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## SHORT COMMUNICATION

# Pigment composition in three *Dinophysis* species (Dinophyceae) and the associated cultures of *Mesodinium rubrum* and *Teleaulax amphioxeia*

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Despite the discussion around the nature of plastids in *Dinophysis*, a comparison of pigment signatures in the three-culture system (*Dinophysis*, the ciliate *Mesodinium rubrum* and the cryptophyte *Teleaulax amphioxeia*) has never been reported. We observed similar pigment composition, but quantitative differences, in four *Dinophysis* species (*D. acuminata*, *D. acuta*, *D. caudata* and *D. tripos*), *Mesodinium* and *Teleaulax*. *Dinophysis* contained 59–221-fold higher Chl *a* per cell than *T. amphioxeia* (depending on the light conditions and species). To explain this result, several reasons (e.g. more chloroplasts than previously appreciated and synthesis of new pigments) are suggested.

**KEYWORDS:** *Dinophysis*; *Mesodinium*; *Teleaulax*; pigments; HPLC

Photosynthetic *Dinophysis* species contain plastids of cryptophycean origin (Schnepf and Elbrächter, 1999), but there continues to be a major controversy around their nature, whether there are only kleptoplastids or permanent ones (García-Cuetos *et al.*, 2010; Park *et al.*, 2010; Kim *et al.*, 2012a).

Numerous protists have acquired photosynthetic capacity through stable endosymbiosis or temporary sequestration of chloroplasts (Nowack and Melkonian, 2010), but in some cases the nature of such association is not fully understood (Johnson, 2011). In *Dinophysis*, kleptoplasty from *Mesodinium rubrum* remains the most likely hypothesis based on molecular data and on the acquisition and turnover trends of the “stolen” plastids (Hackett *et al.*, 2003; Minnhagen and Janson, 2006; Park *et al.*, 2010; Minnhagen *et al.*, 2011). The suggestion that plastids in *Dinophysis* may be permanent, could also be supported by particular chloroplast features in *D. acuminata* (García-Cuetos *et al.*, 2010), though recent studies have demonstrated that plastids are structurally modified in *D. caudata* after enslavement from *Mesodinium* (Kim *et al.*, 2012a). In the case of *M. rubrum*, sequestration of plastids from cryptophytes has also been established (Gustafson *et al.*, 2000; Hansen *et al.*, 2012). Some authors (Hansen and Fenchel, 2006) have noted that *Mesodinium* plastids were larger than in cryptophytes but major structural modifications like *Dinophysis* have not been found (García-Cuetos *et al.*, 2010).

Photosynthetic pigments are chemotaxonomic markers of phytoplankton due to their selective distribution among taxa (Jeffrey *et al.*, 2011). Pigment types are useful to trace the origin of secondary and tertiary plastids in dinoflagellates, and they often correlate with other phenotypic and/or molecular traits (Zapata *et al.*, 2012). However, despite the current debate about the nature of plastids in *Dinophysis*, pigment composition in the three-culture system of *Dinophysis* has not been reported. HPLC pigment analyses were only performed in field samples from the Baltic Sea dominated by *D. norvegica* (Meyer-Harms and Pollehne, 1998), which showed a cryptophyte-like signature (alloxanthin and Chl *c*<sub>2</sub>).

The achievement of cultivating *Dinophysis* (Park *et al.*, 2006) in a three-culture system (including the ciliate *Mesodinium rubrum* fed with cryptophytes, typically *Teleaulax* sp.) opened the possibility of further work on the ecology and physiology of this genus. Based on this finding, the culture of other photosynthetic *Dinophysis* species has been described (reviewed in Reguera *et al.*, 2012).

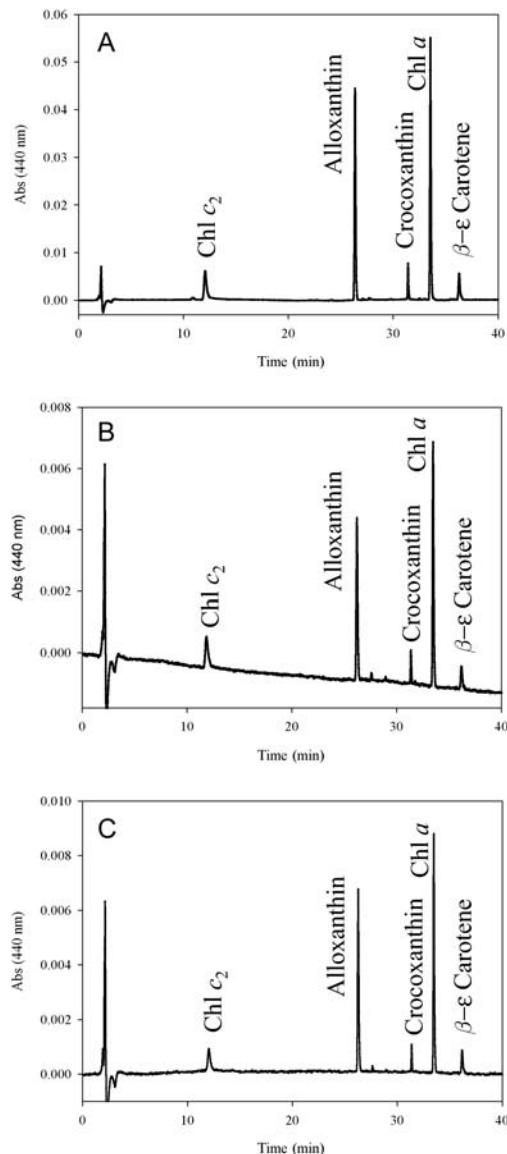
We provide the first HPLC pigment data in cultures of three different *Dinophysis* species, their direct prey (*M. rubrum*) and the *T. amphioxeia* strain ingested by the ciliate. Our interest was to investigate whether pigment

composition could reveal distinct fingerprints indicative of different plastid types in *Dinophysis* and/or *Mesodinium*.

Three *Dinophysis* species isolated in NW Spain (*D. tripos* [VGO1062, Oct 2009], Station B1, Ría de Vigo; 42° 21,40'N 8° 46,42'W; *D. caudata* [VGO1064, Apr 2010], and *D. acuta* [VGO1065, Oct 2010]; Station P2, Ría de Pontevedra; 42° 8,22' N, 8° 51,36' W) were cultured in diluted (1/20) L1-Si medium (Guillard and Hargraves, 1993) at 32 salinity, 12:12 L:D cycle at two light intensities (LL:70 and HL:200  $\mu\text{mol photons m}^{-2}\text{s}^{-1}$ ), in 50 mL glass flasks. The ciliate *Mesodinium rubrum* (AND-A0711; LCCRRPP, Spain) fed with the cryptophyte *Teleaulax amphioxeia* (AND-A0710) was added periodically as prey. Ciliate and cryptophyte strains were isolated in 2007 in the course of weekly sampling of the Andalusian Monitoring Programme (Huelva, SW Spain).

The three-culture system (*Dinophysis*/*Mesodinium*/*Teleaulax*) was maintained for at least a year, before running the experiments shown in this study. Samples for pigment analyses were taken <5 days after being inoculated into fresh medium, to ensure that *Dinophysis* cultures were actively growing and feeding on *Mesodinium* until sampling. To avoid contamination from *Mesodinium* or *Teleaulax*, *Dinophysis* cells were gently rinsed several times with fresh medium through a 20- $\mu\text{m}$  mesh immediately before filtration, and inspected by light microscopy. The same approach was used for *Mesodinium* using a 1- $\mu\text{m}$  mesh to reduce *Teleaulax* densities until they were removed. For cell counts in growth experiments, Lugol's fixed samples (final concentration 2%) were enumerated in a 1-mL Sedgwick-Rafter counting chamber (*Dinophysis* and *Mesodinium*) and a Neubauer-type hemocytometer (*Teleaulax*) in an inverted microscope. An additional aliquot for cell counts was taken after rinsing *Dinophysis* and *Mesodinium* cultures (immediately before filtering samples for HPLC analysis), to estimate pigment content per cell. Doublings per day (*k*) and growth rates (*r*) were calculated from the equations  $k = \log_2 (N_t/N_0)/\Delta t$  and  $r = k \times 0.6931$ , respectively. Filtration, extraction procedures and HPLC pigment analyses were performed following Zapata *et al.* (Zapata *et al.*, 2000). Pigments were identified by the comparison of the spectral information and retention time of chromatographic peaks against a library of chlorophylls and carotenoids isolated from phytoplankton cultures (Zapata *et al.*, 2000).

HPLC pigment analyses in all the organisms showed Chl *c*<sub>2</sub>, Chl *a*, alloxanthin, crocoxanthin and  $\beta,\epsilon$ -carotene as dominant compounds (Fig. 1). All these pigments have been previously reported in cryptophytes (Jeffrey *et al.*, 2011). We did not detect monadoxanthin, a carotenoid found in some cryptophytes within the genera *Rhodomonas*, *Chroomonas* and *Cryptomonas* (Pennington *et al.*, 1985). Thus, we did not find any pigment signature that



**Fig. 1.** HPLC chromatograms in low light conditions ( $70 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ ) of (A) the cryptophyte *Teleaulax amphioxieia* AND-0710, (B) the ciliate *Mesodinium rubrum* AND-0711 and (C) the dinoflagellate *Dinophysis tripos* VGO1062.

could trace a different plastid (or the synthesis of any different pigment) in *Dinophysis* relative to *M. rubrum* and *T. amphioxieia*. However, in both light conditions assayed, *Dinophysis* spp. and *Mesodinium* displayed different pigment ratios to Chl *a*, specifically higher Chl *c*<sub>2</sub>, when compared with *T. amphioxieia* (Table I). Carotenoid ratios were similar in LL and HL conditions in all studied organisms, with lower alloxanthin ratios in *D. acuta*, particularly in HL.

Chl *a* per cell ratios were also determined in both light conditions in the study organisms (excepting *Mesodinium* and *D. tripos* in HL). The available data showed that *Mesodinium* in LL contained  $\sim 60$  times

more Chl *a* per cell than *Teleaulax*. Each *Teleaulax* cell contains a single plastid, whereas *Mesodinium* has been reported to harbour 6–36 plastids (Hansen *et al.*, 2006). Therefore, our estimates of Chl *a* per cell are somewhat greater but comparable with a simple calculation on a plastid basis. We estimated  $28.3 \pm 0.4 \text{ pg Chl } a \text{ per } Mesodinium \text{ cell}$  (from the molar ratios in Table I), somewhat lower than previous estimates of  $\sim 70 \text{ pg Chl } a \text{ cell}^{-1}$  (10 days after the addition of cryptophytes; Gustafson *et al.*, 2000). However, the *Mesodinium* strain in that study was also larger ( $22\text{--}29 \mu\text{m}$  by  $22\text{--}36 \mu\text{m}$ ) than ours ( $10.4\text{--}14.6 \mu\text{m}$ ,  $n = 16$ ).

The chlorophyll *a* content per cell of *Dinophysis* spp. was much greater than expected if the plastids contained the same amount of pigments as in *Teleaulax*. In LL, estimates ranged from 144- to 221-fold higher than in *Teleaulax* (Table I), with increasing values from *D. acuta* to *D. caudata* and *D. tripos*. Such variability in Chl *a* per cell among *Dinophysis* spp. can be explained by their relative sizes (*D. acuta* is smaller ( $50\text{--}95 \mu\text{m}$ ) than *D. caudata* ( $70\text{--}110 \mu\text{m}$ ) and *D. tripos* ( $95\text{--}120 \mu\text{m}$ ) (Reguera, 2003). In HL, the Chl *a* per cell estimates in *Dinophysis* were just 59–70 higher (*D. tripos* values missing) than in *Teleaulax*, where a parallel decrease in Chl *a* per cell was not observed.

In the available literature there are few references to the number of plastids in *Dinophysis* spp. García-Cuetos *et al.* (García-Cuetos *et al.*, 2010) only reported the presence of two axial clusters of stellate compound chloroplasts in *D. acuminata*. Kim *et al.* (Kim *et al.*, 2012b) mentioned 15–30 plastids in *D. caudata* after ingesting *Mesodinium*, but this number appeared to increase 21 h. after feeding on a single prey. We do not have any estimates about the number of plastids in our *Dinophysis* spp. but if the Chl *a* per cell in *Teleaulax* were extrapolated (59–221 times higher in *Dinophysis* spp.), it would probably overestimate their true number of plastids. Even if more kleptoplasts than previously appreciated or their structural modifications (different thylakoid arrangement, elongation and clustering into stellate compound chloroplasts; Kim *et al.*, 2012b), could help to explain these results, it seems that new synthesis of pigments could happen in *Dinophysis*. The ability to synthesize new pigments and replicate plastids has been confirmed in *Mesodinium* (Johnson *et al.*, 2006; Moeller *et al.*, 2011) and explained by the maintenance of functional prey nuclei (Johnson, 2011). However, *Dinophysis* does not harbour a “kleptonucleus” and a different mechanism should operate. In this sense, Wisecaver and Hackett (Wisecaver and Hackett, 2010) demonstrated that *Dinophysis* contains nuclear-encoded genes for plastid function which would allow a temporary control and regulation of kleptoplasts.

**Table I:** Growth rates ( $\text{day}^{-1}$ ) and accessory pigment ratios to Chl *a* (mole:mole) in the studied organisms

Species (light treatment)	Growth rate ( $\text{day}^{-1}$ )	Chl $c_2$	Alloxanthin	Crocoxanthin	$\beta, \epsilon$ -car	Chl <i>a</i> per cell
<i>Teleaulax amphioxeia</i> (LL)	$0.98 \pm 0.06$	$0.094 \pm 0.003$	$0.767 \pm 0.027$	$0.104 \pm 0.005$	$0.054 \pm 0.002$	$0.53 \pm 0.01$ (1)
<i>Teleaulax amphioxeia</i> (HL)	$1.57 \pm 0.25$	$0.094 \pm 0.001$	$0.866 \pm 0.033$	$0.109 \pm 0.001$	$0.058 \pm 0.002$	$0.64 \pm 0.09$ (1.2)
<i>Mesodinium rubrum</i> (LL)	$0.38 \pm 0.06$	$0.166 \pm 0.001$	$0.736 \pm 0.180$	$0.109 \pm 0.008$	$0.048 \pm 0.007$	$31.7 \pm 5.4$ (60.2)
<i>Mesodinium rubrum</i> (HL)	$0.21 \pm 0.03$	$0.220 \pm 0.007$	$0.800 \pm 0.050$	$0.109 \pm 0.007$	$0.043 \pm 0.002$	<i>n.a.</i>
<i>Dinophysis caudata</i> (LL)	$0.08 \pm 0.01$	$0.141 \pm 0.020$	$0.747 \pm 0.044$	$0.088 \pm 0.001$	$0.048 \pm 0.006$	$86.7 \pm 9.2$ (164.3)
<i>Dinophysis caudata</i> (HL)	$0.29 \pm 0.07$	$0.119 \pm 0.004$	$0.856 \pm 0.043$	$0.072 \pm 0.004$	$0.054 \pm 0.010$	$45.1 \pm 5.2$ (70.5)
<i>Dinophysis acuta</i> (LL)	$0.17 \pm 0.10$	$0.162 \pm 0.001$	$0.659 \pm 0.029$	$0.085 \pm 0.004$	$0.048 \pm 0.007$	$76.0 \pm 6.2$ (144.1)
<i>Dinophysis acuta</i> (HL)	$0.27 \pm 0.19$	$0.136 \pm 0.033$	$0.571 \pm 0.038$	$0.068 \pm 0.010$	$0.035 \pm 0.008$	$37.8 \pm 8.7$ (59.1)
<i>Dinophysis tripos</i> (LL)	$0.17 \pm 0.10$	$0.153 \pm 0.006$	$0.686 \pm 0.025$	$0.084 \pm 0.007$	$0.043 \pm 0.004$	$116.7 \pm 36.7$ (221.3)
<i>Dinophysis tripos</i> (HL)	$0.40 \pm 0.09$	$0.124 \pm 0.006$	$0.713 \pm 0.021$	$0.054 \pm 0.002$	$0.039 \pm 0.001$	<i>n.a.</i>

Chl *a* per cell ratios are femtomoles per cell. Below (between parenthesis) the average Chl *a* per cell in each condition versus average Chl *a* per cell in *T. amphioxeia* (LL). All values are expressed as average  $\pm$  S.D ( $n = 3$ ). *n.a.* data not available.

The variable pigment ratios to Chl *a* in *Dinophysis* or *Mesodinium* relative to *Teleaulax* could be due to photoacclimation or physiological changes in their kleptoplastids. The molecular machinery and functional control over the original *T. amphioxeia* plastids are no longer the same in these new hosts (Johnson *et al.*, 2007; Wisecaver and Hackett, 2010), even if photoacclimation in *Mesodinium* has been recently confirmed (Moeller *et al.*, 2011). Similar studies are not yet available in *Dinophysis*, however, Kim *et al.* (Kim *et al.*, 2012b) reported different plastid colour in *D. caudata* when maintained in LL (reddish,  $10 \mu\text{mol photons m}^{-2}\text{s}^{-1}$ ) and HL (green,  $160 \mu\text{mol photons m}^{-2}\text{s}^{-1}$ ). Once shifted back from HL to LL, kleptoplastids could not regain the reddish colour and the authors suggested some photo-damage not repaired by *D. caudata* itself. In our study, we could not detect any significant changes in plastid colour. These changes reported by Kim *et al.* (Kim *et al.*, 2012b) could be associated with a higher degradation of phycobiliproteins (hydrophilic compounds not analysed by HPLC) relative to Chl *a*, which turns reddish cultures (like *T. amphioxeia* AND-0710) into pale green when reaching the late stationary phase.

In our study, the lower Chl *a* content in HL versus LL cultures of *D. acuta* and *D. caudata* could be induced by photodamage or photoacclimation, as shown in *Mesodinium* (Moeller *et al.*, 2011). But it would also agree with the kleptoplastid hypothesis and the apparent inability of *Dinophysis* to replicate plastids (Minnhagen *et al.*, 2011), where faster growing HL cells would dilute their plastids content relative to LL ones.

Regarding the effects of irradiance on accessory pigment ratios, the role of alloxanthin in cryptophytes as light harvesting or photoprotective pigment has not been yet elucidated. But with a few exceptions (Schlüter *et al.*, 2000), higher irradiance promotes increasing ratios of alloxanthin to Chl *a*, suggesting a photoprotective function (Laviale and Neveux, 2011). Chlorophylls *c*

are light harvesting compounds and, overall they tend to decrease in HL conditions relative to Chl *a* (Rodríguez *et al.*, 2006). Both effects were observed in our study (excepting lower alloxanthin:Chl *a* in *D. acuta* HL and higher Chl  $c_2$ :Chl *a* in *Mesodinium* HL).

In conclusion, the presence of another type of plastid (of cryptophyte nature) in *Dinophysis* and *Mesodinium* could not be ruled out from this study. This is so because putative permanent plastids would be phylogenetically related with cryptophytes yielding a similar (if not identical) pigment signature in HPLC analyses.

The selective retention of two types of cryptophyte plastids by *Dinophysis* has been demonstrated previously (Park *et al.*, 2010), and even from multiple algal origins in the field (Kim *et al.*, 2012b), although the nature of the latter (kleptoplastids or just food) could not be ascertained. Further cross-feeding experiments of *Dinophysis* with *Mesodinium* fed upon cryptophytes with different pigment composition (either lipophilic and/or hydrophilic compounds), would be very interesting to check if the turnover trends of these plastids correlate with major changes in pigment composition in *Dinophysis* and *Mesodinium*.

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