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The function of plastids in the deep-sea benthic foraminifer, *Nonionella stella*

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Abstract

Curiously, the benthic foraminifer, *Nonionella stella,* found in the upper 3 cm of sediments collected off California at a depth of ~600 m, retains chloroplasts. We examined the origin and physiological function of the organelles within the host cell. Transmission electron micrographs, fluorescence and absorption spectra, Western blots, and enzyme assays revealed that the chloroplasts were intact and retained functionality for up to 1 year after sample collection. 16S rDNA gene sequences established that the plastids were derived from diatoms closely related to *Skeletonema costatum* and *Odontella sinensis.* Western blots of three major chloroplast proteins (ribulose bisphosphate carboxylase oxygenase [RuBisCO], the D1 protein, and the fucoxanthin chlorophyll *a* protein complex) confirmed that the organelle retained both nuclear and chloroplast encoded proteins, which suggests that the turnover of the plastid machinery is extremely low. Moreover, the two carboxylating enzymes examined, RuBisCO and phosphoenol pyruvate carboxylase, retained catalytic activity. Three hypotheses regarding the function of sequestered chloroplasts in deep-sea foraminifera were considered: (1) the organelles are photosynthetic under extremely low irradiance levels, (2) the organelles utilize exogenous substrates to generate an electrochemical gradient that permits chemoautrophy in the dark, and (3) they are used for the assimilation of inorganic nitrogen. Our results suggest that the chloroplasts are used to meet the nitrogen requirements of the host. Immunolocalization of the nuclear encoded protein, nitrate reductase, supports this hypothesis. This protein is widely distributed in photoautotrophs but is not encoded in eukaryotic protists such as foraminifera.

Although the retention of algae and algal plastids by marine invertebrates is well documented (Lee and Zucker 1969; Lee and Bock 1976; Muller-Merz and Lee 1976; Leutenegger 1984; Lee et al. 1992; Bernhard and Bowser 1999; Rumpho et al. 2000), the physiological relationship between the photosynthetic component and the heterotrophic host cell is not well understood. By definition, a symbiotic relationship is mutually beneficial, as in coral–zooxanthellae symbiosis, where the algae provides reduced carbon to the host and nitrogen is recycled between the two organisms (Rahav et al. 1989; Falkowski et al. 1993). More than 30 yr ago, ultrastructural analysis of the marine ciliate *Mesodinium rubrum* revealed an ''incomplete symbiosis'' involving two enslaved organelles from different organisms: functional chloroplasts and non-ciliate mitochondria (Taylor et al. 1969, 1971). In recent years, studies of algal chloroplast–host associations have been termed ''symbioses,'' given their long-lived association and the ability of the host to acquire a new metabolic capability, as in the case of ''solar-powered'' Sa-

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coglossan sea slugs (Sanders 1991; Green et al. 2000; Rumpho et al. 2000). In fact, symbiotic relationships involving sequestered chloroplasts were thought to provide similar nutrient feedbacks such as those found in zooxanthellate corals (Lee and Bock 1976; Muller-Merz and Lee 1976). This implies that the algal–host relationship is established to preserve and recycle limited nutrients such as nitrogen. Selection pressure for such an association is highest when both organic and inorganic nutrient supplies are low or difficult to access by host cells. Under such conditions, enslaved chloroplasts can be a rich source of carbohydrate and reduced nitrogen because of the presence of the Calvin cycle in the plastid and a nitrogen assimilatory pathway that reduces nitrate to ammonium (Falkowski and Raven 1997). However, unlike intact algae, plastids do not retain all the genetic information required to replicate or to synthesize any of their lipids and many of their proteins. In some, if not all, hosts, however, chloroplast proteins are synthesized (Pierce et al. 1996; Green et al. 2000). Protein synthesis is essential in an oxygenic photosynthetic organelle, where protein turnover can be significant (Pierce et al. 1996).

Many species of foraminifera retain algae or sequester algal chloroplasts (Lee et al. 1974; Lee and Bock 1976; Muller-Merz and Lee 1976; Leutenegger 1984; Lee and Anderson 1991; Bernhard and Bowser 1999; Chai and Lee 2000; Correia and Lee 2000). Like other chloroplast–host relationships in the photic zone, chloroplasts provide photosynthate to the cell (Lee and Muller 1969; Lee and Zucker 1969; Lee and Bock 1976; Muller-Merz and Lee 1976). The foraminiferal host cell is highly selective regarding the plastids retained. Ultrastructural analysis (Leutenegger 1984) and feeding experiments (Correia and Lee 2000) have suggested that foraminifer–diatom plastid associations are most common; however, some hosts will accommodate plastids from rhodophytes, chlorophytes, chrysophytes, and dinoflagellates (Muller-Merz and Lee 1976; Leutenegger 1984; Chai and Lee 2000). The specificity of plastid acquisition is almost certainly a consequence of the coevolution of a signal-receptor system between the surface of specific algae and the foraminiferal pseudopod (Chai and Lee 2000) and can result in an extremely dense population of virtually clonal plastids within the host cell. However, even though foraminifera may sequester plastids, they retain the ability to feed and digest food (Chai and Lee 2000).

Although plastid retention potentially confers photosynthetic competence, not all host–algal associations are found in the photic zone. Chloroplasts have been found in large numbers in specimens of the benthic foraminifer *Nonionella stella* collected from water depths of ~600 m (Bernhard and Bowser 1999). In terms of biovolume, *N. stella* is the dominant benthic organism found in the hypoxic sediments of Santa Barbara Basin (Bernhard et al. 2000). The role of chloroplasts in this foraminifer is the subject of the present article.

The myriad studies of photic symbiotic relationships involving plastids provide the basis for three hypotheses to explain plastid symbiosis in *N. stella.* (1) Plastids photosynthesize at extremely low light levels and provide organic carbon to the host. (2) In the absence of light, the plastids utilize the chemical gradients of the Santa Barbara Basin sediments and provide organic carbon to the host via a chemoautotrophic pathway that somehow uses the electron transport components of the organelle to derive energy and/or reductant. (3) Given that foraminifera are unable to acquire and assimilate inorganic sources of nitrogen, the plastids are sequestered to help meet the nitrogen requirements of the host. None of these hypotheses propose a symbiotic relationship between host and plastid—rather, the role of the chloroplast is ''unibiotic,'' conferring an advantage to the host but gaining nothing in return.

The possibility of a light driven set of reactions to be used in a nearly aphotic environment is an exciting biological phenomenon. To this end, we have characterized the major molecular and biophysical properties of the sequestered chloroplasts of *N. stella* and present an analysis of the potential utility of the plastids to the host.

Materials and methods

Sample collection—Samples were collected from the Santa Barbara Basin (USA, 34°15′N, 120°02′W, 551–591 m) during three cruises: April 1999, October 2000, and February 2001. Details on collection methods are given in Bernhard et al. (1997). In brief, as soon as possible after a box core was recovered on deck (i.e., within 10 min), bulk surface sediments (~1–3 cm) were placed in 125- or 250-ml opaque high-density polyethylene bottles. Sediments were added to the bottles to a depth of ~2 cm; the bottle was filled with chilled bottom water so that as little air space as possible was present. Bottles with samples were maintained near ambient temperature (5°C) during transport and until sample processing or measurements.

Electron microscopy—Sediments were taken into a refrigerated environmental room (5°C) within 5 min after the recovery of the box core. Sediments containing *N. stella* were fixed in 3% glutaraldehyde buffered with 0.1 mol L^{-1} Na-cacodylate (pH 7.2). Samples were kept at 5°C during transport. Sediments were sieved through a 63-μm screen with the use of chilled 0.1 mol L^{-1} cacodylate buffer, and individual foraminifers were removed from the coarse fraction when observed with a dissecting microscope. Specimens were further processed for ultrastructural analyses by use of protocols described in Bernhard and Bowser (1999). Material was examined with a Phillips 301 transmission electron microscope.

Molecular identification of chloroplasts—Mud samples for DNA extraction were sieved through a 63-μm stainless steel sieve with the use of filtered cold seawater, and individual foraminifera were isolated from the sediments with the aid of a dissecting microscope. Samples were maintained in cold seawater until extraction. Samples were mildly sonicated and washed in phosphate-buffered saline (PBS) three times, to free other small particles or bacteria. DNA extraction protocols followed those of Holzmann and Pawlowski (1996). Briefly, 25–50 foraminifera were sonicated for 5 s on low power in 50-μl buffer (50 mmol L^{-1} Tris [pH 8.4], 2 mmol L^{-1} ethylene diaminetetraacetic acid [EDTA], 0.1% Triton X-100, and 0.5% Na deoxycholate) and incubated for

Table 1. Variables used to calculate ETR in *N. stella* given the measured fluorescence quantum yield and σ. The upper limit of light at depth (I_d) and hence ETR were calculated assuming ideal attenuation of light due only to that of water. The lower limit is based on an attenuation of light due to water and the presence of 1 mg Chl *a* m^{-3} in the upper 40 m. See text for further information. Also, the measured enzyme activities in *N. stella* are shown.

Variable	Lower limit	Nonlimit value	Upper limit
I_d (quanta m^{-2} s^{-1})	$9e^{+13}$		$5.00e^{+16}$
I_o (quanta m^{-2} s^{-1})		$1.2046e^{+21}$	
d (m)		600	
$K440_w$ (m^{-1})		0.0168	
K440 for 1 mg Chl *a* m^{-3} (m^{-1})		0.13	
F_v/F_m		0.22 ($\pm$0.07)	
σ PSII (m^2 quanta^{-1})		$7.8e^{-19}$ ($\pm$1.8)	
Qp (*e* RC^{-1})		0.22 ($\pm$0.07)	
ETR (*e* RC^{-1} s^{-1})	$1.6e^{-5}$		$8.7e^{-03}$
Rubp (μg C mg Chl *a* h^{-1})		0.12 ($\pm$0.04)	
PEP (μg C mg Chl *a* h^{-1})		0.04 ($\pm$0.03)	

1 h at 55°C. Particulate matter was removed by centrifugation, and DNA extract was dialyzed against ultrapure H_2O on a 0.02-μm filter for 1 h. DNA was amplified with universal 16S rDNA primers (27f and 1492r primers, 30 cycles of 30 s at 94°C, 30 s at 52°C, and 45 s at 72°C; Field et al. 1997). Polymerase chain reaction (PCR) products were gel purified (Qiagen) and ligated into the PCR II vector (Invitrogen, TA cloning Kit) and transformed into TOP10F′ cells. Unique clones were screened by restriction-fragment length polymorphism (RFLP) with the use of the tandem tetrameric enzymes MSP I and HAE III (Promega). The DNA was run on a 2.5% metaphor gel (FMC Bioproducts) at 4°C for 1.5 h (75 V). Unique clones were sequenced on an ABI 310 (Perkin Elmer) following the manufacturer's protocol.

Chloroplast proteins—Foraminiferal specimens were picked and cleaned as described above. Samples were disrupted by centrifugation in 50 μl buffer A (8% sodium dodecyl sulfate [SDS], 0.2 mol L^{-1} Na_2CO_3, 4 mmol L^{-1} phenylmethylsulfonyl fluoride, and 50 mmol L^{-1} dithiothreitol [DTT]). Controls of the marine diatom *Thalassiosira pseudonana* were similarly treated. Prior to loading SDS gels, samples were diluted in 1 v of buffer B (4% SDS, 15% glycerol, and 0.05% Bromothymol blue). Gels were run on 12% polyacrylamide gels at 150 V for 1.5 h. Gels were transferred to polyvinylidene fluoride membranes, according to the protocols of Towbin et al. (1979) for 1 h at 60 V. Immunoblots were performed using antibodies against fucoxanthin–chlorophyll *a* protein (FCP) complex from the haptophyte algae *Isochrysis galbana*, ribulose bis-phosphate carboxylase oxygenase (RuBisCO) protein also from *I. galbana* and the D1 protein from a conserved synthesized, amino acid sequence (kindly provided by Autar Mattoo). Immunoblots were visualized by use of the West Pico chemiluminescent substrate (Pierce Chemicals) according to the manufacturer's protocol.

Pigment characterization—Samples of individual foraminifera as well as 1.0 ml of bulk sediment were extracted overnight in 100% acetone and diluted to 90% acetone prior to high-performance liquid chromatography (HPLC), fluorescence, or absorption measurements. HPLC samples were run on a Shimadzu HPLC. Absorption measurements were run on an Aminco DW-2a spectrophotometer. Fluorescence excitation and emission spectra were run on an Aminco-Bowman series 2 luminescence spectrometer. Emission maximum for fluorescence excitation spectra was 680 nm (4-nm slit width); and 77 K spectra were performed in quartz tubes that were slowly cooled over liquid nitrogen, plunged in liquid nitrogen, and equilibrated for 10 min.

Photochemical yields—Measurements of variable fluorescence on foraminiferal samples or control samples of mud without foraminifera diluted in filtered seawater were performed by use of the protocols outlined in Kolber et al. (1998). Samples were incubated in either blue or far-red light, to measure changes in reaction center size and changes in the quantum yield of photochemistry due to photoacclimation. Electron transport rates (ETR, in units of *e* RC^{-1} s^{-1}) shown in Table 1 were calculated from the equation

$$\mathrm{ETR} = \sigma\mathrm{PSII} \cdot I \cdot Qp \quad (1)$$

where σPSII is the cross sectional area of photosystem II (PSII; m^2 quanta^{-1}), I is the irradiance with units of quanta m^{-2} s^{-1}, and Qp is the photochemical quenching (electrons per reaction center). We estimate Qp to be equivalent to the measured *Fv*/*Fm*, given that the samples in this environment are essentially dark adapted. Irradiance estimates were made from theoretical estimates of clear sky irradiance and K_{440}, the attenuation of seawater at 440 nm, as the upper limit of light transmittance and K_{440} with the equivalent of 1 mg m^{-3} Chl *a* in the upper 40 m of the water column as a more realistic estimate (Morel 1988). Light at depth *d* is calculated from Beer's law:

$$I_d = I_0 \exp(-kd) \quad (2)$$

Enzyme assays—Measurements of RuBisCO and PEPc activity were performed on foraminifera after isolation and cleaning (see above). Samples were suspended in ice-cold

permeabilization buffer (50 mmol L^{-1} Bicine [pH 8.0], 1 mmol L^{-1} EDTA, 10 mmol L^{-1} $MgCl_2$, 5 mmol L^{-1} DTT, 1.5 mol L^{-1} glycerol, 10 mmol L^{-1} $NaHCO_3^-$, and 0.2 mg ml^{-1} lysolecithin). Cells were disrupted twice in an ice-cold Yeda press. Cell homogenates were aliquoted and incubated for 30 min at 25°C in the dark and then either ribulose bisphosphate or phospho(enol)pyruvate was added (2.3 and 5.0 mmol L^{-1} final concentration, respectively). On the addition of substrate, 7,400 Bq $NaH^{14}CO_3^-$ (final activity) was added to each sample. Samples (including blank with no substrate) were incubated at 25°C for 30 min. Reaction was stopped by adding 20 μl 6 N HCL. After the samples were counted in a scintillation counter (Beckman LS 6000IC), they were normalized to Chl *a* content. Chl *a* was calculated according to the tri-chromatic equation of Jeffrey and Humphrey (1975). Measurements were done in triplicate. PEP enzyme rates are considered to be the sum of PEP carboxylase and PEP carboxykinase, because no attempt was made to distinguish between the two.

Redox chemistry of the NADP$^+$/NADPH couple—Measurements of NADPH/$NADP^+$ ratios were made under mildly reducing conditions in an agar gel intended to mimic the chemical gradients in Santa Barbara Basin sediments. Aerobic, anaerobic, and reducing conditions were created in sealed tubes or microtiter plates by making agar layers of various redox couples, incubating $NADP^+$, and measuring the fraction of reduced NADPH. The experimental setup involved creating a redox gradient by layering test tubes first with sulfuric agar, made from autoclaved seawater, agar (15 g L^{-1}) Na_2S (final concentration between 1 and 500 μmol L^{-1}) and the redox indicator, resazurin (final concentration, 1.6 μmol L^{-1}). Agar mixture was aseptically added to test tubes after degassing with nitrogen. Tris-buffered seawater (pH 7–8) with $NADP^+$ (final concentration, 1–10 mmol L^{-1}) and resazurin were added to the agar plug after solidification. Samples were bubbled with air (oxic) or nitrogen gas (anoxic) or 500 μmol L^{-1} Na-dithionite was added (reducing, E_{m7} = 610 mV). The ratio of seawater to agar plug varied from 2 : 1 to 16 : 1. In this system, sulfur chemistry is defined by the redox couple

$$\frac{1}{8}SO_4^{2-} + \frac{9}{8}H^+ + e^- = \frac{1}{8}HS^- + \frac{1}{2}H_2O \qquad pe^o = 4.25 \quad (3)$$

The acid-base chemistry of reduced sulfur is governed by

$$HS^- + H^+ = H_2S_{(aq)} \qquad pK_1 = 7 \quad (4)$$

$$S^{2-} + H^+ = HS^- \qquad pK_2 = 13.9 \quad (5)$$

Equation 5 is irrelevant in the biological system we are considering. The relationship between redox poise of a given chemical system, pH, and the $NADP^+$/NADPH couple can be derived from the following:

$$NADP^+ + 2H^+ + 2e^- = NADPH + H^+$$

$$pe^o = -2.0; \qquad E_{m7} = 0.32 \text{ V} \quad (6)$$

$$pe = \frac{1}{n}[\log \text{K-log(red/ox)}] \quad (7)$$

$$E_h = (2.3RT/F)pe \quad (8)$$

Where pe^o is a nondimensional scale of electron activity and *Eh*, the redox potential, is measured in volts (V). Equation 7 is the Nernst equation, which defines the electrical potential of any redox half reaction. From these equations, we can derive the relationship among the redox poise of a system (pe_{sys}), pH, and the NADPH/$NADP^+$ ratio (defined in the following equation as *Q*)

$$pe_{sys} = pe^o - \frac{1}{n}\log[Q] + \frac{1}{n}\log[H^+] \quad (9)$$

If we also assume that the intracellular environment of the chloroplast varies and is not equal to pH 7, the relationship between the pe_w^o (pH = 7) of the NADP+/NADPH half reaction and pe^o is

$$pe_w^o = pe^o + \frac{1}{n}\log[H^+] \quad (10)$$

Measurements of $NADP^+$ reduction were performed spectrophotometrically and fluorometrically on abiotic samples incubated for 24 h. Measurements of NADPH spectra were made on an Aminco DW-2000 spectrophotometer in 1-cm quartz cuvettes. Fluorometric measurements were made on a Molecular Devices plate reader (Spectra max gemini XS; 280 nm excitation, 340 nm emission).

Immunolocalization of nitrate reductase—A polyclonal antibody (Vergara et al. 1998) derived from nitrate reductase obtained from the marine diatom *Thalassiosira weissflogii* was tagged with a green fluorescent probe (Alexa 488; Molecular Probes) according to manufacturer's protocols. Frozen samples of *N. stella* were thawed, rinsed in PBS (pH 7.8), and permeabilized with 1% dimethyl sulfoxide in PBS overnight at 4°C. Samples were then rinsed twice and incubated in a 1 : 200 dilution of antibody with PBS. Both samples were incubated in microtiter plates overnight and rinsed three times in PBS. Samples were mounted on slides and examined under an epifluorescence microscope (Zeiss Axioscop 20). Foraminiferal samples were incubated in a 1 : 200 dilution of the green fluorescent probe with no antibody and cultures of *T. weissflogii* grown with NH_4^+ and challenged with the tagged NR antibody were used as negative controls.

Results

Chloroplast identification—Ultrastructural analysis of the engulfed chloroplasts reveals a centrally located pyrenoid with a single lamella passing through the center of the organelle, a three-layer thylakoid lamellae along the periphery, and interconnections between the lamellae (Fig. 1). All of these morphological features are characteristic of heterokonts and suggest that the original source of the plastids was one or more species of diatoms (Dodge 1973).

Confirmation of the origin of the organelle was obtained by sequencing 16S rDNA genes. A typical RFLP gel from the 16S rDNA PCR product reveals two dominant patterns of clones (Fig. 2). Two 400-bp sequences corresponding to the two patterns were consistently identified in all samples from three different cruises (N.sChl1, accession number

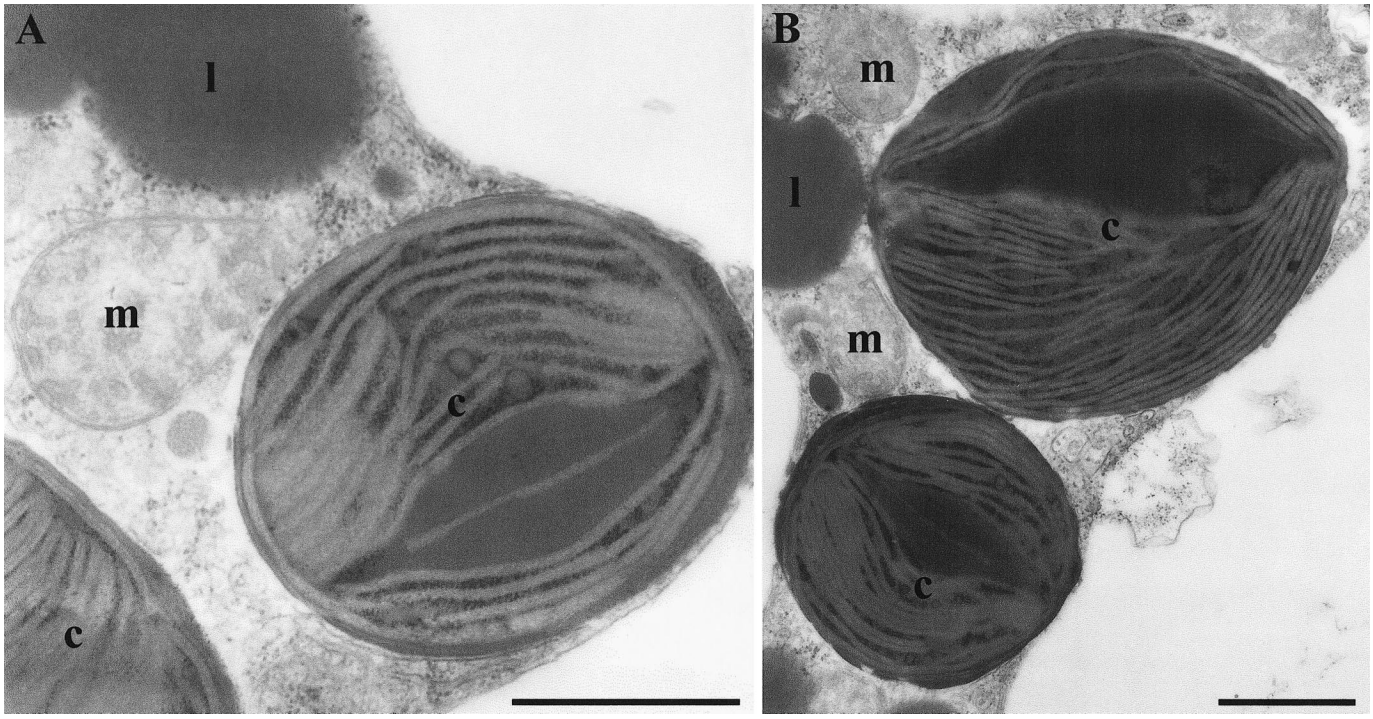

Fig. 1. Transmission electron micrographs of *N. stella* cytoplasm showing plastids. Note the similarity of the chloroplasts (c): the outer lamella and the single pyrenoid with the transverse lamella. l, lipid; m, mitochondrium. Scale bars = 1 μm.

AF495756 and N.sChl2, accession number AF495757). *N. stella* chloroplast sequence 1 (N.sChl1) is phylogenetically related to the cosmopolitan diatom, *Skeletonema costatum* (Fig. 3), whereas N.sChl2 is closely related to *Odontella sinensis* (Fig. 3). Fifty clones from three independent samples of foraminifera were digested, and ~80% of the clones exhibited the two dominant restriction digest patterns shown in Fig. 2. Of the 15 unique clones that were partially sequenced, only two other chloroplast sequences were retrieved from BLAST searches. These were uncultured, unidentified marine 16S rDNA chloroplasts from environmental samples. Despite collecting samples from different years and in the fall and spring, the same diatom sequences and RFLP patterns were found in *N. stella.* The

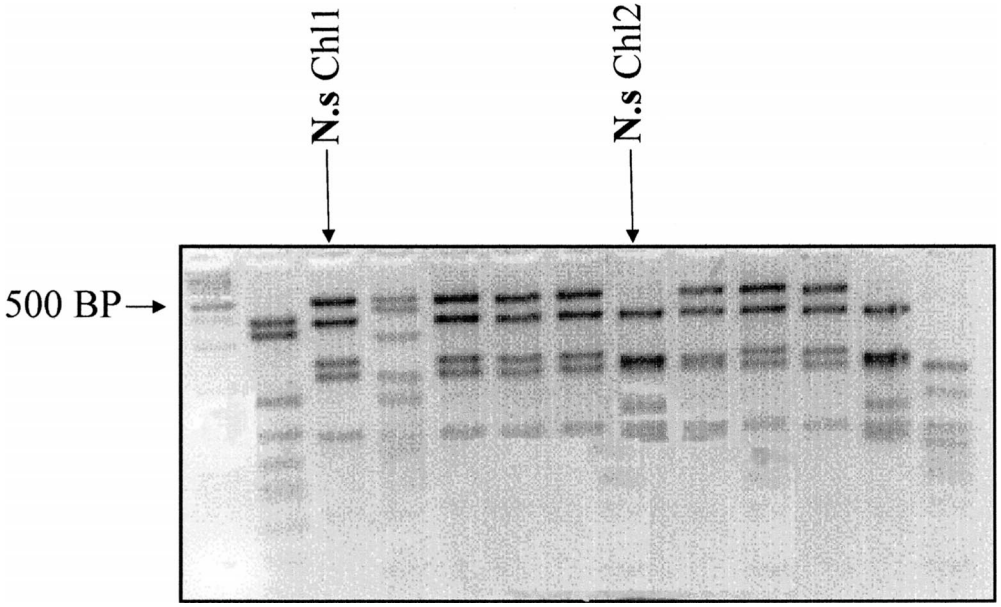

Fig. 2. RFLP of 16S clonal library from *N. stella.* The two dominant patterns denoted N.sChl1 and N.sChl2 are highlighted by arrows. Sequences for these chloroplasts are shown in Fig. 3.

	181										193
Alexandrium fundyense	T	A	C	A	T	G	G	C	A	A	C	A	-
Emiliania huxleyii	C	G	C	A	C	G	G	C	T	C	T	A	C
Skeletonema costatum	T	A	T	A	T	T	T	A	T	C	C	G	-
Odontella sinensis	T	A	T	-	-	-	-	-	-	C	T	G	-
N.sChl1	T	A	T	A	T	T	T	A	T	C	C	G	-
N.sChl2	T	A	T	-	-	-	-	-	-	C	T	G	-

Fig. 3. 16S rDNA sequence comparisons between a representative dinoflagellate, *Alexandrium fundyense*, a representative prymnesiophyte, *Emiliania huxleyii*, and two diatoms, *S. costatum* and *O. sinensis*, as well as the two clones from the present study, N.sChl1 and N.sChl2.

most significant difference between N.sChl1 and N.sChl2 is an 8-bp difference starting at position 184 on the published *S. costatum* sequence (Fig. 3). This feature is also a major sequence difference between *S. costatum* and *O. sinensis* (96.1%, homologous, Fig. 3). The major differences in 16S rDNA sequence between species of phytoplankton representing the three phylogenetically distinct eukaryotic algal taxa with chromophyte plastids, namely diatoms, dinoflagellates, and prymnesiophytes, are also shown in Fig. 3. The dinoflagellate and prymnesiophytes are only 50% homologous to diatoms.

Chloroplast proteins—Western blot analyses of total protein extracts from *N. stella* confirm the presence and similar size of three key chloroplast proteins found in diatoms: the photosytem II reaction center protein, D1, the carboxylating enzyme RuBisCO, and FCP. The FCP, found in chromophytes and some dinoflagellates, is encoded by a gene family that contains at least five genes (Green and Durnford 1996). The two major pigment-binding proteins migrate with apparent molecular masses of ~21.6 and ~23.8 kDa (Fig. 4A). Similar analyses of the diatom *T. pseudonana* are presented for comparison. The FCP antibody cross-reacts with both the foraminiferal chloroplast and the diatom, indicating that the fucoxanthin–Chl *a* protein is the dominant light-harvesting complex. A control antibody for the squash Chl *a/b* protein-binding protein (which cross reacts with chlorophyte algal light harvesting complexes) was also challenged against the diatom and foraminiferal proteins (Fig. 4A). The minor, single-band cross-reaction is presumed to be a Chl *a* protein component of the complex.

The large subunit of RuBisCO migrates with an apparent molecular weight of 53 kDa (Fig. 4B). The D1 component of the PSII structural D1/D2 dimer is present at 32 kDa (Fig. 4C). Both D1 and RuBisCO are encoded in the chloroplast, whereas FCP is encoded in the nucleus of the diatom. The blots presented were from foraminiferal samples that were between 9 and 12 months old.

Pigment characterization—The 77 K fluorescence excitation emission spectra show that the majority of the pigments contained in *N. stella* chloroplasts are located on proteins to form pigment protein complexes containing Chl *a*, *c*, and fucoxanthin. The chlorophyll degradation products, chlorophyllide *a* and pheophytin, are also present. The 77 K

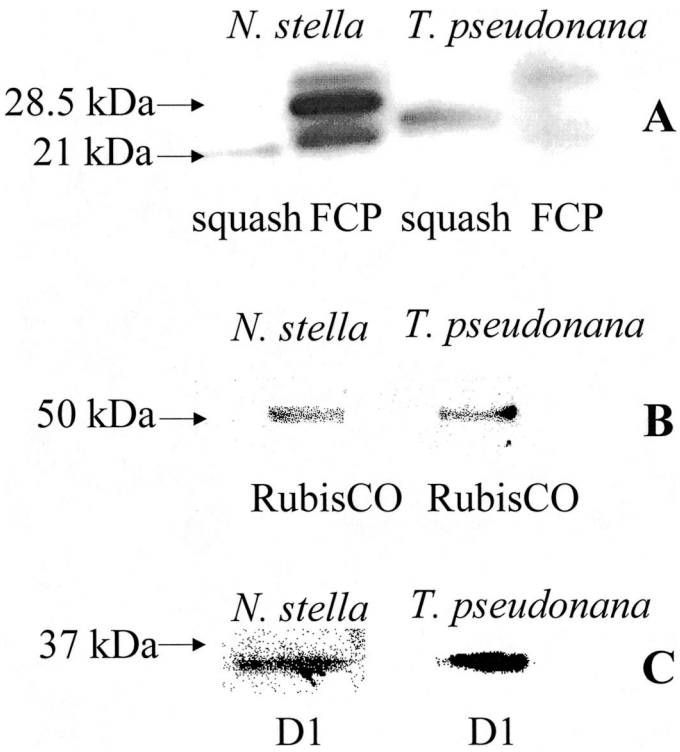

Fig. 4. Digitized images from Western blots of protein extract from *N. stella* and the marine diatom *T. pseudonana*. (A) Proteins challenged with the antibody for the major light harvesting complex of Chromophyte algae, FCP, and the major light-harvesting complex for the Chl *a*, *b*–containing plant, squash. (B) Proteins challenged with the antibody for the carboxylating enzyme RuBisCO. Shown is the cross-reaction with the 53-kDa large subunit. (C) Proteins challenged with the antibody for the 32.5-kDa D1 component of the major structural dimer of PSII.

fluorescence excitation spectrum of isolated *N. stella* (Fig. 5A) exhibits characteristic excitation energy of antenna pigments to Chl *a*. Specifically, there is a high rate of excitation energy in the blue (400–450 nm) that is emitted in the red. The blue absorption corresponds to the Soret bands of chlorophylls a and c and fucoxanthin. The broad maximum between 400 and 500 nm, due to increased light harvesting pigments, is a characteristic of low-light adaptation. The extraction of the pigments and in vitro absorption spectrum of *N. stella* also reveal that the majority of pigments are intact and not degraded (Fig. 5B). In vitro absorption of degraded chlorophylls and other pigments have higher blue : red ratios (>5) and have significant peak shifts to lower wavelengths. The in vitro absorption properties of *N. stella* are characteristic of intact, Chl *a/c*–containing phytoplankton with peaks at 432, 618, and 664 nm, and a broad shoulder at 460–500 nm. The blue : red peak ratio is ~5, also characteristic of nondegraded in vitro absorption properties of diatoms (Johnsen and Sakshaug 1993).

The 77 K emission spectrum and Gaussian deconvolution from ~20 foraminifera is shown in Fig. 6. The major Gaussian curves at 658, 673, and 686 nm correspond to Chl *c*, FCP, and PSII. The 704- and 735-nm peaks can be attributed to photosystem I. Part of the 686-nm peak could also be due

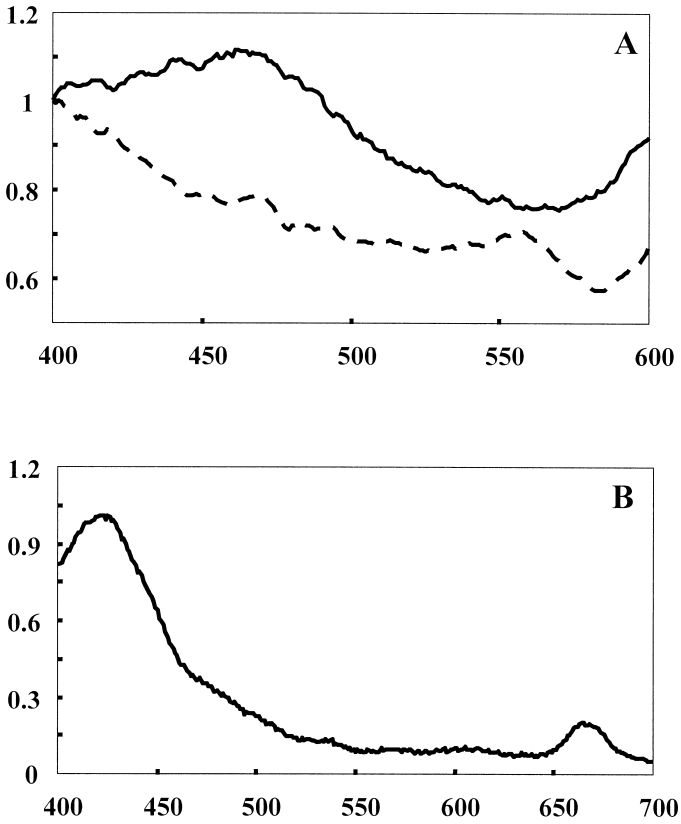

Fig. 5. (A) Fluorescence excitation spectra scaled to 1 at 400 nm (440 ex, 680 em) for *N. stella* (solid line) and Santa Barbara Basin mud (dashed line). (B) In vitro absorption spectrum for a 90% acetone extract of *N. stella.*

to PSI without the light harvesting complex I (Brown 1988). The 77 K emission curve provides further evidence that antenna pigments are capable of transferring light energy to the reaction center chlorophylls, P680 and P700.

Photochemical yields and enzyme activity assays—To assess whether the enslaved plastids retained photochemical activity, we examined the potential for primary charge separation using fast repetition rate fluorometry (Kolber et al. 1998). Foraminifera removed from the mud and placed in cold seawater exhibited a fluorescence transient with a very low quantum yield of photochemistry (Fig. 7, Table 1). Nonetheless, stable charge separation and a small reduction in Q_A were recorded. The calculated effective absorption cross section for PSII photochemistry was ~780 A^2 at 450 nm. Is this limited but viable photochemical potential sufficient to drive solar-based photosynthetic electron transport in Santa Barbara basin sediments?

At 600 m, calculated solar radiation could vary between $9.0e^{13}$ and $5.0e^{16}$ quanta m^{-2} s^{-1} depending on the attenuation coefficient (Table 1). The upper range is only a theoretical maximum and assumes no attenuation of light from any substance other than water. ETR were calculated from the measured absorption cross-section, the quantum yield of photochemistry, and the light flux. From Eq. 1 it is clear that light flux is the dominant variable responsible for the rate of electron flow; theoretical fluxes of light could vary by at least three orders of magnitude, given Beer's law. Thus, ETR in foraminiferal chloroplasts could potentially vary between a maximal rate of one electron every 2 min to approximately one electron every 4 h (Table 1).

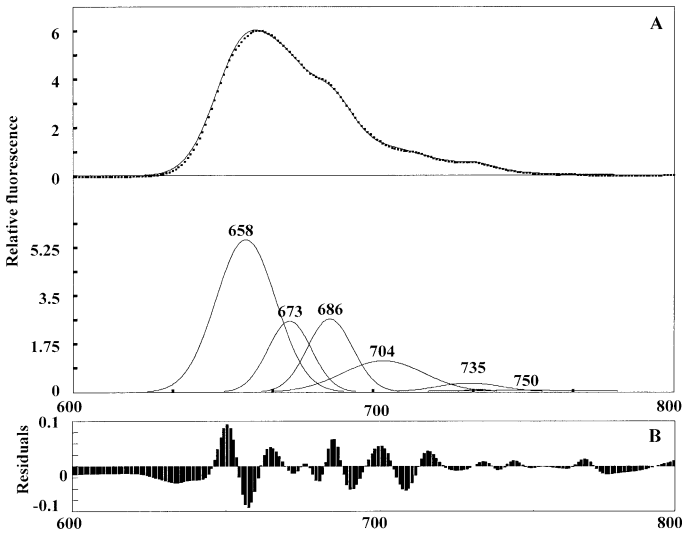

Fig. 6. (A) 77k emission spectrum (squares) of *N. stella* (440 ex) and the corresponding fit of the Gaussian peaks (smooth line). (B) Gaussian curves of the six major components of the emission spectrum and the residuals of the fit. The major components of the spectrum are explained in the text.

N. stella stored at 4°C in the laboratory had measured RuBisCO and PepC activity that averaged 0.12 and 0.04 μmol C (mg Chl *a*) h^{-1} (Table 1). RuBisCO activity is significantly lower than activities published elsewhere (Macintyre et al. 1996), where activities ranged from ~10–20 μmol C (mg Chl *a*) h^{-1}. However, sample preparations, cell breakage, and time factor into the different measurements. Our rates are measured on samples that are ~1 yr old; therefore,

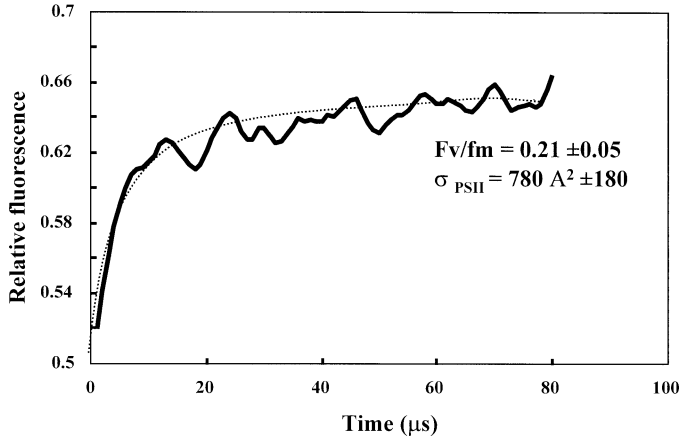

Fig. 7. Fast-repetition rate fluorometric measurement of *N. stella.* The curve is an average of 10 measurements and shows the change in fluorescence after a series of subsaturating blue light-emitting diode flashes, as described in the text. The dotted line is the exponential fit as described in Kolber et al. (1998).

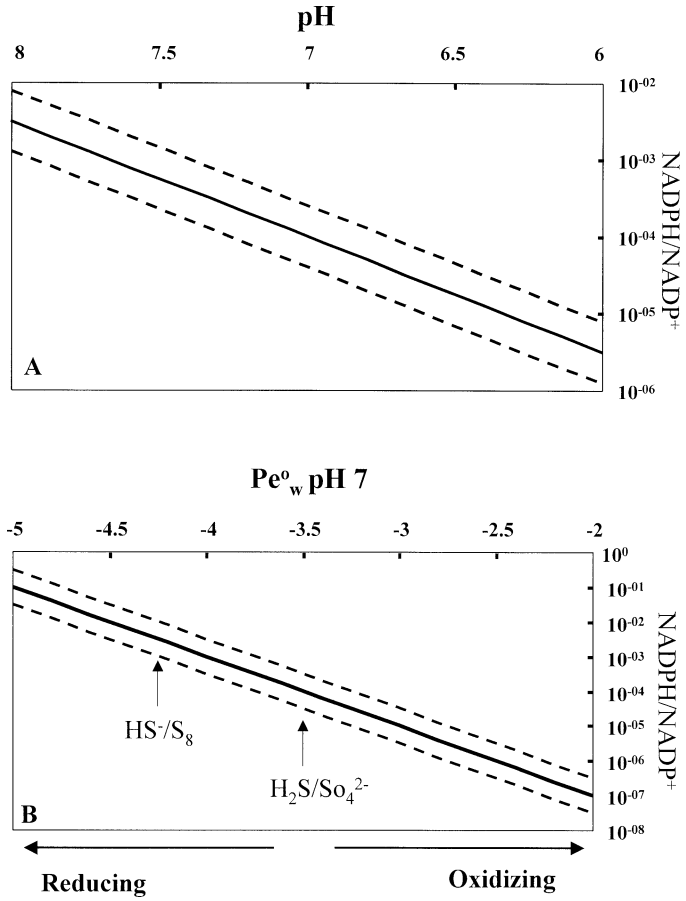

Fig. 8. (A) Relationship between pH and *Q* (NADPH/NADP$^+$) different reducing conditions. Previously published *pe*s for the NADPH/NADP$^+$ couple correspond to the black line. Upper and lower error limits are presented. (B) The relationship between the redox poise of a system and *Q* calculated from the equations in the text.

the half-life of the RuBisCO enzyme activity can be estimated to be at least 50 d.

Abiotic NADPH reduction—To assess whether the plastids can synthesize reducing power for the chemoautotrophic synthesis of organic carbon, we tested the possibility of reducing NADPH under conditions similar to those found in the sediments of the Santa Barbara basin. Our experimental system for testing abiotic NADPH reduction yielded results that did not significantly differ from calculations based on redox potential ($P > 0.1$). The pH of the system was fixed, and the pe^o of our system was equivalent to the H_2S/SO_4^{2-} couple $pe_w^o = -3.5$; therefore, according to Eq. 9, we could verify the pe^o of the NADPH/NADP$^+$ couple (-2.0 ± 0.2). These values are similar to -1.9 and -2.0 reported elsewhere (Nicholls and Ferguson 1992; Morel and Hering 1993). The range of *Q* given different redox couples and pH is plotted (Fig. 8). The error in the measurements is equal to about a decade change in *Q* (NADPH/NADP$^+$); however, we consider this minor at relevant biological pHs equaling between 3 and 30 nmol L^{-1} NADPH in 100 μmol L^{-1} NADP$^+$ at pH 7. The change in *Q* given the published pe^os of -1.9 and -2.0 approximates a 10-nmol L^{-1} change under the same circumstances. The plot of pH and *Q* illustrates the important effect of pH (given a constant pe_{sys}) on both the pe^o of the system and the pe^o of NADPH. For example, given the dominant redox couple H_2S/SO_4^{2-}, as pH increases the pe^o of the system becomes more reducing, thus increasing *Q* according to Eq. 9 (Fig. 8A). Changes in *Q* are also affected in systems where pH is held constant and the dominant redox couple changes (Fig. 8B).

Immunolocalization of nitrate reductase—Samples of *N. stella* cross-reacted with the fluorescent-tagged nitrate reductase antibody, resulting in a green fluorescence signal (Fig. 9A,B). Samples of *T. weissflogii* grown on NH_4^+ had no cross-reaction with the antibody except for a small background signal from the diatom frustule (Fig. 9C). There was a strong cross-reaction of the fluorescent NR antibody with *T. weissflogii* grown with NO_3^-. In these samples, there was a high green fluorescent signal that also corresponded to the red autofluorescent diatom plastid (Fig. 9C).

Discussion

Ultrastructure and sequence identity—The chloroplasts in *N. stella* are intact. However, the mechanisms of chloroplast selection and capture are unknown. Foraminifera are phagotrophs. However, the even distribution of autofluorescence seen in cells of *N. stella* (Bernhard and Bowser 1999) suggests a selective mechanism for retaining plastid integrity. Our sequence data suggest some selective ingestion or digestion of algal material occurs in the foraminiferal host. The majority of chloroplast sequences in our library of 16S rDNA data from *N. stella* were from two diatoms. Feeding and subsequent partial digestion of phytoplankton were limited to the two close relatives of *S. costatum* and *O. sinensis*, designated N.sChl1 and N.sChl2. Chai and Lee (2000) have detected a glycoprotein on the surface of diatoms that foraminifera sequester. Diatoms that did not have the surface protein were not sequestered. Regardless of the specific intracellular or extracellular mechanism responsible for *N. stella* plastid capture, the data from three different cruises are consistent with specificity of plastid endosymbiosis in *N. stella.* Furthermore, there is no relationship between the natural abundances of phytoplankton in a given ecosystem and their presence in foraminifera (Lee et al. 1989; Chai and Lee 2000). The two dominant sequences found in *N. stella* are not the major phytoplankton in the overlying water of the Santa Barbara Basin or in the sediment (Reimers et al. 1990). Furthermore, *O. sinensis* has not been documented in the historical data set (>25 yr) of the Santa Barbara Basin water column (E. L. Venrick pers. comm.).

Bio-optical properties of sequestered plastids—All of our spectroscopic data suggest that the plastids are capable of energy transfer given a photon source. This does not necessarily imply that the plastids are used for photoautotrophy. The differences between excitation spectra of in vivo plastids and Santa Barbara basin mud (which presumably contain plastids and whole phytoplankton) offer proof that the intracellular environment of the foraminifera is a refuge from

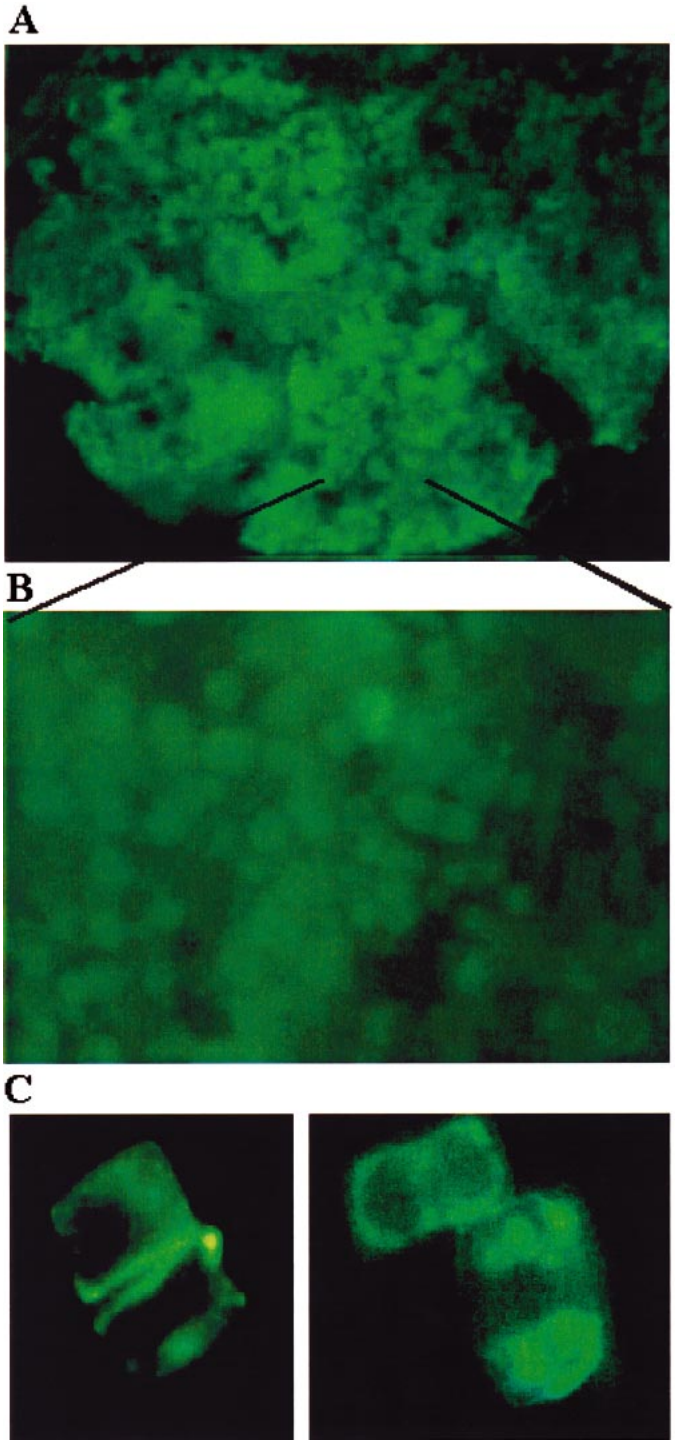

Fig. 9. (A) Epifluorescence micrograph (×400) of one chamber of *N. stella* showing the location of the Alexa 488–tagged nitrate reductase enzyme. (B) As in panel A, except ×1,000 showing individually labeled microbodies that were identified as chloroplasts by their autofluorescence (not shown). (C) Epifluorescence micrograph of *T. weisfloggii* grown in media that contained NH_4^+ as the source of inorganic nitrogen (left panel) and NO_3^- (right panel).

chloroplast degradation. Foraminifera in general have a propensity to enslave chloroplasts; they appear to be particularly good microhabitats for the establishment and maintenance of these chloroplasts (Lee and Anderson 1991). Although hypoxia and the absence of light in the Santa Barbara basin probably help reduce the rate of plastid degradation, the indefinite maintenance of energy transfer capabilities requires nuclear encoded proteins.

Low-temperature excitation spectra measure the relative emission of Chl *a* associated with PSII and PSI throughout the absorption range of pigments. Emission spectra measure the intensity of Chl *a* fluorescence at the wavelength of maximum absorption. The curves indicate the presence of PSI, PSII, FCP, and free pigment. Brown reports for diatoms that the relative yield of PSII fluorescence is ~10 times that of PSI (Brown 1988). The amplitudes of our deconvolution analysis suggest that the ratio of PSII : PSI is ~16. Not surprisingly, there was a signal also associated with free pigment and/or lower vibrational energies of Chl *a* and its degradation products. The measurement demonstrates that a significant fraction of the fucoxanthin-chlorophyll–binding proteins identified via Western blot analyses were capable of transferring light energy to the reaction center chlorophylls of PSI and PSII.

Enzyme activity and longevity of proteins—A significant problem exists for the host of a plastid regardless of plastid function. That is, how are plastid proteins maintained when the majority was derived from nuclear genes? All of our samples, regardless of age, contained intact proteins as determined by Western blot analysis. The same is true for all other proteins we challenged with antibodies. This does not mean that the proteins are imported into the chloroplast from remnant diatom nuclei or from actual foraminifera nuclei containing FCP genes, although this is an intriguing phenomenon. Most likely, the proteins, like the chloroplasts, are extremely stable. Similarly, enzyme assays on two proteins, RuBisCO and pepC, yielded activities that were suggestive of extremely slow turnover of chloroplast proteins. The RuBisCO enzyme is composed of two subunits that, in diatoms, are both encoded in the chloroplast. Therefore, if the host organism was utilizing the chloroplast for carbon reduction, de novo synthesis of the protein would have to occur or the pool of enzymes captured would have to remain functional. Although chloroplast gene expression in sequestered chloroplasts has been reported (Green et al. 2000), it has never been demonstrated that the organelles replicate within the host and are thus able to live indefinitely from generation to generation.

The D1 protein is the 32-kDa product of the psbA gene. The D1/D2 dimer is the major structural complex of PSII and is encoded in the chloroplast. The D1 component is rapidly degraded and resynthesized in the light, and the turnover is strongly correlated to irradiance (Tyystjarvi and Aro 1996). Data from pea thylakoid preparations show that D1 protein turnover is very slow under anaerobic or reducing conditions (Nedbal et al. 1990). Similar processes occur in diatoms (J.G. unpubl. data). Therefore, although it is possible that D1 protein turnover proceeds and is synthesized de novo, the parsimonious explanation is one of environmental

protection. The extremely low photon fluxes combined with the decreased pe_w^o (more reducing conditions) reduce the turnover pressure and the protein remains stable. Unless the pe_w^o of the chloroplast were to remain oxidizing in the mildly reducing environment of the mud, the plastoquinone pool ($pe_w^o = -0.5$) would be almost completely reduced. This would have a similar effect on electron transport as the herbicide DCMU, which blocks electron transport from Q_A to Q_B. DCMU-treated chloroplasts have significantly decreased D1 turnover rates (Kirilovsky et al. 1994). It is likely that the sequestered chloroplasts are stable for at least a year. Even if de novo protein synthesis does not occur, the chloroplasts could be a favorable metabolic addition to the host. The upper limit of sequestered chloroplast survival in these environments is unknown.

Chloroplast function: the light hypothesis—Our measurements of the quantum yield of photochemistry and the calculations of light flux preclude the possibility of photosynthesis and oxygen evolution from the solar radiation at depth in the Santa Barbara Basin. It should be emphasized that without another source of light flux, the theoretical upper limit of electron transport is much lower than that half time for the back reactions in PSII (Radmer and Kok 1973; Diner and Joliot 1977). Hence, given the basic biophysical constraints on oxygenic photosynthesis, solar-driven photosynthetic electron transport is not viable in situ in the Santa Barbara basin sediments. More specifically, oxygen evolution occurs after the accumulation of four positive charges in the Mn cluster of the oxygen evolution complex. After this accumulation, termed the S_4 state, two molecules of water are oxidized to O_2. The penultimate S-state before oxygen evolution, the S_3 state, has a half-time of decay ~3 s (Radmer and Kok 1973). Thus, it is highly unlikely that an ETR of $8.7e^{-03}$ s^{-1} (or one electron every 120 s) would enable the system to ever proceed beyond S_3. The lower photon flux density limits of photosynthetic growth have been thoroughly reviewed (Raven et al. 2000). The minimum photon flux required for photosynthesis in the Santa Barbara Basin must be at least 100-fold greater.

It is likely that the biological signals we measured from the chloroplasts are a product of environmental stability resulting from no light, relatively cold constant temperatures, and some synergism between host and chloroplast. Although the chloroplasts, especially their light-sensitive pigments and proteins, remain preserved and functional other aspects of photosynthetic regulation, especially photoacclimation, are lost. Chloroplasts poised to photosynthesize would change the fluorescent characteristics, the quantum yield of photochemistry, and the size of the PSII reaction center when exposed to blue and red light regimes. However, this did not occur.

The oxidation of sulfide is a chemiluminescent reaction and is potentially responsible for the reports of light production in deep-sea hydrothermal vent environments (Van Dover et al. 1988; Pelli and Chamberlain 1989; Tapley et al. 1999). Chemiluminescence is positively correlated with sulfide concentration and requires oxygen. In the Santa Barbara Basin, sulfide concentrations can reach 250 μmol L^{-1} at sediment depths of 7 cm where there is no oxygen; foraminifera have only been found to depths of 3 cm where sulfide concentrations typically range from nmol L^{-1} to μmol L^{-1} M concentrations (Bernhard and Reimers 1991; Kuwabara et al. 1999). Therefore, sulfide oxidation is not responsible for a significant light flux (capable of generating oxygen via photosynthesis) in the Santa Barbara Basin.

Chloroplast function: the sulfur hypothesis—Sulfur chemistry has additional potentially important biological roles in the Santa Barbara Basin ecosystem. The spontaneous oxidation of sulfide in the presence of oxygen is highly favorable ($K = 10^{32}$) and yields damaging oxygen and sulfide radicals (Tapley et al. 1999). Sulfide is extremely toxic. It disrupts mitochondrial respiration and the $NADH/O_2$ redox reaction by poisoning the cytochrome c complex (Hochachka and Somero 1984). Given the success of *N. stella* in this environment, it is possible that it has adapted to sulfide toxicity by substituting the chlororespiratory pathway for mitochondrial respiration during periods of high intracellular sulfide concentrations. Metabolically, this means that the chloroplast is the site of adenosine triphosphate (ATP) production and is not also incapacitated by sulfide. Chlororespiration is an aphotic process that utilizes stored reductant (NADPH) to reduce the plastoquinone (PQ) pool and create the electrochemical gradient necessary to generate ATP (Bennoun 1982). We think this is unlikely, because sulfide toxicity or a high a concentration of sulfide is spatially and/or temporally separate from the presence of oxygen in the sediments. Unless there is an oxidase in the chloroplast similar to the plastid terminal oxidase found in green algae (Cournac et al. 2000), there is not a sufficiently strong oxidizer to complete the oxidation of PQ; therefore, a sulfide driven chlororespiratory pathway could not occur. Furthermore, our measurements of abiotic NADPH reduction given the Santa Barbara Basin and cellular redox environment suggest that this type of process would not be renewable. In the event that there is a simultaneous presence of sulfide and oxygen the foraminifera would be exposed to oxidative radicals. The generation of H_2O_2 via sulfide oxidation could explain the large number of peroxisomes present in *N. stella* (Bernhard and Bowser 1999). In general, *N. stella*, a eukaryote, has adapted to a complex chemical environment where few eukaryotes thrive. *N. stella* survives multiple but temporally and spatially separate problems: sulfide toxicity, anoxia, and sulfur and oxygen radical generation.

Chloroplast function: the nitrogen hypothesis—We have noted the presence and activity of the carboxylating enzyme RuBisCO in the sequestered chloroplasts of *N. stella.* The strategy of sequestering chloroplasts in photic environments and living off of the reduced carbon from photosynthesis is well documented; however, in an oligotrophic environment this would certainly lead to nitrogen starvation. The coral–zooxanthellae symbiosis is a model system for effective nitrogen cycling, especially given that the assimilation of NH_4^+ by glutamate dehydrogenase (GDH) may not be favored in numerous marine invertebrates (Muscatine et al. 1979; Moyes et al. 1985). The Santa Barbara Basin is an organically rich environment; however, if we assume that periods of denitrification occur in the upper sediments of the

Santa Barbara Basin, foraminifera might not be able to meet their nitrogen requirements. Concentrations of NH_4^+, NO_3^-, and NO_2^- in the upper centimeters of Santa Barbara Basin sediments vary substantially from undetectable to ~100 μmol L^{-1} (Bernhard and Reimers 1991).

Diatoms assimilate inorganic nitrogen via the glutamine synthetase and glutamate 2-oxo-glutarate amidotransferase (GOGAT) pathway after the reduction of NO_3^- and NO_2^- (Zehr and Falkowski 1988; Zehr et al. 1988). Therefore, given an unfavorable host GDH pathway or high fluxes of NO_3^- and NO_2^- from overlying water, glutamate could be provided to the host by the chloroplast nitrogen assimilation pathway. In fact, blocking GOGAT activity in zooxanthellae results in NH_4^+ release from corals, which suggests that the role of GDH is more closely tied to respiration than to NH_4^+ assimilation (Rahav et al. 1989). The presence of the diatom nuclear encoded enzyme nitrate reductase in *N. stella* supports this hypothesis. The protein is essential for the assimilation of NO_3^- for use as a nitrogen source for amino acid biosynthesis.

The protist *N. stella* is a modern analog to the endosymbiotic origin of chromophyte algae. Chromophytes are a diverse group of heterokont and haptophyte algae that are the result of secondary symbioses between a protistan heterotroph and a eukaryotic alga (Delwiche and Palmer 1997; Delwiche 2000). The modus operandi of plastid capture and retention is unknown. However, some metabolic challenges were overcome through the incorporation of the chloroplast. It is easy to assume that the primary metabolic advantage would be photoautotrophy. However, this is not the case with *N. stella*. In heterotrophic (nonphotosynthetic) algae that contain plastids, a major metabolic advantage is the ability to grow on oxidized forms of nitrogen. In fact, in the modern ocean, this is almost a requirement for all photoautotrophic growth (the exceptions are the diazotrophs) (Falkowski 1997).

Recently, the marine diatom *Phaeodactylum tricornutum* was engineered to grow heterotrophically (Zaslavskaia et al. 2001). This is a potential model system of our described adaptation of the chloroplast. The *P. tricornutum* mutant (Glut1-17) has the same growth rate on glucose in the light or dark, which implies a reduced role of the chloroplast (Zaslavskaia et al. 2001). Much like *N. stella*, the quantum yields of photochemistry and electron transport rates in *P. tricornutum* are very low. In the dark, the chloroplast ceases to function autotrophically, yet it is maintained. Instead of capturing chloroplasts, the *P. tricornutum* mutant has the genetic machinery to synthesize them, despite not using them in photosynthesis. The mutant's only source of nitrogen is NO_3^-, which is imported into the chloroplast after reduction to nitrite. The chloroplast is the site of nitrate reduction and the formation of glutamine. Thus, in these mutants and all heterotrophic, plastid-containing algae growing on NO_3^-, one of the major roles of the chloroplast is NH_4^+ assimilation.

N. stella is a unique organism because it sequesters plastids in a relatively deep benthic environment unsuitable for photosynthesis. However, there are many other organisms that have plastids and do not photosynthesize. These organisms appear in the photic zone and are also genetically engineered in the lab. These exceptions help us conclude that, in oxidized environments or in environments depleted in organic nitrogen, the chloroplast becomes increasingly important to meet the metabolic requirements of the cell, regardless of the light regime.

Even more important, the case of enslaved chloroplasts in *N. stella* raises the important distinction between a symbiosis and a ''unibiosis.'' All chloroplast enslavements confer some ecological or even evolutionary advantage on the host cell and are thus at least unibiotic. Only in the specific case in which the enslaved plastid is able to survive and replicate from generation to generation is it appropriate to call the relationship symbiotic. It remains to be seen whether the plastid–*N. stella* relationship is truly symbiotic.

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