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Feeding deterrence properties of apo-fucoxanthinoids from marine diatoms. II. Physiology of production of apo-fucoxanthinoids by the marine diatoms *Phaeodactylum tricornutum* and *Thalassiosira pseudonana*, and their feeding deterrent effects on the copepod *Tigriopus californicus*

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Abstract The marine diatoms *Phaeodactylum tricornutum* and *Thalassiosira pseudonana* have been shown to produce apo-fucoxanthinoid compounds which act as feeding deterrents against the harpacticoid copepod *Tigriopus californicus*. The amounts and types of apo-fucoxanthinoids produced were species specific. *Th. pseudonana* produced small quantities of apo-12′-fucoxanthinal and apo-13′-fucoxanthinone only during senescence, while *P. tricornutum* produced much greater quantities of these two compounds during both log and senescence phases, in addition to producing a third compound, apo-10-fucoxanthinal, only during senescence. For both species, production of apo-fucoxanthinoids increased as the cells entered senescence phase due to phosphate limitation. The amounts of apo-fucoxanthinoids necessary to reduce feeding in *T. californicus* by 50% ranged from 2.22 to 20.2 ppm. This range was approximately 1000 times lower than the total apo-fucoxanthinoid concentration in *P. tricornutum*. The amounts of apo-fucoxanthinoids necessary to cause a 50% mortality in a population of *T. californicus* ranged from 36.8 to 76.7 ppm. Thus, these compounds are present in concentrations which may have ecological significance in the control of bloom formation and grazing. The production of apo-fucoxanthinoids may be a phenomenon common to many diatoms, particularly as they enter senescence due to nutrient limitation.

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Introduction

Certain marine phytoplankton produce chemical feeding deterrents which reduce or inhibit zooplankton grazing (Targett and Ward 1991). A new bioassay, developed for detecting and measuring feeding deterrent activities in phytoplankton (Shaw et al. 1994), uses the tidepool-dwelling harpacticoid copepod, *Tigriopus californicus*. This bioassay has identified several apo-fucoxanthinoid compounds as responsible for feeding deterrence in the diatom *Phaeodactylum tricornutum* (Shaw et al. 1995), also found in tidepools (Lewin 1958). These compounds are related to the xanthophyll pigment fucoxanthin found in diatoms (Shaw et al. 1995). However, little is known about the factors which affect the production of apo-fucoxanthinoids by *P. tricornutum* or the concentrations of these compounds necessary to produce a feeding deterrent response in a grazer.

Two theories have been proposed to explain the production of feeding deterrents by phytoplankton. Huntley et al. (1986) proposed that feeding deterrents allowed slow growing species, such as dinoflagellates, to attain bloom concentrations. This theory would predict that maximum production of feeding deterrents should occur during the logarithmic phase of phytoplankton growth. A second theory for the production of feeding deterrents suggests that they are produced when the phytoplankton cells are stressed by an environmental limitation, are no longer actively growing, and are therefore susceptible to grazing pressure (Hansen et al. 1992). Using this theory, production of feeding deterrents would be expected to increase during the senescent phase of phytoplankton growth and would be correlated with a limiting growth factor (e.g. nutrient limitation). Thus, the time at which feeding deterrents are produced during the cell growth cycle is important in determining their ecological significance.

The nature of the stresses to which a phytoplankton cell is exposed is also important in controlling the production of these protective compounds. For example, Trick et al. (1984) showed that phosphate-limited cultures of *Prorocentrum minimum* produced twice as much β-diketone as nitrogen-limited cultures. *Alexandrium tamarense* (=*Protogonyaulax tamarensis*) and *Alexandrium* (=*Protogonyaulax*) *catenella* both produced more paralytic shellfish poisoning (PSP) toxins under phosphate limitation (Boyer et al. 1985, 1987). The brown tide organism, *Aureococcus anophagefferens*, was shown to increase its toxicity to the blue mussel, *Mytilus edulis*, under conditions of both high light and high temperature (Tracey et al. 1990), while conditions of low temperature caused *Protogonyaulax catenalla* to increase its total toxin content (Boyer et al. 1985). Therefore, it is important not only to observe when a protective substance is being produced, but the nature of the environmental conditions which may be responsible for this production.

Bioactive compounds produced by phytoplankton, such as the apo-fucoxanthinoids, can have both lethal and sub-lethal (e.g. feeding deterrence) effects on other organisms (Reish and Oshida 1987). In the present study, the production of apo-fucoxanthinoid feeding deterrents by two diatoms, *Phaeodactylum tricornutum* and *Thalassiosira pseudonana*, was measured in batch cultures, where growth was eventually terminated by phosphate starvation. The lethal effects of these compounds were measured by determining the concentration that produced a 50% mortality in the harpacticoid copepod *Tigriopus californicus*, and the sub-lethal effects were determined by measuring the concentration that reduced feeding by 50%.

Materials and methods

Phytoplankton cultures

Cultures of the diatoms *Thalassiosira pseudonana* and *Phaeodactylum tricornutum* were grown and harvested to yield cellular extracts suitable for quantitative bioassays and high performance liquid chromatography (HPLC) analysis. Unialgal cultures and natural sea water were obtained from the same sources as described in Shaw et al. 1995. Sea water used for culturing was filtered through activated charcoal to remove organics (Craigie and McLachlan 1964) and then filtered through a 0.8 μm Millipore filter to remove particulates. The sea water was sterilized by autoclaving. All cultures were grown using full ES enriched natural sea water (Harrison et al. 1980) at 19 °C under continuous irradiance (≈224 μmol m^{-2} s^{-1}), bubbled with air filtered through GF/F filters, and stirred at ≈60 rpm.

Three 12 litre cultures were grown simultaneously. Space limitations in the water-cooled tank used for culturing permitted only three cultures to be grown in a single experiment. Therefore, two replicate *Phaeodactylum tricornutum* cultures and only one *Thalassiosira pseudonana* (control) culture were used.

Culture growth was measured by in vivo fluorescence. Cell densities were determined microscopically. Cell volumes were calculated from microscopic measurements of dimensions of cells preserved in Lugol's iodine solution.

The initial and final culture pH's were measured. Initial and final nitrate and phosphate concentrations were measured using a Technicon Autoanalyzer and standard methods described in Davis et al. (1973).

Extraction and HPLC analysis

Phytoplankton cells were harvested as described in Shaw et al. 1995. The dried cell extract from each sample was dissolved in 7.0 ml of methanol, and 1.0 ml aliquots of this solution were quantitatively transferred to seven glass vials (volume ≈7 ml). These samples were dried under a stream of nitrogen gas and stored at −16 °C. Exposure of samples to light was avoided during these procedures.

The extracellular material was collected by passing the filtrate from the harvested cells through two C-18 sep-paks connected in series. The sep-paks were then rinsed with 20 ml of water, and the retained compounds were eluted with 20 ml of ethyl acetate. The ethyl acetate was evaporated under a stream of nitrogen gas and the dried sample was stored at −16 °C. Exposure of samples to light was avoided during these procedures.

For HPLC analysis, the cellular extract was dissolved in 10 ml of water/methanol (80:20) and applied to a C-18 sep-pak. The sep-pak was rinsed with 20 ml water, and retained pigments were eluted with 10 ml ethyl acetate. The ethyl acetate was evaporated under a stream of nitrogen gas. Just prior to injection on the HPLC column, the sample was redissolved in 0.8 ml of methanol and 0.2 ml of water. The extracellular extract was processed in the same manner.

The HPLC method was modified from Wright et al. (1991). The solvent system was as follows: Solvent A consisted of 80:20 methanol/0.5 *M* ammonium acetate (aqueous; pH 7.2 v/v); Solvent B consisted of 90:10 acetonitrile (210 nm ultraviolet cut-off grade) /water (v/v); Solvent C consisted of ethyl acetate (HPLC grade). The HPLC was operated in gradient mode. The program used is shown in Table 1. The Water's HPLC system used was equipped with a photodiode array (PDA) detector and an Econsil C-18, 5 μm (length = 250 mm, i.d. = 4.6 mm) reversed phase column. The injection volume for all samples was 25 μl. Data was processed using Water's Millenium software. Pure fucoxanthin and semi-synthetic apo-10′-fucoxanthinal, apo-12′-fucoxanthinal, apo-12-fucoxanthinal, and apo-13′-fucoxanthinone were used to calibrate this new system (Table 2).

The total intracellular apo-fucoxanthinoid concentration was calculated and expressed in terms of relative intracellular apo-12′-fucoxanthinal concentration, using the equation:

$$Cc = C1 + a \times C2 + b \times C3, \quad (1)$$

where Cc = total relative intracellular apo-12′-fucoxanthinal concentration (gl^{-1} cell volume); C1 = intracellular apo-12′-fucoxanthinal concentration (gl^{-1} cell volume); C2 = intracellular

Table 1 Program for the high-performance liquid chromatography (HPLC) solvent system gradient used for quantifying apo-fucoxanthinoids

Time (min)	Flow rate (ml min^{-1})	% A	% B	% C	Conditions
0	1.0	100	0	0	Injection
4	1.0	0	100	0	Linear gradient
18	1.0	0	20	80	Linear gradient
21	1.0	0	100	0	Linear gradient
24	1.0	100	0	0	Linear gradient
35	1.0	100	0	0	Equilibration
36	0	100	0	0	Shut down; Linear gradient

Table 2 Data on apo-fucoxanthinoid standards for quantitative HPLC assays

Compound	Retention time (min)		Observed peaks (nm)	Wavelength for analysis (nm)
	major peak	shoulder		
Apo-10′-fucoxanthinal	9.05	9.58	424, 445	418
Apo-12′-fucoxanthinal	7.71	7.31	395, 418	393
Apo-10-fucoxanthinal	13.2	–	439	439
Apo-12-fucoxanthinal	13.0	14.0	417	407
Apo-13′-fucoxanthinone	5.25	–	333	329
Fucoxanthin	14.8	–	467	446

apo-13′-fucoxanthinone concentration ($g l^{-1}$ cell volume); C3 = intracellular apo-10-fucoxanthinal concentration ($g l^{-1}$ cell volume); a = (IC_{50} for apo-12′-fucoxanthinal)/(IC_{50} for apo-13′-fucoxanthinone); b = (IC_{50} for apo-12′-fucoxanthinal)/(IC_{50} for apo-12-fucoxanthinal). Since there was no pure semi-synthetic apo-10-fucoxanthinal available for the IC_{50} bioassays, the IC_{50} value for apo-12-fucoxanthinal (a very similar compound) was substituted.

In order to determine if apo-fucoxanthinoids were produced abiotically under typical culture conditions, or if they were produced as artifacts during sample preparation for HPLC analysis, fucoxanthin was incubated in the presence of light and oxygen, and incubated samples were prepared for HPLC as described above. Fucoxanthin was isolated by the method described by Haugen and Liaaen-Jensen (1989) from *Fucus distichus* collected from Copper Cove, West Vancouver, British Columbia in March. A total of 28 mg of pure fucoxanthin was suspended in 500 ml of ES enriched (Harrison et al. 1980) natural sea water. This mixture was incubated in a 1 litre flask for 6.75 d at 19 °C with continuous irradiance of ≈ 224 μmol m^{-2} s^{-1}. The solution was vigorously bubbled with air filtered through a GF/F filter and stirred at 60 rpm.

Four times over the course of the incubation, 50 ml of solution was removed from the flask and passed through a C-18 sep-pak. The sep-pak was rinsed with 20 ml of water, and retained compounds were eluted with 20 ml of ethyl acetate, which was then evaporated under a stream of nitrogen gas. The dried sample was stored at −16 °C. Exposure of samples to light was avoided during these procedures. Just prior to injection on the HPLC column, the sample was redissolved in 0.8 ml of methanol and 0.2 ml of water. The HPLC system used for analysis of these samples was the same as the one described above.

Copepod cultures

A culture of the harpacticoid copepod *Tigriopus californicus* was maintained to provide a continuous supply of organisms for the bioassay as described in Shaw et al. 1995. Copepods used for bioassay were fed exponentially growing cultures of *Thalassiosira pseudonana*, which was used as a control in the feeding deterrent experiments.

Bioassay procedure

Solutions of compounds and crude extracts to be quantitatively bioassayed were prepared as follows. A weighed amount of the compound or extract was dissolved in 1 ml of autoclaved sea water and sonicated (1 min in bath sonicator at 60 Hz) to facilitate mixing. Five ml of an exponentially growing culture of the diatom *Thalassiosira pseudonana* was added to the dissolved material. Clumps were removed from the cell culture prior to use in the bioassay by filtration (forced through a 200 μm screen at a rate of ≈ 50 ml min^{-1} using a syringe; repeated four times).

This mixture was placed in a glass vial (volume ≈ 7 ml and five male C6 copepods (*Tigriopus californicus*) were added. Copepods were preconditioned in autoclaved sea water without food for 24 h prior to the bioassay. The vial was stoppered loosely with cotton wool and placed in a wire rack. The assay was incubated for 24 h at 18 °C with an irradiance of ≈ 100 μmol m^{-2} s^{-1} and a 18 h light:6 h dark cycle. Although the food organism used in this bioassay (*Thalassiosira pseudonana*) was grown under continuous light prior to the bioassay, it was felt that any physiological changes in this culture as a result of the transition to a light:dark cycle which might affect copepod feeding would be small compared to the effects produced by the addition of a feeding deterrent compound to the bioassay mixture. At the end of 24 h, the number of live copepods and the number of fecal pellets in each sample were enumerated.

Semi-synthetic apo-fucoxanthinoid compounds were produced from fucoxanthin as described by Shaw et al. (1995). Two separate bioassay experiments were run as follows. Experiment #1 included apo-10′-fucoxanthinal at concentrations of 0.5, 2, 6, 25, and 100 ppm; apo-12′-fucoxanthinal at concentrations of 0.6, 2, 7, 28, and 113 ppm; and five controls (0 ppm). Experiment #2 included apo-12-fucoxanthinal at concentrations of 0.1, 0.4, 2, 6, and 25 ppm; apo-13′-fucoxanthinone at concentrations of 0.1, 0.4, 2, 6, and 25 ppm; and five controls (0 ppm).

Fecal pellet production rate (pellets h^{-1} $copepod^{-1}$) was calculated from the fecal pellet count for each sample. The IC_{50} value (value at which feeding is inhibited by 50%) was calculated from the data set for each apo-fucoxanthinoid compound using two methods, the classical PROBIT analysis and curve fitting analysis. A curve was fit to the data set generated from the bioassays of each of the four apo-fucoxanthinoids using the program Sigmaplot 5.0. These curves were of the general equation:

$$F = F_m \times e^{-kc^a}, \quad (2)$$

where c = the concentration of the feeding deterrent (ppm); F = fecal pellet production rate (pellets h^{-1}); F_m = the maximum fecal pellet production rate (pellets h^{-1}) determined by measuring the average fecal pellet production rate for the control (c = 0); k, a = constants. The IC_{50} value was then calculated by setting $F = 0.5 \times F_m$. The IC_{50} values of the apo-fucoxanthinoids were tested for significant differences using a two-tailed *t*-test.

The survivorship was also measured for each sample and a curve was fit to each of the apo-fucoxanthinoid data sets as described above using the general equation:

$$S = 100 \times e^{-kc^a}, \quad (3)$$

where c = the concentration of the feeding deterrent (ppm); S = percentage of subjects which survived at the end of 24 h; k, a = constants. The LC_{50} value was then calculated by setting S = 50. The LC_{50} values of the apo-fucoxanthinoids were tested for significant differences using a two-tailed *t*-test.

The samples of crude cell extracts were quantitatively bioassayed as described above with the following changes. Only two male C6

copepods were added to the bioassay mixture in each vial. Six replicates were done for each culture sample. The assay was incubated for 23 to 23.5 h at 18 °C with an irradiance of $\approx 100\ \mu mol\ m^{-2}\ s^{-1}$ and a 18 h light:6 h dark cycle.

The total feeding deterrent activity in the cell extracts was expressed as total intracellular apo-fucoxanthinoid concentration (in terms of relative intracellular apo-12′-fucoxanthinal concentration) using the following equations:

$$Cb = \{[\ln(Ft/Fc)]/ - 0.4456\}^{2.569} \quad (4)$$

$$Cc = (4.2E9 \times Cb)/(D \times S \times V), \quad (5)$$

where Cb = total relative intracellular apo-12′-fucoxanthinal concentration in bioassay medium ($g\ l^{-1}$); Ft = fecal pellet rate of sample (pellets h^{-1}); Fc = fecal pellet rate of control (pellets h^{-1}); Cc = total relative intracellular apo-12′-fucoxanthinal concentration ($g\ l^{-1}$ cell volume); D = cell density of the culture (cells ml^{-1}); S = volume of culture used for sample (ml); V = cell volume (fL $cell^{-1}$). The equation for Cb was derived from the curve fitting analysis used to calculate the IC_{50} value for apo-12′-fucoxanthinal. The value 4.2E9 is a dilution factor. The values of Cb were tested for significant differences using a one-tailed *t*-test.

Results

Determination of IC_{50} values of apo-fucoxanthinoids

Four pure semi-synthetic apo-fucoxanthinoid compounds were tested for their feeding deterrent activity against *Tigriopus californicus*: apo-10′-fucoxanthinal, apo-12′-fucoxanthinal, apo-12-fucoxanthinal, and apo-13′-fucoxanthinone. The IC_{50} value (value at which feeding is inhibited by 50%) was calculated for each apo-fucoxanthinoid compound using both PROBIT analysis and curve fitting analysis. Both methods generated the same IC_{50} value; however, the curve fitting analysis also generated an equation which could later be used for estimating the amounts of apo-fucoxanthinoids in crude samples. Therefore, only the results from the curve fitting analysis are presented here (Table 3). At $\alpha = 0.001$, apo-13′-fucoxanthinone had a significantly higher IC_{50} value than apo-12-fucoxanthinal or apo-12′-fucoxanthinal. At $\alpha = 0.1$, apo-13′-fucoxanthinone had a significantly higher IC_{50} value than all the other three apo-fucoxanthinoids. The IC_{50} values of apo-10′-, apo-12′-, and apo-12-fucoxanthinal were not significantly different at $\alpha = 0.001$; however, at $\alpha = 0.1$, apo-12-fucoxanthinal had a higher IC_{50} than apo-12′-fucoxanthinal.

Determination of LC_{50} values of apo-fucoxanthinoids

Two semi-synthetic apo-fucoxanthinoid compounds were tested for lethality against *Tigriopus californicus*: apo-10′-fucoxanthinal and apo-12′-fucoxanthinal. There was not enough semi-synthetic apo-10-fucoxanthinal or apo-13′-fucoxanthinone available to perform LC_{50} analyses. The LC_{50} value (value at which there is 50% mortality) was calculated in the same manner as for the IC_{50} value. The results from the curve fitting analyses are given in Table 4. At $\alpha = 0.01$, apo-10′-fucoxanthinal had a significantly higher LC_{50} value than apo-12′-fucoxanthinal.

Detection of apo-fucoxanthinoids using HPLC

Using an HPLC method developed for detecting and quantifying apo-fucoxanthinoid compounds (based on the system used by Wright et al. 1991), crude cellular extracts from *Phaeodactylum tricornutum* were examined. This HPLC analysis led to the discovery of another apo-fucoxanthinoid, identified as apo-10-fucoxanthinal based on its elution time on HPLC and its spectral characteristics. As no pure semi-synthetic apo-10-fucoxanthinal was available, the HPLC could not be calibrated for quantitative analysis of this compound. However, apo-10-fucoxanthinal is very similar to apo-12-fucoxanthinal, differing only in having a chain length which is two carbon units longer. Based on this similarity, quantitative amounts of apo-10-fucoxanthinal were estimated using the calibration from apo-12-fucoxanthinal.

Growth of phytoplankton cultures for apo-fucoxanthinoid analysis

Three 12 litre batch cultures, one culture of *Thalassiosira pseudonana* and two cultures of *Phaeodactylum tricornutum*, were grown. The cultures were sampled (4 litre sample size) at approximately 2 d after inoculation and again (3 litre sample size) at approximately 6 d after inoculation for apo-fucoxanthinoid analysis. The growth data for these three cultures are given in Table 5. Although cultures #2 and #3 were intended to be replicate *P. tricornutum* cultures, culture #2

Table 3 Constants and IC_{50} values from the equation $F = F_m \times e^{-kc^a}$ when fitted to the data sets for each of the four apo-fucoxanthinoid compounds

Compound	F_m	k	a	IC_{50} (ppm)	±1 SD range
Apo-10′- fucoxanthinal	1.09	0.593	0.197	2.22	0.198–18.3
Apo-12′-fucoxanthinal	1.09	0.445	0.390	3.11	1.36–6.63
Apo-12-fucoxanthinal	1.76	0.122	0.914	6.69	6.24–7.17
Apo-13′-fucoxanthinone	1.76	1.30E-6	4.39	20.2	18.7–21.7

had a slightly faster growth rate than culture #3, and entered senescence slightly earlier.

The three cultures did not appear to be carbon-limited, as indicated by culture pH, which did not change throughout the culture growth. The N:P ratios at the end of the experiment (Day 6) were as follows: culture #1 = 111; culture #2 = 3830; culture #3 = 493. Wynne and Rhee (1986) determined the cellular N:P ratio to be 33:1 for *Thalassiosira pseudonana* and 37:1 for *Phaeodactylum tricornutum*. Thus all three cultures were phosphate-starved in senescence.

Table 4 Constants and LC_{50} values from the equation $S = 100 \times e^{-kc^a}$ when fitted to the data sets for each of the two apo-fucoxanthinoid compounds

Compound	k	a	LC_{50} (ppm)	±1 SD range
Apo-10′-fucoxanthinal	0.00661	1.07	76.7	64.2–90.8
Apo-12′-fucoxanthinal	2.30E-7	4.14	36.8	30.6–42.8

Table 5 *Thalassiosira pseudonana* and *Phaeodactylum tricornutum* calculated growth constants for batch cultures

Species, culture	Growth rate, μ (d)	Time at which cultures entered senescence (d)
Th. pseudonana, culture #1	1.47	2.5
P. tricornutum, culture #2	1.34	2.5
P. tricornutum, culture #3	1.38	2.7

The average cell volume was calculated from the cell dimensions measured microscopically. The cell volume was 160 μm^3 for *Thalassiosira pseudonana* and 37 μm^3 for *Phaeodactylum tricornutum*.

HPLC analysis of apo-fucoxanthinoid production

The intracellular production and extracellular release of apo-fucoxanthinoids and fucoxanthin by the diatoms *Thalassiosira pseudonana* and *Phaeodactylum tricornutum* during both log (Day 2) and senescence (Day 6) phase were studied using HPLC (Fig. 1). As the present study was preliminary, with $n = 2$ for *Phaeodactylum tricornutum* and no replicates for the control, *Thalassiosira pseudonana* (due to culture tank space restrictions), results are simply expressed as ranges. Nine general observations were made (Fig. 1). (1) No apo-10′-fucoxanthinal or apo-12-fucoxanthinal was present either intracellularly or extracellularly in any of the samples. (2) No apo-fucoxanthinoids were detected extracellularly. (3) The intracellular apo-10-fucoxanthinal concentration in senescent *P. tricornutum* cells ranged from 4.97 to 9.38 $g l^{-1}$ and was much greater than all other apo-fucoxanthinoid concentrations in either log or senescent phase *P. tricornutum* or *Th. pseudonana* cells. (4) In log phase *P. tricornutum* cells, the intracellular apo-13′-fucoxanthinone concentration ranged from 0.0342 to 0.0229 $g l^{-1}$ and was greater than in all other samples. (5) In *P. tricornutum*, the intracellular fucoxanthin concentration was higher than any of the apo-fucoxanthinoid concentrations during log phase (ranging from 8.57 to 8.58 $g l^{-1}$), but was in the same concentration range as apo-10-fucoxanthinal during senescence (6.69 to 6.73 $g l^{-1}$ for fucoxanthin compared with 4.97 to 9.38 $g l^{-1}$ for apo-10-fucoxanthinal). (6) The intracellular fucoxanthin

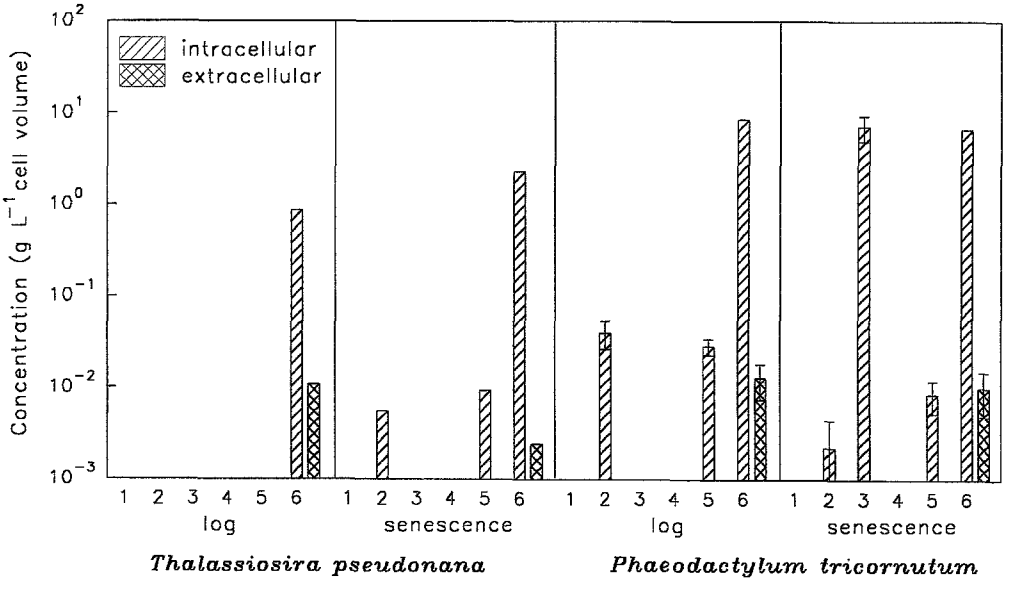

Fig. 1 Intracellular and extracellular concentrations of apo-fucoxanthinoids and fucoxanthin as calculated from high performance liquid chromatography (HPLC) analysis. The concentration axis is in log units to allow both fucoxanthin and the apo-fucoxanthinoids to be shown in the same plot. Compounds are numbered as follows: *1* apo-10′-fucoxanthinal; *2* apo-12′-fucoxanthinal; *3* apo-10-fucoxanthinal; *4* apo-12-fucoxanthinal; *5* apo-13′-fucoxanthinone; and *6* fucoxanthin. *Error bars* represent ranges around the average

concentration in *P. tricornutum* was lower in senescent cells (6.69 to 6.73 $g l^{-1}$) than in log phase cells (8.57 to 8.58 $g l^{-1}$). (7) The intracellular fucoxanthin concentrations in both log and senescent phase *P. tricornutum* cells (8.57 to 8.58 $g l^{-1}$ for log phase and 6.69 to 6.73 $g l^{-1}$ for senescent phase) were higher than in the corresponding log and senescent phase cells of *Th. pseudonana* (0.860 $g l^{-1}$ for log phase and 2.30 $g l^{-1}$ for senescent phase). (8) There were no apparent trends, either by species or by growth phase, in the amounts of fucoxanthin released per unit cell volume. (9) The amount of fucoxanthin released per unit cell volume (0.00751 to 0.0183 $g l^{-1}$ for log phase and 0.00487 to 0.0151 $g l^{-1}$ for senescent phase) was much less than the total intracellular fucoxanthin (8.57 to 8.58 $g l^{-1}$ for log phase and 6.69 to 6.73 $g l^{-1}$ for senescent phase) for *P. tricornutum*.

The total intracellular apo-fucoxanthinoid concentration for each individual sample is shown in Fig. 2. Averaging the replicate *Phaeodactylum tricornutum* cultures, the values (in $g l^{-1}$ cell volume) are as follows: log phase *Thalassiosira pseudonana* = 0; senescent phase *Th. pseudonana* = 0.00698; log phase *P. tricornutum* = 0.0442 (range 0.0298 to 0.0585); senescent phase *P. tricornutum* = 3.34 (range 2.31 to 4.37). Two general observations were made from these data. (1) The total intracellular apo-fucoxanthinoid concentration from senescent phase *P. tricornutum* cells was greater than the total intracellular concentrations from either *Th. pseudonana* cells (log or senescent phases) or log phase *P. tricornutum* cells. (2) The total intracellular concentration from log phase *P. tricornutum* cells was greater than the total intracellular concentration from log phase *Th. pseudonana* cells.

Apo-fucoxanthinoids did not appear to be produced abiotically under the culturing conditions used in these experiments or as artifacts during sample preparation for HPLC analysis. Fucoxanthin samples incubated in sea water in the presence of light and oxygen contained no detectable apo-fucoxanthinoids when analyzed by HPLC. The total fucoxanthin concentration decreased from 0.056 $g l^{-1}$ at $t = 0$ to 0.043 $g l^{-1}$ at $t = 6.75$ d. A number of unidentified compounds, which were clearly not any of the five apo-fucoxanthinoids identified as feeding deterrents, did increase over the duration of the experiment.

Analysis of apo-fucoxanthinoid production using quantitative bioassay

The results from the quantitative bioassay for feeding deterrent activity in crude cellular extracts from the diatoms *Thalassiosira pseudonana* and *Phaeodactylum tricornutum* are shown in Fig. 2. All the samples were significantly different ($\alpha < 0.05$) except for the following pairs: *Th. pseudonana* (culture #1) Day 6 and *P. tricornutum* (culture #3) Day 2; *P. tricornutum* (culture #2) Day 2 and *P. tricornutum* (culture #2) Day 6; *P. tricornutum* (culture #2) Day 2 and *P. tricornutum* (culture #3) Day 6; *P. tricornutum* (culture #2) Day 6 and *P. tricornutum* (culture #3) Day 6.

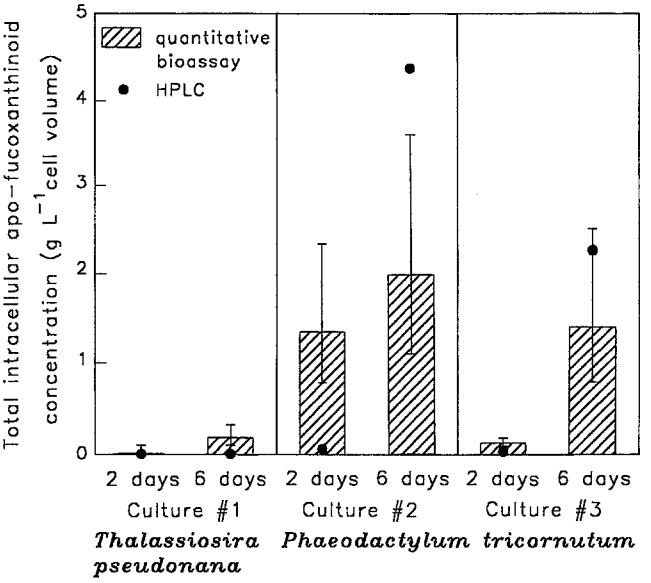

Fig. 2 Total intracellular apo-fucoxanthinoid concentration calculated relative to apo-12′-fucoxanthinal from both quantitative bioassay and HPLC results. *Hatched bars* are values calculated from the quantitative bioassay results and *filled circles* are values calculated from the HPLC results. The measurement at Day 2 is in log phase growth and the measurement at Day 6 is in senescence phase. For the quantitative bioassay results, $n = 6$, and for the HPLC results, $n = 1$. *Error bars* represent $\pm$ 1 SD

On Day 2, cultures #1 and #3 were still in log phase growth, while culture #2 had entered senescence. Thus, the maximum intracellular apo-fucoxanthinoid concentration was produced in the *Phaeodactylum tricornutum* culture #2 during both Days 2 and 6 and the *P. tricornutum* culture #3 during Day 6. The average intracellular apo-fucoxanthinoid concentration for senescent *P. tricornutum* cells (Day 6, cultures #2 and #3) was 2.03 $g l^{-1}$ ($\pm$ 1 SD = 0.491). This value was not significantly different ($\alpha = 0.45$) from the average value 3.34 $g l^{-1}$ measured by HPLC.

The total intracellular apo-fucoxanthinoid concentration from senescence phase *Phaeodactylum tricornutum* cells was significantly ($\alpha = 0.05$) greater than the apo-fucoxanthinoid concentration from senescence phase *Thalassiosira pseudonana* cells. The intracellular apo-fucoxanthinoid concentration from log phase *P. tricornutum* cells was also significantly ($\alpha = 0.05$) greater than the apo-fucoxanthinoid concentration from log phase *Th. pseudonana* cells. In the *P. tricornutum* culture #3, the intracellular apo-fucoxanthinoid concentration from the senescence phase cells was significantly ($\alpha = 0.05$) greater than the apo-fucoxanthinoid concentration from log phase cells. The intracellular apo-fucoxanthinoid concentration from senescence phase *Th. pseudonana* cells was greater ($\alpha = 0.05$) than the

apo-fucoxanthinoid concentration from log phase *Th. pseudonana* cells. The total intracellular apo-fucoxanthinoid concentration measured by HPLC for the individual samples followed the same trend as the bioassay results, with some variations.

Discussion

Many species of marine phytoplankton have been shown to reduce or completely inhibit feeding in various zooplankton (Ali 1970; Chotiyaputta and Hirayama 1978; Huntley 1982; Verity and Stoecker 1982; Egloff 1986; Huntley et al. 1986; Sykes and Huntley 1987; Ward and Targett 1989; Uye and Takamatsu 1990). Recently, a new bioassay for feeding deterrents has facilitated the isolation of feeding deterrent compounds from the marine diatom *Phaeodactylum tricornutum* (Shaw et al. 1994). These compounds have been structurally characterized as apo-fucoxanthinoids, compounds related to the xanthophyll pigment fucoxanthin found in diatoms (Shaw et al. 1995). However, little was known about the specific activity and production of these compounds.

IC_{50} and LC_{50} values of apo-fucoxanthinoids

The IC_{50} values of four apo-fucoxanthinoid compounds were determined using the copepod *Tigriopus californicus*. These values ranged from 2.22 to 20.2 ppm. Of the four compounds tested, apo-13′-fucoxanthinone had the highest IC_{50} (lowest feeding deterrent activity). Apo-13′-fucoxanthinone is a ketone, while the other three apo-fucoxanthinoids tested are aldehydes. These results suggest that the structures of the feeding deterrent compounds may be related to their activity. There is a need for further study of the biological activity of apo-fucoxanthinoids to determine the precise nature of this relationship. All four apo-fucoxanthinoids were active at very low levels, with IC_{50} values 1000 times lower than the total intracellular apo-fucoxanthinoid concentrations.

The LC_{50} values of two apo-funcoxanthinoid compounds were also determined using the copepod *Tigriopus californicus*. The LC_{50} values ranged from 36.8 to 76.7 ppm. Apo-10′-fucoxanthinal had a higher LC_{50} than apo-12′-fucoxanthinal.

Production of apo-fucoxanthinoids by two diatoms

A preliminary study of intracellular production and extracellular release of apo-fucoxanthinoids and fucoxanthin by the diatoms *Thalassiosira pseudonana* and *Phaeodactylum tricornutum* during both log and senescence phase using HPLC produced a number of interesting results. Although apo-10′-fucoxanthinal and apo-12-fucoxanthinal had been previously isolated from *P. tricornutum* (Shaw et al. 1995), none of the cultures in this experiment contained these compounds, either intracellularly or extracellularly. A possible explanation for this is that the large scale cultures used to produce material for isolation and structural determination of the apo-fucoxanthinoids were grown under a light:dark cycle, while the cultures in this experiment were grown under continuous irradiance. It is quite likely that the particular "suite" of apo-fucoxanthinoids produced by *P. tricornutum* may vary significantly with the culture conditions. This is supported by the fact that a fifth compound, apo-10-fucoxanthinal, was present in very large quantities, but had not been isolated in previous work on *P. tricornutum* grown in large scale batch cultures.

No apo-fucoxanthinoids were detected by HPLC in the extracellular medium. Apparently, apo-fucoxanthinoids are not secreted into the medium by the phytoplankton cells tested. A small amount of fucoxanthin was present in the extracellular medium, probably from cell death and lysis. This fucoxanthin was not oxidatively cleaved in the presence of light and oxygen to form the known apo-fucoxanthinoid feeding deterrents. Even when present in very high concentrations ($0.056\ g l^{-1}$) in the fucoxanthin incubation experiment, fucoxanthin did not degrade to form this suite of apo-fucoxanthinoids. These negative results also indicated that the HPLC methodology was not producing apo-fucoxanthinoids as artifacts.

An unexpected result of the HPLC analysis was the presence of apo-fucoxanthinoids in the diatom *Thalassiosira pseudonana*. Previous research (Shaw et al. 1994) showed that *Th. pseudonana* had no feeding deterrent activity, and thus it was selected as the control for this set of experiments. Even though the copepods had been preconditioned by feeding with log phase *Th. pseudonana* cells, quantitative bioassays indicated that cell extracts from senescent phase *Th. pseudonana* cells did produce a feeding deterrent response. This result was further supported by the presence of apo-fucoxanthinoids as detected by HPLC. However, log phase *Th. pseudonana* cells contained no detectable apo-fucoxanthinoids. In the previous studies using *Th. pseudonana*, cells were bioassayed in late log phase, and this explains why the bioassay results were negative. The production of apo-fucoxanthinoids may be a phenomenon common to many diatoms, with the level of production and the specific suite of compounds being dependent on the species (perhaps species-specific enzymes which have variable levels of activity). The preliminary experiments in the present study indicated that *Th. pseudonana* has lower intracellular apo-fucoxanthinoid concentrations than *Phaeodactylum tricornutum*.

Both diatom species appeared to have increased total apo-fucoxanthinoid production during senescence.

This production may be a function of cessation of active growth and the start of cell aging. Thus, this process might be analogous to the increase in carotenoid degradation products in aging tissues (e.g. leaves) of higher plants (Gross 1991). The cultures entered senescence due to phosphate limitation. An increase in feeding deterrents under phosphate limitation would agree with observed increases in other biologically active compounds in phytoplankton under phosphate limitation (Trick et al. 1984; Boyer et al. 1985, 1987; Carlsson 1990; Reguera and Oshima 1990). From an ecological viewpoint, the production of feeding deterrent compounds during senescence should prevent overgrazing of the phytoplankton population during a period of time when growth rate is less than grazing rate.

The average total intracellular apo-fucoxanthinoid concentration for *Phaeodactylum tricornutum* calculated from the HPLC analysis was in the same range as the average total intracellular apo-fucoxanthinoid concentration calculated from the quantitative bioassay. Thus, there is reasonable agreement between the total feeding deterrent activity and the amounts of the apo-fucoxanthinoids present in the cell which are responsible for this activity. However, a closer look at the results for the individual samples shows that although both analyses yield the same trend, there are some variations (Fig. 2). These variations may be a result of: (1) small inaccuracies in the measurement of the relative activities of the apo-fucoxanthinoids in the IC_{50} experiments or of the activities of the crude cellular extracts; (2) the presence of additional feeding deterrent compounds (apo-fucoxanthinoids or other unidentified compounds) not measured by the HPLC method; (3) altered solubility behavior of the active compounds in crude extracts; or (4) synergistic effects between the apo-fucoxanthinoids or with other compounds in the crude extract.

Ecological and commercial significance of apo-fucoxanthinoid production

Based on the above conclusions, several general statements can be made. It appears that some aging diatom cells produce apo-fucoxanthinoid compounds intracellularly. Certain diatoms produce more of these apo-fucoxanthinoid compounds than others. In *Phaeodactylum tricornutum*, the intracellular concentration of these apo-fucoxanthinoids is high enough to act as a feeding deterrent for grazers when the cells are ingested.

Apo-fucoxanthinoids may be released into the "phycosphere" surrounding the cell or be bound onto the surface of the cell. Compounds closely associated with the cell surface would not be distinguishable from intracellular compounds using the methods developed in this research. However, contact chemoreception by grazers, followed by subsequent particle rejection could reduce grazing of the phytoplankton cells without ingestion of the cells. Thus predation could be deterred without sacrificing individual cells (Targett and Ward 1991).

The bioassay organism, *Tigriopus californicus*, is a very hardy, tidepool-dwelling harpacticoid copepod. It is not commonly associated with phytoplankton blooms; however, the diatom *Phaeodactylum tricornutum* is a benthic diatom often found in tidepools. If a deterrent compound produces a response in a hardy organism, it is likely to have a much stronger effect on more sensitive organisms. The effects of apo-fucoxanthinoids on other species of zooplankton need to be examined further. However, if some species are more sensitive to apo-fucoxanthinoids than *T. californicus*, then low levels of apo-fucoxanthinoids may have a significant impact on some grazers in the natural environment.

Although *Phaeodactylum tricornutum* is not an important bloom-forming species in the marine environment, this research suggests that the production of apo-fucoxanthinoid compounds may be common to many diatoms, and may be of ecological significance in natural blooms of diatoms entering senescence. Diatoms which produce larger amounts of apo-fucoxanthinoids may have a competitive edge in certain ecological situations (e.g. under conditions of nutrient limitation when grazing pressures are high). If the presence of these apo-fucoxanthinoid feeding deterrents causes grazers to selectively prey on certain phytoplankton species and not others, then the production of apo-fucoxanthinoids may play an important role in phytoplankton species succession.

The results of this research may be of commercial value. In mariculture, where phytoplankton are used as a food source for organisms such as oyster larvae, it is important to know which species of phytoplankton are good food sources. This work clearly indicates that *Phaeodactylum tricornutum* is a poor food choice for some species. As well, any diatom cultures entering senescence may be of reduced value as a food source if they produce apo-fucoxanthinoids. Attempting to grow commercially important invertebrates using phytoplankton which produce feeding deterrents would lead to decreased growth rates and increased mortality in the mariculture species, which would reduce the net profit of the "crop". A better understanding of which species produce feeding deterrents and what factors affect the production of these feeding deterrents would improve culturing practices in mariculture.

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