 13.1 ± 7.9 fmol $cell^{-1}$ SD during the exponential phase, corresponding to 3.4 pg $cell^{-1} \pm 2.1$ pg $cell^{-1}$ SD. Their level increased to 24.7 ± 8.5 fmol $cell^{-1}$ SD in the stationary phase (Student's *t*-test, $p < 0.05$) and remained stable until the declining phase of growth (21.6 ± 8.8 fmol $cell^{-1}$ SD, Fig. 1c). During the exponential growth phase, the dominant PUFAs were hexadecatetraenoic and hexadecatrienoic acids (5.9 ± 3.4 fmol $cell^{-1}$ SD and 4.7 ± 2.7 fmol $cell^{-1}$ SD, respectively), accounting for 44.8% and 35.6% of the three PUA precursors, while eicosapentaenoic acid was present in lower amounts (2.6 ± 2.1 fmol $cell^{-1}$ SD, i.e., 19.6% of the three PUA precursors). In the stationary phase of growth, cell

Table 2
Particulate organic nitrogen (PON), phosphate (POP), or carbon (POC), molar ratios of N:P, and C:N of *S. marinoi* cultures in exponential or stationary phases of growth under nutrient-replete (replete), nitrate-limiting (N-limited), or phosphate-limiting (P-limited) conditions. EXP is exponential, STAT is stationary

		Replete	N-limited	P-limited
EXP	PON (pg $cell^{-1}$)	2.35 ± 0.16	0.87 ± 0.09	1.55 ± 0.21
	POP (pg $cell^{-1}$)	0.39 ± 0.04	0.25 ± 0.05	0.12 ± 0.02
	POC (pg $cell^{-1}$)	12.85 ± 1.35	6.15 ± 0.19	8.42 ± 1.34
	N:P	13.40 ± 1.12	7.71 ± 0.98	28.44 ± 1.73
	C:N	6.40 ± 0.76	8.28 ± 1.02	6.31 ± 0.33
STAT	PON (pg $cell^{-1}$)	2.29 ± 0.23	0.96 ± 0.08	1.69 ± 0.20
	POP (pg $cell^{-1}$)	0.34 ± 0.04	0.32 ± 0.03	0.08 ± 0.01
	POC (pg $cell^{-1}$)	15.71 ± 2.17	10.63 ± 1.06	15.61 ± 1.42
	N:P	16.68 ± 1.05	6.75 ± 1.10	45.82 ± 2.50
	C:N	8.02 ± 1.13	12.91 ± 0.68	10.83 ± 0.41

Data are means ± standard deviations ($n = 9$ for the nutrient-replete conditions, $n = 3$ for the nutrient-limited conditions).

Table 1
Values of Forward Angle Light Scatter (FALS), Right Angle Light Scatter (RALS), red fluorescence from chlorophyll (RED), all expressed in units relative to the beads used as internal standard (r. u.), percentage of SYTOX Green positive cells (SYTOX+, non-viable) and chlorophyll *a* (Chl *a*) during different phases of *S. marinoi* under standard conditions (replete) and under nitrate-limiting (N-limited) or phosphate-limiting (P-limited) conditions. EXP is exponential, STAT is stationary, DECLIN is declining

Growth phase	Initial nutrient conditions	FALS (r. u.)	RALS (r. u.)	RED (r. u.)	SYTOX+ (%)	Chl *a* (pg $cell^{-1}$)
EXP	Replete	10.53 ± 1.74	0.83 ± 0.15	79.04 ± 5.16	0.7 ± 0.1	0.76 ± 0.17
EXP	N-limited	6.33 ± 1.51	0.50 ± 0.04	41.61 ± 13.76	1.4 ± 0.4	0.16 ± 0.02
EXP	P-limited	4.25 ± 0.48	1.14 ± 0.12	78.81 ± 8.37	0.8 ± 0.3	0.24 ± 0.09
STAT	Replete	9.50 ± 1.38	1.09 ± 0.30	116.26 ± 12.67	3.6 ± 0.8	1.01 ± 0.14
STAT	N-limited	5.76 ± 0.51	1.03 ± 0.06	56.05 ± 9.18	9.2 ± 0.8	0.25 ± 0.03
STAT	P-limited	5.19 ± 0.47	1.07 ± 0.03	81.36 ± 10.51	15.9 ± 3.1	0.22 ± 0.02
DECLIN	Replete	10.12 ± 1.69	1.61 ± 0.26	55.67 ± 6.25	9.3 ± 1.5	0.80 ± 0.15

Data are means ± standard deviations (for the nutrient-replete conditions, $n = 9$ for cell densities, FALS, RALS, RED, and SYTOX+; $n = 4$ for Chl *a*; for the nutrient-limited conditions, $n = 3$).

content in terms of hexadecatrienoic and eicosapentaenoic acids increased in absolute values to 10.1 ± 4.3 fmol cell^{-1} SD and 8.0 ± 4.2 fmol cell^{-1} SD, respectively. The total percent contribution of both PUFAs was 40.6% and 32.5%, respectively. In contrast, the absolute amount of hexadecatetraenoic acid remained nearly constant (6.7 ± 1.9 fmol cell^{-1} SD), but this resulted in a lower percent contribution (26.9%). During the declining phase, the amount of PUFAs did not change significantly as compared to the early stationary phase (Student's t-test, $p > 0.05$, Fig. 1c).

2.2. Growth under nitrogen or phosphorus limitation

For the nutrient-limitation experiments, only samples from the exponential and stationary phases of growth were compared to the control due to the high variability in conditions of cultures in the declining phase. Both N- and P-limited cultures in the exponential phase of growth exhibited similar growth rates to the control (0.91 ± 0.04 d^{-1} SD and 0.88 ± 0.06 d^{-1} SD, respectively) but reached the stationary phase at lower cell concentrations ($0.29 \pm 0.04 \times 10^6$ cells ml^{-1} SD and $0.22 \pm 0.02 \times 10^6$ cells ml^{-1} SD, respectively, Fig. 1a).

It is important to note that for these experiments, cultures were pre-conditioned by growing them in nutrient-limited medium. For this reason, we observed differences with the control even in the exponential phase. Cells in the exponential phase of growth had 39.9% (±14.4% SD) lower FALS in N-limited cells and 59.7% (±4.5% SD) lower FALS in P-limited cells relative to the control. Values did not vary significantly with the age of the culture (Table 1). RALS values were lower in N-limited and higher in P-limited cells in the exponential phase, while no significant difference with the control was observed in the stationary phase. Red fluorescence values were 47.4% (±18.7% SD) lower in exponential N-limited cells and no difference was observed in P-limited cells as compared to the control, while in the stationary phase, nutrient-limited cells showed lower values (51.8 ± 7.0% SD and 30.0 ± 7.3% SD in N- and P-limited cells respectively). Cell viability was not affected by nutrient-limited conditions in the exponential phase, while a higher proportion of SYTOX Green-positive cells was observed in the stationary phase as compared to the control (Table 1).

POC, PON, and particulate organic phosphorus (POP) per cell and the N:P ratio were strongly affected by N- and P-limitation (Table 2) so as Chl *a* content, which was reduced by 79% and 69% in exponential phase, respectively (Table 1). Reduction in Chl *a* content, ratio between organic nutrient content are typical responses of nutrient-limited cells which had undergone metabolic modifications under nutrient stress (for review, Wilhelm et al., 2006).

Total PUAs produced per cell in exponentially growing cultures were slightly higher under P-limitation (1.61 ± 0.59 fmol cell^{-1} SD), as compared to the control (1.23 ± 0.37 fmol cell^{-1} SD), mainly due to an increase in heptadienal, but these differences were not significant (Student t-test, $p > 0.05$). Under N-limitation PUAs produced per cell were significantly lower ($p < 0.001$) due to a decrease of both heptadienal and octadienal (Fig. 2a,b).

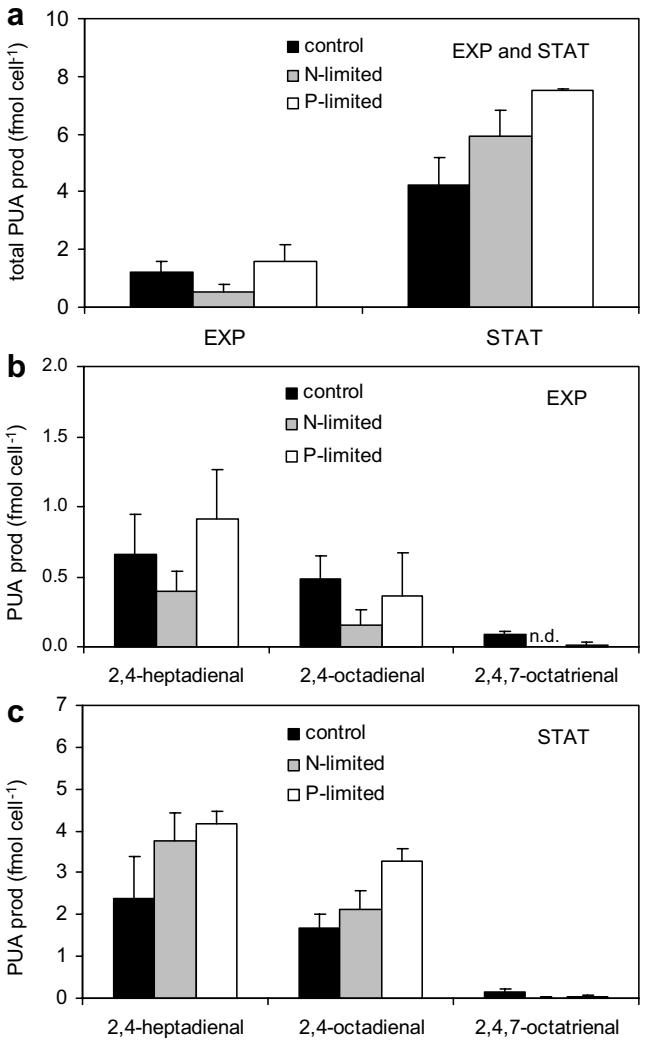

Fig. 2. (a) Total PUA production per cell in exponential (EXP) and stationary (STAT) phases of growth in nutrient-replete (control), N-limited, and P-limited cultures of *S. marinoi*. (b) Composition in terms of different PUAs produced at the three culture conditions in exponential phase of growth, (c) Composition in terms of different PUAs produced at the three culture conditions in stationary phase of growth. Error bars are standard deviations. N. d. not detected.

PUA production increased dramatically when cultures attained the stationary phase, by 10.5 times in N-limited and 4.6 times in P-limited cultures, in comparison to the exponential phase ($p < 0.001$ for both). PUAs produced were as high as 7.49 ± 0.08 fmol cell^{-1} SD in P-limited and 5.90 ± 0.94 fmol cell^{-1} SD in the N-limited cultures (Fig. 2a). The increase in PUAs in cultures in the stationary phase was mainly attributable to an increase in heptadienal (3.8 ± 0.7 fmol cell^{-1} SD and 4.2 ± 0.15 fmol cell^{-1} SD in N- and P-limited cells, respectively) and octadienal production in P-limited cells (3.3 ± 0.1 fmol cell^{-1} SD) (Fig. 2c). Octatrienal was only present in traces in all cultures (Fig. 2b and c).

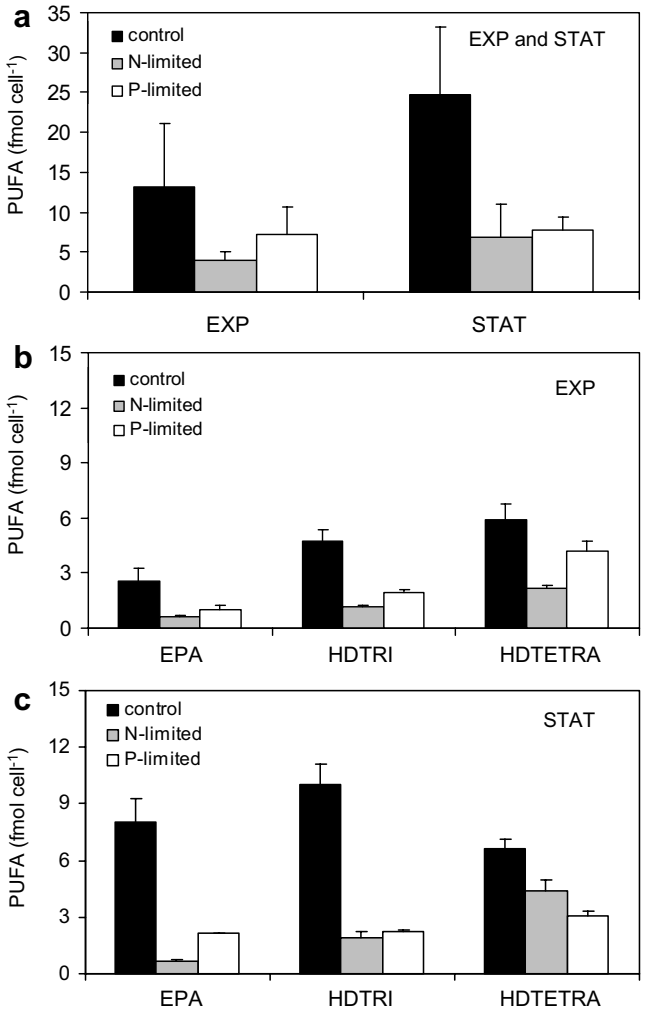

Fig. 3. (a) PUA-precursor PUFAs in exponential (EXP) or stationary (STAT) phases of growth in nutrient-replete (control), N-limited, and P-limited cultures of *S. marinoi*. (b) PUFA relative composition in the exponential, and (c) in the stationary phases of growth. Different PUFAs are indicated in the legend. EPA is eicosapentaenoic acid, HDTRI is hexadecatrienoic acid, and HDTETRA is hexadecatetraenoic acid. For all plots, data are means of replicates from 9 independent experiments for the control and from 3 independent experiments for the nutrient-limited cultures. Error bars are standard deviations.

All differences between average values in the exponential phase and those in the stationary phases of growth were statistically highly significant (Student *t*-test, $p < 0.001$ for heptadienal and octadienal, $p < 0.01$ for octatrienal).

Average content per cell of the three PUA precursor fatty acids was strongly affected by nutrient limitation, being much lower in both N- and P-limited exponential and stationary cultures as compared to the control (Fig. 3a). No increase of these PUFAs occurred between exponential and stationary growth phases in nutrient-limited cultures ($p > 0.1$). N- and P-limited cultures in the stationary phase contained 6.9 ± 4.2 fmol cell^{-1} SD and 7.7 ± 1.7 fmol cell^{-1} SD of the three PUFAs, respectively, as compared to 24.7 ± 8.5 fmol cell^{-1} SD of the control cultures (Fig. 3a). The proportion of the three precursors of aldehydes was similar in exponential N- and P-limited cultures, with hexadecatrienoic and hexadecatetraenoic acids being the dominant fatty acids (Fig. 3b). The trend was similar to the controls in stationary P-limited cultures, with eicosapentaenoic acid increasing and hexadecatrienoic acid decreasing their relative contributions (from 13.7% to 28.8%, and from 58.8% to 43.6%, respectively). The trend was opposite in N-limited cultures, but variations were not significantly different (Fig. 3c, $p > 0.1$).

3. Discussion

The increasing potential for PUA production with age of the culture or under nutrient conditions suggests the endogenous and/or environmental control of diatom toxicity. Culture conditions and bloom phase are thus very important when assessing the effect of a diatom diet upon copepod fitness or reproduction. PUAs have been shown to have teratogenic effects on predators by inducing abortions, malformations and reduced larval growth (Ianora et al., 2004). In a broader survey of different diatom species and isolates in the stationary growth phase, PUA levels ranging from 0.01 fmol cell^{-1} to 9.81 fmol cell^{-1} have been observed in ca. 30% of the investigated diatoms, while 70% lacked these oxylipins (Wichard et al., 2005a). *S. marinoi*, which releases 4.21 fmol cell^{-1} total PUAs during the stationary phase, belongs thus to the group of strong producers hitherto investigated. In the laboratory, most often exponentially growing cultures were used to test for possible teratogenic effects of diatom cells (Poulet et al., 1994 onwards). Our results suggest that these previous lab experiments may therefore have potentially underestimated the impact of diatom PUAs on copepod reproduction, and that the potential number of species that have deleterious effects may be higher.

As a consequence of the results presented here, caution should be taken when PUA production in nature is inferred from culture data as this value may change depending on the culture condition. Laboratory experiments should be consequently carefully designed to avoid an interfering influence of strongly fluctuating levels of PUA.

Interestingly, highest PUA production has been so far observed in some of the most common bloom-forming genera like *Thalassiosira* or *Skeletonema* (Wichard et al., 2005a). During the evolution of a bloom at sea, phytoplankton species such as diatoms are thought to utilize chemical signals or defense metabolites to enhance their resistance against grazers and pathogens. This holds especially true at peak cell concentrations, when the probability to be grazed or infected increases. Several studies have invoked the role of a chemical defense in diatoms based on the production of PUAs to explain the observed impairment of copepod reproduction during blooms (e.g. Miralto et al., 1999b; Ianora et al., 2004). The modulation of PUA production with age of the culture supports the hypothesis of a direct link between toxin production and cell physiological

state that is related to the level and type of nutrient limitation, and also to the previous nutrient conditions that cells experience. If the same happens during blooms, grazers feeding at the end of a bloom would be more affected than early-bloom grazers. This implies that diatom cells at the end of the bloom are better defended chemically against grazers than at the beginning of a bloom, and even more if the bloom is triggered by pulses of nutrients injected in nutrient-depleted surface layers, provided that all other factors are available. If this is an active process, triggered by higher cell densities and a consequently higher need for defense, or if this is simply related to general senescence of cells and production of aldehydes as metabolic byproducts, still needs to be elucidated. PUAs have also been found to be toxic for invertebrates other than copepods (reviewed in Caldwell et al., 2004) and other phytoplankton including diatoms (Casotti et al., 2005; Ribalet et al., in preparation), calling for a putative allelochemical and/or infochemical role in aquatic ecosystems.

PUA production increased with age of the culture in our experiments as cells entered the stationary phase of growth. Berges et al. (2001) predict that phosphate should be the first nutrient to be depleted in the f/2 medium used in this study, which could consequently trigger a drop in EPA per diatom cell (Harrison et al., 1990). This was not our case, as addition of phosphate in control cultures at the onset of the stationary phase did not resume an increase in cell concentration, suggesting that this was not the factor limiting growth. Both polar and neutral lipids have been observed to increase in diatoms under nitrogen or silica deficiency, suggesting that nitrogen deficiency does not affect lipid synthesis directly, but reduces intracellular energy demand on storage lipids by causing cell division to stop (Dempster and Sommerfeld, 1998 and references therein).

Previous work shows that toxin production by phytoplankton species is either constitutive or increases with culture age, related to limitation of different factors (e.g., Legrand et al., 2003). In general, these studies focused on structurally complex toxins, which require pathways involving a multitude of specialized enzymes and energy-rich cofactors such as NADPH or ATP for their biosynthesis. In strong contrast, the wound-activated production of polyunsaturated aldehydes in diatoms does not require energy-rich factors but rather relies on a few key enzymes, which could also be involved in primary metabolism and on the availability of free PUFAs (Pohnert, 2005). Our data suggest that both enzyme expression or activity and availability of precursor PUFAs can be limiting under different experimental conditions. In *S. marinoi* more than 95% of the relevant PUA-precursor fatty acids are bound in phospho- and galactolipids (Berge et al., 1995) and would thus be available to the lipases releasing free fatty acids as substrates for processing to PUAs (Pohnert, 2002; d'Ippolito et al., 2004). In the nutrient-replete cultures, it is unlikely that PUA synthesis was limited by PUFA cell content, since between 5 and 10 fold excess PUFAs were available in the total fatty acid pool, depending on the growth phase (Fig. 1c). A possible explanation for this might be that, with age of the culture, either PUFA release or their further transformation was prevented due to insufficiently active lipases or lipoxygenases. Even given a high variability in triacylglycerol content (1.7–14% of total lipids) in *S. marinoi* (Berge et al., 1995 and citations therein), it can be excluded that PUA-precursor fatty acids were predominantly bound to these inaccessible lipids and were thus not available to the relevant enzymes. The amount of precursor fatty acids and the PUA production do not follow similar patterns during the growth of the culture (Fig. 1). In addition, the relative increase of specific precursor fatty acids (e.g., EPA) does not show a parallel relative increase of the corresponding PUA (e.g., heptadienal) derived from these acids (Fig. 1). It can thus be concluded that it is not the precursor availability but rather the enzyme activity that controls the absolute and the relative amount of PUA produced during normal growth conditions. In contrast, under limiting conditions, the PUFA pool was depleted, while the potential for wound-activated PUA production significantly increased. Thus in this situation a substrate limitation can be assumed (Fig. 3b and c). Apparently, in diatoms under nutrient limitation a larger pool of active enzymes involved in PUA production is available and/or most PUFAs are accessible for the phospho- and galactolipases initiating the transformations.

Chrysophytes in freshwater ecosystems, which can be considered equivalent to marine diatoms for their dominant ecological role and for their ability to produce similar PUAs, show species-specific responses in quantity and quality of PUAs produced as a function of growth phase and nutrient conditions (Watson and Satchwill, 2003). And also in crysophytes no clear link has been observed between PUAs and PUFA content, implying that lipid composition and/or enzyme specificity are both important in PUA chemistry. It is interesting to note that PUA production in both freshwater chrysophytes and marine diatoms is modulated by nutrient stress, suggesting a common mechanism controlling lipid metabolism.

Our data suggest that PUA production is a highly dynamic process, which responds to environmental (e.g. nutrients, but also light or hydrodynamics) or endogenous (e.g. cell cycle, self-regulatory mechanisms) triggers. Decadienal appear to be involved in culture in stress surveillance and signaling for population control in diatoms (Vardi et al., 2006). This system triggers cell death once the signal is propagated among single individuals within a population. If this holds true in the natural environment, nutrient stress may act as an upstream signal for bloom termination, by switching the cell response towards cell death.

4. Conclusions

A strong dependence of PUA production on culture age and N- or P-limitation in cultures of *S. marinoi* has been

observed, suggesting a direct link between toxin production, physiological conditions and nutrient stress. This has direct implications for natural conditions, since nutrient limitation is often, if not always, the factor triggering the end of a bloom at sea (Cullen, 1991). In many coastal areas, where most algal blooms occur, N:P ratios have changed considerably compared to the Redfield ratio due to high and erratic inputs of anthropogenic N and P. Our results show that these changes may strongly affect the production of PUAs and therefore enhance their impact on the trophic food web.

5. Experimental

5.1. Biological material

Axenic cultures of the marine diatom *Skeletonema marinoi* (Sarno and Zingone), strain CCMP 2092 (separated from *S. costatum*), were cultured in 2-l polycarbonate bottles with air bubbling in a growth chamber (Hereaeus). Axenicity was confirmed at the start and the end of every experiment on any replicate by inoculating 1 ml of culture in 0.1% peptone agar in medium. One-month old natural seawater amended with f/2 nutrients (Guillard, 1975) was used as medium. The cultures were maintained at 17 °C on a 12 H light-12H dark cycle under a photon flux density of 150 μmol quanta $m^{-2}\,s^{-1}$ provided by white fluorescent tubes (Phillips TLD 36W/950). Cell concentrations were monitored daily by flow cytometry (FACScalibur, Becton Dickinson), equipped with standard optics (Casotti et al., 2005). Growth rates were calculated as μ (d^{-1}) according to:

$$\mu = (\ln N_1 - \ln N_0)/t \quad (1)$$

where N_0 and N_1 represent cell densities at the start and the end of the growth period, and t the time considered (usually 1 d).

To investigate production of PUAs per cell during growth under standard conditions, several independent batch cultures were sampled in mid-exponential, early stationary, and early declining phases of growth, as assessed from the growth curve (Fig. 1). These phases have been chosen since they represent critical steps in the evolution of an algal culture, being characterized by different dominant cell processes (growth, senescence and mortality) that are known to affect cell metabolism. All samples were taken 6 h after the onset of the light period in order to avoid interference from circadian variability.

To study the effect of nutrient limitation on PUA production, separate cultures in triplicates were grown in f/2 medium with an N:P ratio of 24.5:1 ($NO_3^- = 883$ μM, $PO_4^{3-} = 36$ μM, considered to be nutrient-replete), 4:1 ($NO_3^- = 145$ μM, $PO_4^{3-} = 36$ μM, considered to be nitrogen-limiting), and 80:1 ($NO_3^- = 883$ μM, $PO_4^{3-} = 11$ μM, considered to be phosphorus-limiting). Cultures were grown continuously for at least 6 generations in the limiting medium and then diluted 5 times in the same N- or P-limiting medium and allowed to grow until stationary phase of growth in polycarbonate flasks with air bubbling. Samples were taken at the exponential and at the early stationary growth phases for chemical and flow cytometric analyses. For these experiments, the declining phase was not sampled due to the high fragility of dying cells which easily break during filtration and manipulation, liberating PUAs before addition of the derivatizing agent (see below).

5.2. Cell properties and viability

A Becton-Dickinson FACScalibur flow cytometer equipped with an air-cooled 488 nm argon-ion laser at 15 mW power was used. The sheath fluid was natural seawater filtered onto 0.22 μm polycarbonate filters (Nuclepore). Both the sheath fluid and sample velocities (40 μl min^{-1}) were kept constant during all of the analyses. Optical properties of cells, as measured by flow cytometry in different culture conditions, were compared using forward angle light scatter (FALS) and right angle light scatter (RALS). Red fluorescence (RED) was collected through a 650 long-pass filter and was also used as a proxy for chlorophyll cell content. All values were expressed as units relative to the beads used as internal standards (3.7 μm Coulter FlowSet Fluorospheres). Data acquisition (10^4 cells on average for each sample) and analysis were performed using CellQuest software (Becton-Dickinson). As a trigger signal, RED was used with a threshold at channel 52.

Percentages of viable cells were assessed using the vital stain SYTOX Green (Molecular Probes) using flow cytometry (Casotti et al., 2005). Optimal final concentration (500 nmol L^{-1}) and time of incubation (10 min) were assessed experimentally. The green fluorescence of stained cells was collected through a 530/30-nm bandpass filter. Only SYTOX Green-negative cells were considered for calculation of PUA content, as it was assumed that inactive cells would not be able to activate processes leading to PUA production.

5.3. Analyses of PUA, PUFA, particulate organic nutrients, POC, PON and chlorophyll

The quantification of PUAs was assessed following a slightly modified protocol as described by Wichard et al. (2005b). For each data point, three independent cultures were harvested, each by gentle filtration on Versapore filters of 0.8 μm pore size (Pall Corporation), and resuspended in 2 ml of their original growth medium by carefully pipetting. The resulting cell suspensions, containing approximately 30 million cells, were divided in four parts of equal volume. One subsample was used to determine cell concentrations by flow cytometry. The three additional subsamples were used for further chemical analyses to obtain additional technical replication for each replicate culture. Then 1 ml of the derivatizing agent *O*-2,3,4,5,6-

pentafluorobenzyl hydroxylamine hydrochloride (25 mM PFBHA-HCl in 100 mM Tris/HCl pH 7.0, Fluka) was added to each subsample together with 5 µl of the internal standard (1 mM benzaldehyde in methanol, Fluka). Each subsample was sonicated for 1 min (Branson Sonifier 250) to disrupt cell integrity and to trigger PUA production. The cell fragments were incubated for 30 min at 17 °C, enough time to reach a stationary level of PUA (Pohnert, 2000) and to allow for quantitative derivatization (Wichard et al., 2005b). Samples were immediately frozen in liquid nitrogen and stored at −80 °C until further analysis. For extraction, the frozen subsample was treated with 0.5 ml methanol. After thawing, the oxime-derivatives were extracted with 1 ml hexane and subsequently analyzed using gas-chromatography coupled with mass spectroscopy (Finnigan Trace GC-MS, Thermo Finnigan) according to Wichard et al. (2005b). The limit of quantification was 5 ng mL^{-1} and the coefficient of variation between subsamples originating from the same replicates averaged 12%. The means of the technical replicates were used for the determination of the standard deviation for the repeated measurements with the three independent cultures.

Quantification of the polyunsaturated fatty acids (PUFAs) which have been identified as PUA-precursors in *S. marinoi*: eicosapentaenoic (20:5*n*−3, EPA), hexadecatrienoic (16:3*n*−4, HDTRI), and hexadecatetraenoic acid (16:4*n*−1, HDTETRA), was performed after *trans*-esterification following the procedure of Rodriguez-Ruiz et al. (1998). For replication, three independent cultures were filtered each onto separate GF/F filters (Whatman), frozen in liquid nitrogen and stored at −80 °C until further analysis. Once thawed, the filters were rinsed with 2 ml of the methylation mixture (methanol:acetyl chloride, 20:1 v:v) and vortexed. After addition of [$^2H_{27}$] myristic acid as internal standard (10 µg ml^{-1} final concentration), the sample was sonicated for 10 s (Braun Sonicator 1000 l). Then 1 ml of hexane was added and the samples were sealed and heated for 10 min at 100 °C in pressure resistant glass vials. After re-cooling in an ice bath, 1 ml of distilled water was added and the samples were vortexed for 1 min. The hexane layer was separated and concentrated under an argon stream. Samples were taken up in 50–100 µl hexane and analysed by GC-MS with a EC5 column. The temperature program was 60 °C (1 min), 30 °C min^{-1} to 120 °C, 5 °C min^{-1} to 250 °C, and 20 °C min^{-1} to 300 °C (2 min). The fatty acid methyl esters were identified by comparison with synthetic (HDTETRA, Pohnert et al., 2004), or commercially available standards (EPA). Hexadecatrienoic acid was identified from its mass spectrum. Because of fatty acid-depending response factors, calibration curves were determined for each PUFA. The response factor of hexadecatrienoic acid was assumed to be similar to that of γ-linolenic acid (18:3*n*−6). Ions used for quantification were monitored in the selected ion extraction mode according to Dodds et al. (2005).

Samples for particulate C and N (POC and PON, respectively) from each independent culture were filtered on acidified pre-combusted (450 °C, 24 h) GF/F filters and stored at −80 °C until analysis, using a CHN elemental analyzer (Perkin Elmer 2400). Organic phosphorus (POP) was measured colorimetrically after reaction with a 1% potassium persulfate solution for 1 h at 121 °C (Parsons et al., 1984).

Samples for chlorophyll *a* (Chl *a*) concentrations were filtered from independent cultures through GF/F filters and immediately frozen in liquid nitrogen. Once thawed, filters were mechanically ground in 100% methanol and the extract injected into a Beckman System Gold HPLC as outlined in Casotti et al. (2005).

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