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ALLOXANTHIN IN DINOPHYSIS NORVEGICA (DINOPHYSIALES, DINOPHYCEAE) FROM THE BALTIC SEA[1]

Bettina Meyer-Harms[2] *and Falk Pollehne*[3]

Institut für Ostseeforschung, Sektion Biologische Meereskunde, Seestr. 15, D-18119 Rostock, Germany

ABSTRACT

The pigment composition of Dinophysis norvegica *Ehrenberg from the central Baltic Sea differs from the normal pigment pattern found in dinoflagellates, which contain peridinin as a typical marker pigment. In* D. norvegica *isolated by cell fractionation of field samples, the major carotenoid was alloxanthin, a typical cryptomonad pigment. No evidence was found that the presence of alloxanthin was due to a recent phagotrophic uptake of cryptomonads, so the presence of alloxanthin in* D. norvegica *may be a consistent feature of a permanent endosymbiosis.*

Key index words: alloxanthin; Baltic Sea; Dinophysis norvegica; *HPLC; phytoplankton pigments*

Dinophysis norvegica is a large dinoflagellate of about 45 × 70 μm, which may cause diarrhetic shellfish poisoning in humans (Sechet et al. 1990, Rao et al. 1993). It is common in northern European marine waters during summer (Drebes 1974) and was the predominant dinoflagellate species in the central Baltic Sea during two cruises in June/July 1993 and 1994. Population maxima occurred beneath surface blooms of cyanobacteria (*Anabaena* sp., *Aphanizomenon flos aqua*) at depths of 10–15 m and 18–25 m, in 1993 and 1994, respectively. *Dinophysis norvegica* cooccurred with *Prorocentrum minimum* in 1993 and with *Gymnodinium simplex* in 1994. The maximum distribution of *D. norvegica* was noted as a peak in the chlorophyll (chl) *a* concentration, which was occasionally greater than that of the surface cyanobacterial blooms (Carpenter et al. 1995). Subsurface maxima of *D. norvegica* and other dinoflagellates have been observed before in the northern Baltic Sea (Kuosa 1990) and appear to be a recurrent feature during summer. Carpenter et al. (1995) suggest that low grazing pressure and a negligible sinking rate may be major factors allowing a slow-growing population to accumulate. The fluorescence of *D. norvegica,* and of certain other species in this genus, is in the yellow-orange range (580 nm) when excited by blue light (Lessard and Swift 1986, Hallegraeff and Lucas 1988, Schnepf and Elbrächter 1988). Pigment extractions show that this fluorescence is due to the presence of phycoerythrin (Geider and Gunter 1989) normally associated with cyanobacteria or cryptomonads (Jeffrey and Vesk 1997). Ultrastructural observations of thylakoid arrangement show that chloroplasts in *D. norvegica* appear to be derived from cryptomonad-type endosymbionts rather than from cyanobacteria (Schnepf and Elbrächter 1988, Berland et al. 1995, Carpenter et al. 1995). To test this hypothesis, we analyzed the composition of taxon-specific marker pigments separated by high-performance liquid chromatography (HPLC) in *D. norvegica* and the other dinoflagellates and cryptophytes (exclusively *Rhodomonas* sp.) that cooccurred with *D. norvegica* in the same body of water.

MATERIALS AND METHODS

General sampling procedure. Sampling was done on the R/V *Professor Albrecht Penck* (28 June–2 July 1993) and R/V *Alexander von Humboldt* (18–22 July 1994) at a site east of Gotland (57°20′ N, 20° E) in the central Baltic Sea. Water samples were collected from different depths using a rosette water sampler equipped with 12-L bottles.

For analysis of plant pigments, 1 L of seawater was filtered through a Whatman GF/F glass-fiber filter and stored in a −80° C freezer for 2 weeks until analysis in the laboratory.

For determination of phytoplankton composition in the water column, 250 mL of seawater was fixed with 1% Lugol's solution and counted under the inverted microscope (Utermöhl 1958) at 400× magnification after settling in a Hydro-Bios counting chamber. Phytoplankton counts were converted into carbon equivalents via biovolume calculations using a volume to carbon conversion coefficient of 0.11 $pg \cdot \mu m^{-3}$ according to Strathmann (1967).

Sampling D. norvegica. Water samples were collected from the depth where *D. norvegica* were most abundant. *Dinophysis norvegica* were separated from other species by size fractionation. The water samples were first passed through a 60-μm mesh to eliminate larger organisms from the water and then through a 30-μm mesh to concentrate *D. norvegica.* The concentrated cells were finally rinsed into 30 mL of filtered seawater. Subsamples of these concentrates were immediately counted at 400× magnification in a Bürker counting chamber under a light microscope in order to verify the separation. No other phytoplankton species were found in any of the concentrated samples of *D. norvegica.* The concentrated samples were then filtered onto GF/F filters and stored at −80° C for further pigment analysis. A total of 30 filters was analyzed for the two sampling periods in 1993 and 1994.

Sampling phytoplankton species cooccurring with D. norvegica. The filtrate, originating from the *D. norvegica* concentration procedure, was further passed through a 10-μm mesh to isolate the phytoplankton organisms occurring together with *D. norvegica.* The identification of the phytoplankton was done according to Pankow (1990), directly on-board with unpreserved samples from the 10-μm mesh concentration which were counted at 400× magnification in a Bürker counting chamber under a light microscope. The dinoflagellates (*Prorocentrum minimum, Gymnodinium simplex,* and *Katodinium rotundatum*) and the cryptophyte *Rhodomonas* sp. were identified. Parallel phytoplankton samples were fixed with Lugol's solution and counted in the laboratory under the inverted microscope. Both methods showed identical species composition, so it is unlikely that artifacts occurred during mash fractionation and preservation.

Live cells from the dinoflagellates *Prorocentrum minimum, Gym-*

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[2] Present address: Plymouth Marine Laboratory, Prospect Place, West Hoe, Plymouth PL1 3 DH, United Kingdom; e-mail bmh@wpo.nerc.ac.uk.

[3] Author for reprint requests: falk.pollehne@io–warnemuende.de.

TABLE 1. *Spectral characteristics of chlorophylls (chl* c_1*, chl* c_2*) and carotenoids (peridinin, alloxanthin) isolated from HPLC fractions of field samples of* D. norvegica *and cultures of* Prorocentrum minimum, Gymnodinium simplex, Katodinium rotundatum, *and* Rhodomonas *sp. The latter were isolated from field samples and cooccured together with* D. norvegica *in the same water body on the summer cruises of 1993 and 1994 in the Gotland Sea. FI is the goodness of fit index between reference spectra in the diode array library and the absorption spectra of the chlorophyll and carotenoid peaks from the different species.*

Pigments	Phytoplankton species	Solvent	Absorption maxima (nm) I	II	III	Band ratios	FI
I:III Band ratio							
chl c_1	*Rhodomonas* sp.	Acetone	446.2	578.1	630.0	8.02	
		HPLC eluant	440.4	576.2	629.1		0.95–0.99
chl c_2	*Rhodomonas* sp.	Acetone	448.2	581.3	630.1	9.02	
	D. norvegica		448.0	581.1	630.8	8.93	
	Rhodomonas sp.	HPLC eluant	446.0	580.1	630.0		0.99
	D. norvegica		446.0	580.0	630.0		0.99
Peridinin	*P. minimum*	Acetone		474.0		0	
	G. simplex			474.0		all solvents	
	K. rotundatum			474.1			
	P. minimum	Ethanol		474.2			
	G. simplex			474.0			
	K. rotundatum			474.1			
	P. minimum	HPLC eluant		472.4			0.99
	G. simplex			473.0			0.99
	K. rotundatum			473.6			0.99
% III:II Band ratio							
Alloxanthin	*Rhodomonas* sp.	Acetone		454.1	484.3	49.96	
	D. norvegica			454.0	484.0	50.03	
	Rhodomonas sp.	Ethanol		453.0	482.0	29.3	
	D. norvegica			453.4	482.2	29.1	
	Rhodomonas sp.	HPLC eluant		454.2	483.6		0.99
	D. norvegica			454.2	483.6		0.99

nodinium simplex, and *Katodinium rotundatum* and the cryptophyte *Rhodomonas* sp. were isolated from the concentrated (>10 μm) samples for further culture in the laboratory according to the method described by Drebes (1974). In both years, the isolated cells were grown in batch cultures in F/2 medium (Guillard and Ryther 1962) under 80 μmol photons·m^{-2}·s^{-1} in a 12:12 h LD cycle provided by white fluorescent tubes (Philips) at 12° C. For each species (*Prorocentrum minimum, Gymnodinium simplex, Katodinium rotundatum,* and *Rhodomonas* sp.), subsamples from the batch cultures in the log-growth phase were filtered onto GF/F filters and analyzed by HPLC to determine their specific pigment composition. For each species, six filters were measured in 1993 and 1994, respectively.

Extraction of samples. The frozen filter containing the algal sample was cut into small slices and homogenized in 5 mL 100% methanol buffered with 2% ammonium acetate in a glass homogenizer (25 mL capacity, held in a ice bath) with a motorized teflon grinder pestle, until no pigments were visible on the filter (<1 min). The filter plus extract was transferred in a centrifuge tube and centrifuged for 3 min at 700 × *g* at 0° C. The extract was filtered over a GF/F glass fiber filter (ϕ 10 mm) and 100 μL was injected directly onto the HPLC column.

HPLC technique. The photosynthetic pigments of the different phytoplankton groups were analyzed by HPLC using the method by Kraay et al. (1992). A RSil C_{18} column (Bio-Rad RSL), HPLC-grade solvents (Merck) A (0.5 M ammonium acetate in methanol and water [85:15, v/v]), B (acetonitrile and water [90:10, v/v]), and C (100% ethyl acetate) were used with the following linear tertiary gradient system (time, %A, %B, %C): (0 min, 60, 40, 0), (2 min, 0, 100, 0), (7 min, 0, 80, 20), (17 min, 0, 50, 50), (21 min, 0, 30, 70), (28.5 min, 0, 30, 70), (29.5 min, 0, 100, 0), (30.5 min, 60, 40, 0) and a flow rate of 0.8 mL·min^{-1}. The instrument used was a Merck-Hitachi liquid chromatograph equipped with a L6200A gradient pump with system controller (interface module D-600), a photodiode array detector (L4500), and an F-1050 fluorescence spectrophotometer. Pigment detection was done at 436 nm for all chlorophylls and carotenoids and 405 nm for pheophytin *a* and pheophorbide *a*. The HPLC system was calibrated with pigment standards prepared from different phytoplankton cultures by transferring HPLC fractions to standard solvents (acetone, ethanol) and comparing their visible absorption spectra with spectra given in the literature (Jeffrey 1968, 1989, Foppen 1971, Davies 1976, Gieskes and Kraay 1983, Wright and Shearer 1984, Jeffrey and Wright 1987, 1994, Wright et al. 1991, Kraay et al. 1992, Jeffrey et al. 1997). A Kontron UVIKON 941 spectrophotometer was used for UV-visible spectroscopy of purified pigments.

Identification of pigments. Pigments were identified by retention time and comparison of on-line collected absorption spectra (between 300 and 700 nm) with those of the spectral library (see above). The diode array detector we used gave a goodness of fit index (FI) from 0 (no conformity) to 1 (100% conformity) between ''reference'' spectra in the diode array library and the spectra of HPLC peaks from the fractionated field samples of *D. norvegica* and from the cultured *Prorocentrum minimum, Katodinium rotundatum, Gymnodinium simplex,* and *Rhodomonas* sp. isolated from the field (see Table 1). In addition, we calculated band ratios and measured absorption maxima from the pigment spectra detected in the dinoflagellates and the cryptophyte (see above). Values from the literature were used for comparison of band ratios and absorption maxima (Jeffrey 1969, Jeffrey et al. 1975, 1997, Cheng et al. 1974, Gieskes and Kraay 1983, Wright and Shearer 1984, Jeffrey and Wright 1987, Wright et al. 1991, Kraay et al. 1992, Jeffrey et al. 1997).

RESULTS

The three dinoflagellates (*Prorocentrum minimum, Katodinium rotundatum,* and *Gymnodinium simplex*) that cooccurred with *D. norvegica* in the Gotland Sea and were cultured in the laboratory displayed the typical pigment pattern for dinoflagellates with peridinin as the marker carotenoid (Table 1). Figure 1a shows an example chromatogram from cultured

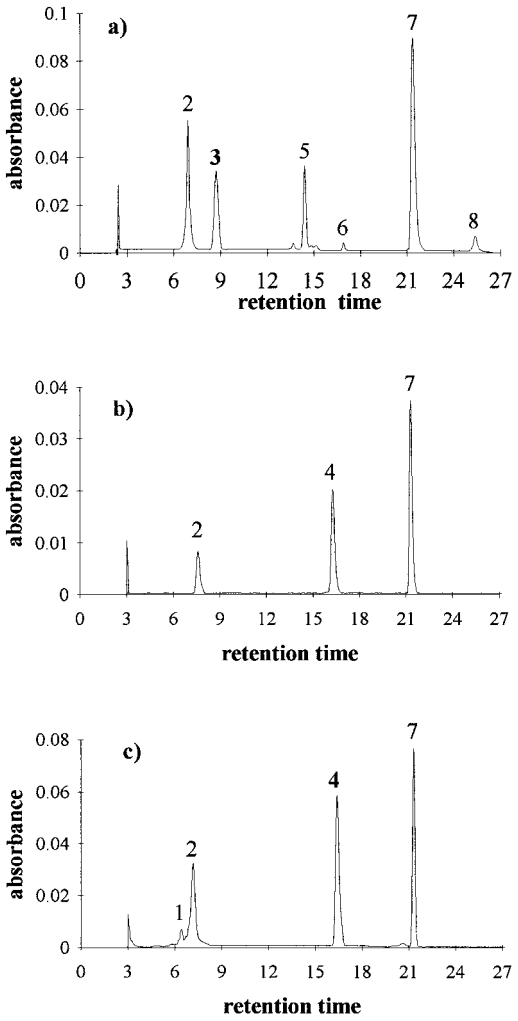

FIG. 1. Pigment chromatograms at 436 nm for both chlorophylls and carotenoids. 1, chl c_1. 2, chl c_2. 3, peridinin. 4, alloxanthin. 5, diadinoxanthin. 6, diatoxanthin. 7, chl *a*. 8, β,β-carotene. (a) Batch-cultured *Prorocentrum minimum* isolated from the Gotland Sea with the characteristic carotenoid peridinin (3) and chl c_2 (2). (b) *Dinophysis norvegica* concentrated by size fractionation of field samples from the Gotland Sea with the dominant carotenoid alloxanthin (4) and chl c_2 (2). (c) *Rhodomonas* sp. isolated from the Gotland Sea with the dominant carotenoid alloxanthin (4), chl c_1 (1), and chl c_2 (2).

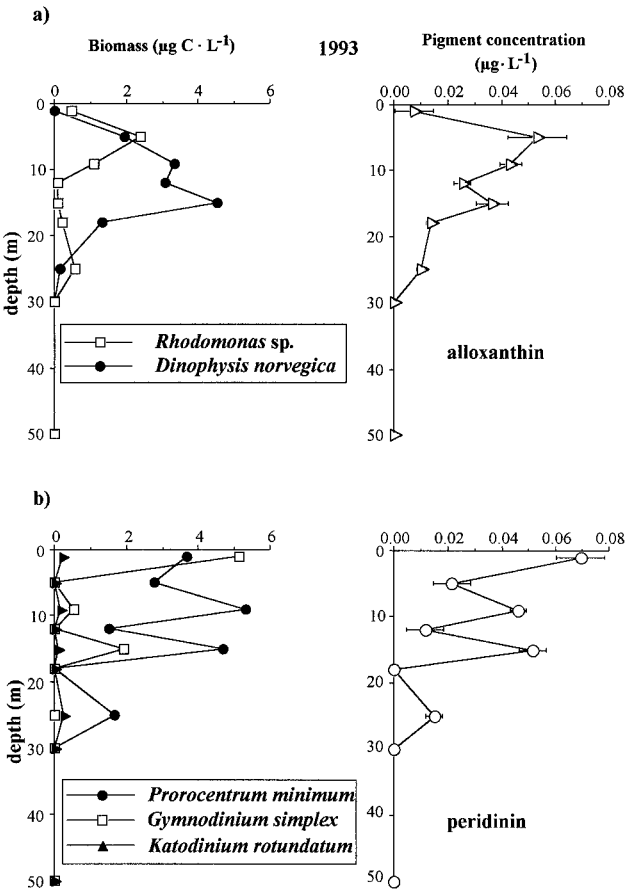

FIG. 2. Vertical profiles of cell carbon and pigment distribution in the Gotland Sea in 1993 (28 June–2 July). (a) *Rhodomonas* sp. and *D. norvegica* (left) and alloxanthin (right, n = 5). (b) *P. minimum, G. simplex, K. rotundatum* (left), and peridinin (right, n = 5).

P. minimum, with chl c_2, peridinin, diadinoxanthin, diatoxanthin, chl *a,* and β,β-carotene as major pigments. The pigment composition of the dinoflagellate *D. norvegica* differed from this typical pattern. No peridinin could be detected but the characteristic carotenoid of cryptomonads, alloxanthin, was found (Fig. 1b, c, Table 1). The cryptophyte *Rhodomonas* sp. showed the typical carotenoid alloxanthin (Fig. 1c), and the only difference in pigment composition between *Rhodomonas* sp. and *D. norvegica* was found in the chl *c* types (Fig. 1b, c). Trace amounts of chl c_1 could be detected in addition to chl c_2 in *Rhodomonas* sp. but not in *D. norvegica.* The spectral characteristics (Table 1) of the purified pigments (chl c_1 and c_2, peridinin, and alloxanthin) are close to those published (for references, see ''Identification of pigments'' section above). The high FI of 0.99 (Table 1) for the absorption spectra of the pigments detected in *D. norvegica,* the other dinoflagellates, and *Rhodomonas* sp. shows a good conformity with those of ''reference'' spectra in the diode array library.

The discrepancies between the vertical profile of cell carbon for field samples of the cryptomonad *Rhodomonas* sp. and the concentrations of the group-specific pigment alloxanthin in both years are explained by the presence of alloxanthin in *D. norvegica* (Figs. 1b, 2a, 3a). In 1993, the low cell counts of *Rhodomonas* sp. at 15 m water depth did not correspond with the peak of alloxanthin in the same depth (Table 2, Fig. 2a right). This anomaly was more pronounced in 1994. The cell counts of *Rhodomonas* sp. were highest in samples from 1 to 16 m depth (12,250–20,420 cells·L^{-1}, Table 2), but the pigment concentration of alloxanthin showed two marked peaks at 18 and 25 m water depth (Fig. 3a right), where the abundance of counted cryptophyceans was lowest, with 960 and 110 cells·L^{-1}, respec-

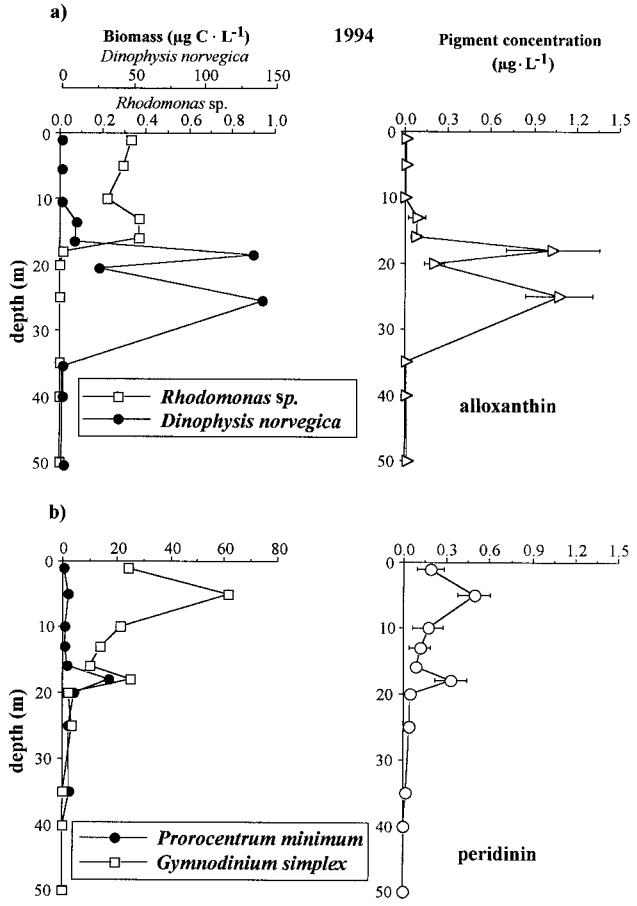

FIG. 3. Vertical profiles of cell carbon and pigment distribution in the Gotland Sea in 1994 (18–22 July). (a) *Rhodomonas* sp. and *D. norvegica* (left) and alloxanthin (right, n = 5). The pigment concentration for alloxanthin at 1, 5, and 10 m depth was 0.0053, 0.0047, and 0.0036 $\mu \cdot L^{-1}$, respectively. (b) *P. minimum, G. simplex* (left), and peridinin (right, n = 5).

tively (Table 2), and where *D. norvegica* was most abundant (18 m: 68,540, 25 m: 71,520 cells·L^{-1}, Figs. 2a, 3a). The distribution of cell carbon of the dinoflagellates *P. minimum, G. simplex,* and *K. rotundatum* agreed very well with the distribution of peridinin concentration in both years of the study (Figs. 2b, 3b).

DISCUSSION

In recent years, pigment analysis has been routinely and extensively used for the determination of phytoplankton composition in field samples (Millie et al. 1993). HPLC has proven effective in rapidly separating and distinguishing chlorophylls, chlorophyll-degradation products, and carotenoids (Wright et al. 1991). This method has many advantages when compared to microscopic methods. Microscopic evaluation takes considerable time and does not allow characterization of the physiological status of the taxa (Millie et al. 1993). In addition, it is difficult to identify small phytoplankton species in the light microscope correctly. However, the determination of algal groups by pigment analysis also has its limitations, because there are exceptions to the general assumption that certain pigments are characteristic for distinct taxonomic groups (Jeffrey and Vesk 1997 and references therein). The problem lies in the presence of endosymbionts. For example, according to Jeffrey and Vesk (1997), several dinoflagellates have no trace of peridinin but have pigments characteristic of their endosymbionts (e.g. chrysophytes, prymnesiophytes, or green algae). It is therefore important also to check the species composition microscopically if symbionts are suspected.

Schnepf and Elbrächter (1988) and Carpenter et al. (1995) suggested that the chloroplasts in the dinoflagellate *D. norvegica* originated from cryptomonads because of fluorescence spectra suggesting the presence of phycoerythrin and because of the ultrastructural characteristics of their thylakoids. Evidence in support of this hypothesis was also given by Vesk and coworkers (1996), who found (by gold labeling) that phycoerythrin was located in the thylakoids. These results are supported strongly by the pigment data found in this study.

Alloxanthin, which is the characteristic pigment of cryptophytes (Jeffrey and Vesk 1997), was the dominant carotenoid in *D. norvegica* fractionated from field populations, whereas no trace of peridinin, the characteristic pigment of dinoflagellates (Jeffrey and Vesk 1997), could be detected. Chl c_2, one of the key pigments of cryptophytes, dinoflagellates, and diatoms was also found in *D. norvegica* (Jeffrey and Vesk 1997). It is known that this pigment can be found in cryptophytes in addition to chl c_1 (Kraay et al. 1992, this study), or it can be replaced by chl c_3 in dinoflagellates, when fucoxanthin is present (Jeffrey et al. 1975, Withers et al. 1977, Jeffrey 1989), and in diatoms (Stauber and Jeffrey 1988).

So far, only limited information is available on the pigment composition of *Dinophysis.* Hallegraeff and Lucas (1988) found that *Dinophysis* species (*D. acuminata* or *D. fortii*) in Tasmanian waters contained peridinin exclusively as the dominant carotenoid. Maestrini et al. (1996) reported both peridinin and alloxanthin for *D. acuminata* from Antifer harbor (Normandy, France), but whether this peridinin originated only from *Dinophysis* cells is unclear, since *Prorocentrum micans* was also present.

Hence, a key question is whether alloxanthin in *D. norvegica* originates from recently ingested cells like *Rhodomonas* sp. or whether it is produced by the chloroplasts themselves. Our observations and data from cruises in 1993 and 1994 strongly support the second hypothesis as explained by the following points:

- Alloxanthin concentrations in both years were strongly related to *D. norvegica* abundance (Figs. 2, 3). Peak abundance of *Dinophysis* was 30 times higher in 1994 than in 1993, and concentrations

TABLE 2. *Mean number of cells per liter (L^{-1}) during the five-day sampling period of dinoflagellates and cryptophytes (*Rhodomonas *sp.) in the Gotland Sea in 1993 (a) and 1994 (b) (n = 5; ± standard deviation in %).*

	Dinoflagellates (cells·L^{-1} ± SD%)				Cryptophytes (cells·L^{-1} ± SD%)
Depth (m)	*Prorocentrum minimum*	*Gymnodinium simplex*	*Katodinium rotundatum*	*Dinophysis norvegica*	*Rhodomonas* sp.
a. 1993					
1	30,630 ± 55	6230 ± 13	6030 ± 20	3 ± 26	26,550 ± 58
5	23,140 ± 39	7 ± 15	4 ± 50	1000 ± 46	132,020 ± 55
9	44,280 ± 64	640 ± 33	4080 ± 47	1700 ± 24	61,260 ± 52
12	12,540 ± 70	3 ± 70	9 ± 13	1580 ± 48	5720 ± 61
15	39,100 ± 30	2320 ± 18	2730 ± 41	2320 ± 42	5450 ± 39
18	3 ± 22	7 ± 15	0	680 ± 36	13,080 ± 41
25	14,000 ± 68	0	7260 ± 16	80 ± 66	31,070 ± 31
30	0	0	0	0	0
50	0	0	0	0	0
b. 1994					
1	3720 ± 29	29,410 ± 20		0	18,380 ± 44
5	18,790 ± 77	75,150 ± 27		0	16,340 ± 47
10	6530 ± 40	26,130 ± 54		3 ± 33	12,250 ± 61
13	7600 ± 58	16,910 ± 40		5100 ± 26	20,420 ± 52
16	12,270 ± 43	12,250 ± 47		4590 ± 37	20,420 ± 57
18	144,100 ± 35	30,740 ± 44		68,540 ± 42	960 ± 69
20	34,890 ± 31	2690 ± 26		13,460 ± 45	4 ± 25
25	17,020 ± 35	4250 ± 33		71,520 ± 23	110 ± 36
35	19,610 ± 29	0		480 ± 41	0
40	60 ± 90	0		520 ± 18	0
50	70 ± 42	0		990 ± 12	0

of alloxanthin showed a comparable increase at the depth where *D. norvegica* was most abundant (Fig. 3).

- Carpenter et al. (1995) published ultrastructural observations of *D. norvegica* from the cruise in 1993, as well as from earlier studies in 1991 and 1992 in the Gotland Sea: no food vacuoles were found in these cells. These results support observations of Berland et al. (1995) and Maestrini et al. (1996) on *D.* cf. *acuminata.* Furthermore, if *D. norvegica* were ingesting *Rhodomonas* cells as food, chl *a* degradation products (pheophytin *a,* pheophorbide *a*) might be expected (Knight and Mantoura 1985) in *D. norvegica,* but they were not detected. Feeding and digestion could have been extracellular, as described by Jacobson and Andersen (1986) for heterotrophic dinoflagellates. However, this was not observed in *D. norvegica* (see Carpenter et al. 1995).
- Observations using fluorescence microscopy during the cruise in 1994 on freshly isolated *D. norvegica* from different stations and depths showed that excitation with blue or green light produced an even, bright orange fluorescence within the cells (Reckermann, pers. commun.). A more heterogeneous pattern of fluorescence would be expected within the cells if the fluorescence were caused by ingested cryptomonads.

One of the major drawbacks in understanding the life history of *Dinophysis* is that, to date, all efforts to culture *Dinophysis* have been unsuccessful (Lucas and Vesk 1990, Jacobson and Andersen 1994, Maestrini et al. 1995, Vesk et al. 1996, Andersen and Drebes, pers. commun.). Knowledge gained from field experiments such as ours may lead to more successful culture attempts in the future.

Our results support the hypothesis that endosymbionts of cryptomonad origin are responsible for the presence of alloxanthin as the dominant carotenoid in *D. norvegica.* Where there are high abundances of both *D. norvegica* and cryptomonads, care should be taken when interpreting pigment signatures.

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