MIXOTROPHY AND NITROGEN UPTAKE BY *PFISTERIA PISCICIDA* (DINOPHYCEAE)\(^1\)

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The nutritional versatility of dinoflagellates is a complicating factor in identifying potential links between nutrient enrichment and the proliferation of harmful algal blooms. For example, although dinoflagellates associated with harmful algal blooms (e.g. red tides) are generally considered to be phototrophic and use inorganic nutrients such as nitrate or phosphate, many of these species also have pronounced heterotrophic capabilities either as osmotrophs or phagotrophs. Recently, the widespread occurrence of the heterotrophic toxic dinoflagellate, *Pfisteria piscicida* Steidinger et Burkholder, has been documented in turbid estuarine waters. *Pfisteria piscicida* has a relatively proficient grazing ability, but also has an ability to function as a phototroph by acquiring chloroplasts from algal prey, a process termed kleptoplastidy. We tested the ability of kleptoplastidic *P. piscicida* to take up \(^2\)H-labeled NH\(_3\), NO\(_3\), and urea, or glutamate. The photosynthetic activity of these cultures was verified, in part, by use of the fluorochrome, primulin, which indicated a positive relationship between photosynthetic starch production and growth irradiance. All four N substrates were taken up by *P. piscicida*, and the highest uptake rates were in the range cited for phytoplankton and were similar to N uptake estimates for phototrophic *P. piscicida*. The demonstration of direct nutrient uptake by kleptoplastidic *P. piscicida* suggests that the response of the dinoflagellate to nutrient enrichment is complex, and that the specific pathway of nutrient stimulation (e.g. direct stimulation through enhancement of phytoplankton prey abundance vs. direct stimulation by saprotrophic nutrient uptake) may depend on *P. piscicida*'s nutritional state (phototrophy vs. phototrophy).

**Key index words:** cryptophytes; fish kills; harmful algal bloom; kleptoplastidy; nutrient loading; *Pfisteria piscicida*; primulin; starch; toxic dinoflagellates

*Pfisteria piscicida* Steidinger et Burkholder (Dinophyceae) is one of several myzocytotically feeding "heterotrophic" dinoflagellates with a reported capability for kleptoplastidy, the process by which functional chloroplasts are retained from algal prey (Larsen 1992, Laval-Feuto 1992, Schnepl and Elbrächter 1992, Leutitus et al. 1999). Although this form of mixotrophy has been acknowledged in dinoflagellates for a number of years, studies examining the role and regulation of phototrophic and phagotrophic nutrition in kleptoplastidic species are rare (e.g. Fields and Rhodes 1991, Skovgaard 1998), at least partly a consequence of the difficulty in recognizing and culturing kleptoplastidic dinoflagellates (Schnepl et al. 1989, Schnepl and Elbrächter 1992, Leutitus et al. 1999). Schnepl and Elbrächter (1992) and Skovgaard (1998) suggested that dinoflagellates with an inconsistent chloroplast number per cell may be kleptoplastidic, and further speculated that the ability is more common in dinoflagellates than previously considered. It is also possible that problems associated with maintaining seemingly "phototrophic" dinoflagellates in a laboratory culture may be related, in some cases, to the unrecognized need to replenish prey supply. Awareness of the prevalence of these and other mixotrophic protists has grown dramatically in recent years (Stoecker 1998), stressing the need to understand their physiological ecology and role in microbial food web function.

Stoecker's (1998) classification scheme for mixotrophic protists presented a conceptual model for kleptoplastidic "protozoa" (e.g. some siliates, sarcodines, and dinoflagellates) based on their relative dependency on phototrophic vs. phagotrophic nutrition, and the response of these processes to light.
dissolved inorganic nutrients, and particulate food availability. In this model: (a) carbon, nitrogen, and phosphorus are acquired primarily through phagotrophy; (b) growth is linked to the low occurrence of the prolonged absence of prey; and (c) photosynthesis is thought to be used to supplement carbon nutrition by covering respiratory demands during periods when prey are abundant. The model is based primarily on information from studies with carcharodon or ciliates. Recently, Skovgaard (1998) suggested that photosynthesis in kelpoplastic Gymnodinium graciletinum is used primarily to enhance survival under food limitation, based on the short (~2 day) turnover time of kleptoplankton.

Kleptoplankton also has been demonstrated in Phaeodactylum tricornutum (Levin et al., 1999), an ichthyotoxozoid. It is believed that, over the last decade, has been implicated as a causative factor of fish kills in North Carolina estuaries and the Chesapeake Bay (Burkholtter et al., 1992, 1995, Burkholtter and Glasgow, 1997, Maryland Department of Natural Resources 1997, University of Maryland Center for Environmental Science 1997). Unlike G. graciletinum, a longer kleptochloroplant retention time (at least 9 days) was observed in P. tricornutum, but Levin et al. (1999) considered this and the low photosynthetic rates (0.14 pg C-cell-1 h-1) to be low or even absent in the photosynthetic capacity because of the limiting growth irradiance used in their experiment. Even at that low irradiance, however, a population doubling was measured. The photosynthetic activity was not essential for cell division. The growth, or even maintenance, of photosynthetically active P. tricornutum populations implies that nutrients were taken up selectively in support of photosynthesis. The photosynthetic activity of P. tricornutum to acquire nutrients directly has implications toward the hypothesized role of nutrient loading in promoting the dinoflagellate's growth and toxic activity (Burkholtter et al., 1995, 1997, 1998, Burkholtter and Glasgow 1997, Levin et al., 1999). It is recognition that P. tricornutum can function as a phytotankton, or not just as a heterotrophic protist, stresses consideration of nutrient stimulatory mechanisms analogous to those that affect phytoplankton. In this study, we tested the hypothesis that Kleptoplanktonic P. tricornutum can take up N nutrients directly.

MATERIALS AND METHODS

The Phaeodactylum tricornutum isolate was obtained from the New River estuary, North Carolina. This identification was confirmed by the plate configuration (Steidinger et al., 1996), using scanning electron microscopy (Glasgow, J., unpubl.). The nontoxic zoospores used in this experiment were derived from toxic zoospore cultures maintained in fish aquaria. Before beginning the experiment, toxic zoospores were transferred to media (0.5-KCl enriched seawater, Guillard 1975) but without Si containing Rhodomonas sp. Karent COMPT75 (Cryophyceae), an 8-μm diameter cryophyte previously shown to be a preferred prey species and source of kleptochloroplants for P. tricornutum (Glasgow et al., 1998, Levin et al., 1999). This culture was maintained at 25°C, 15 ppt, and a light-dark cycle of 12:12 h (80 μE m-2 s-1), and inspected microscopically several times a day for P. tricornutum feeding behavior.

After 5 days of maintenance, P. tricornutum was observed to shift from a lag phase, when growth was very slow, to a period of active feeding phase when the majority of the population was observed to rapidly ingest cryophytes (referred to as a "warming up" response) or feeding Daphnia (Glasgow et al., 1998, Levin et al., 1999). This feeding event was followed by a reduction in grazing activity, decreased cryophyte abundance, and increased P. tricornutum abundance. Fluxes after the feeding event, aliquots (75 mL) from this culture were gently transferred to 18 125-mL flasks. Six of these were sampled immediately (designated "initial" treatment), and 18 were placed in the dark. After being kept for 15 h in the dark, the 12 flasks were incubated at a relatively low irradiance (70 μE m-2 s-1) for 3 h, after which six flasks were sampled (designated "low light" treatment), and the remaining six placed under a relatively high irradiance (350 μE m-2 s-1) and sampled 3 h later (designated "high light" treatment). The principle of this experimental design was to allow comparison of the effect of light on both the experimental population, (containing recently ingested chloroplasts), (b) "low light" populations (kleptoplankton cultures examined after a brief pulse in growth irradiance). These three populations were considered to be kleptoplankton based on microscopic inspections indicating that grazing on cryophytes did not occur after the initial feeding event, and examination of DAPI-stained samples indicating that plastids containing P. tricornutum did not contain cryophyte nuclei (Levin et al., 1999).

The above cultures were sampled for P. tricornutum and Rhodomonas cell counts, particulate nitrogen, dissolved nitrogen (NO2, NO3), dissolved phosphorus, urea, and ammonia. Samples were filtered through 0.45 μm polycarbonate membrane filters, NO2 and NO3 concentrations were determined colorimetrically using a Technicon autoanalyzer, and urea was measured by the urease method (Parsons et al. 1984). Dissolved nutrients were measured in the filtrate from sterile 0.45 μm polycarbonate membrane filters after filtration onto precombusted Whatman GF/C filters. Flasks, and one last measurement was made using a Coulter Equipment Bright line hemacytometer (depth 0.5 mm). For particulate nitrogen, water was filtered onto precombusted Whatman GF/C filters, frozen, and a later measurement was made using a Coulter Equipment CHN analyzer (Parsons et al. 1984). Dissolved nutrients were measured in the filtrate from sterile 0.45 μm polycarbonate membrane filters. NO2 and NO3 concentrations were determined colorimetrically using a Technicon autoanalyzer, and urea was measured by the urease method (Parsons et al. 1984). Nutrient concentrations were determined by reverse-phase high-performance liquid chromatography (Lindroth and Mopper 1979). Nutrient uptake rates were determined using an in vivo techniques, following Gibert and Capone (1995). Labeled substrates were added at an initial concentration of 10 μg at N L-1, and samples were incubated for 30 min. Samples were then collected using a Fleetland MAT 251 mass spectrometer, coupled with a Varian 3400 elemental analyzer system.

Cell morphological observations were determined using image analysis (Optronics image analysis system with Flashpoint software) on 4,6-diamidino-2-phenylindole (DAPI) or DAPI-stained samples.
Fig. 1. The mean and standard deviation of *Pfiesteria piscicida* (white) or *Rhodomonas* sp. (black) abundance in the initial, low light, or high light cultures.

Free-living cryptophyte chloroplasts and zoospore kleptochloroplasts. Such is a photosynthetic product of cryptophytes that is produced within the periplasmic space (Santore 1985), and a previous study demonstrated the accumulation of starch within *P. piscicida* kleptochloroplasts (Lewitus et al. 1993).

In another experiment, we tested the relationship between the primulin-fluorescent area within *Cryptomonas* sp. HP9001 (Cryptophyceae) cells and the cellular nonstructural carbohydrates in batch cultures grown at three irradiances (211, 104, or 8 \(\mu\)E-m\(^{-2}\)-s\(^{-1}\)) and sampled from exponential growth phase. Samples were harvested by centrifugation, resuspended in a phosphate buffer, pH 6.8 (Lewitus and Caron 1990), and cell material extracted by cell disruption, using a Biospec Products Mini-Beadbeater-8\(^{TM}\) (two 65-s runs on “Homogenize” setting using 0.5-mm diameter zirconia/silica beads). After centrifugation (microcentrifuge, 16,000 X g), the supernatant and pellet were analyzed for total nonstructural carbohydrates, using 3% perchloric acid extraction and a colorimetric assay based on the phenolsulfuric acid reaction (Burke et al. 1992). Absorbance was converted to concentration in milligram glucose equivalents per milliliter using a standard curve (\(r = 0.992\)).

A test (significance level of 0.05) was used in all comparisons of means referred to below.

RESULTS

Mean *P. piscicida* population abundance in “initial” cultures (those sampled 5 h after the feeding event) was nearly 100-fold greater than that of *Rhodomonas* (Fig. 1). Cryptophyte abundance did not change significantly during the experiment, remaining below 200 cell-ml\(^{-1}\). Cultures incubated under “low” or “high” light contained ca. 40% lower zoospore abundances than initial cultures, but cell numbers did not vary with light treatment.

Primulin-stained samples were analyzed for three variables (Figs. 2, 3): (1) “Primulin-pigment,” the area within the plastid-containing zoospore vacuole characterized by primulin fluorescence (yellow to white area of epibeca in Fig. 2), (2) “Pfiesteria pigment,” the area within the plastid-containing zoospore vacuole characterized by pigment (phycocerythrin) autofluorescence (reddish orange area in Fig.
Fig. 4. The relationship between the primulin fluorescence area per cell and nonstructural carbohydrate content per cell in Gymatosoma sp. H9801 cultures. After total nonstructural carbohydrate extraction, carbohydrate concentrations were measured in both the dissolved (supernatant) and particulate (pellet) fractions. The carbohydrate content is plotted, based strictly on the particulate extract (A) or based on the sum of particulate and dissolved extracts (B).

2), and (3) "Rhodomonas primulin," the area within the free-living cryptophyte chloroplasts characterized by primulin fluorescence (not included in Fig. 2). In comparison with light treatments, the "Pfiesteria primulin" area per zoospore was nearly 50% greater in high-light than in low-light cultures, while the amount of "Pfiesteria pigment" or "Rhodomonas primulin" area did not vary with treatment (Fig. 3A). The treatment effect on Pfiesteria primulin fluorescence and not Rhodomonas primulin fluorescence is evidence that the positive relationship between growth irradiance and primulin-reactive material (presumably starch) is not a function of prey ingestion. Although the primulin fluorescent area per zoospore was significantly greater in cultures grown at high light, the relative proportion of this area to total cell area did not vary significantly with growth irradiance (Fig. 3B). The average area of high- and low-light cells was 54 and 47 µm², respectively (data not shown). The relatively greater absolute, but not proportional, Pfiesteria primulin area per cell in high vs. low light cultures suggests that the increase in cell size was related to an increase in primulin-stained cell material. Our assumption that this material was starch is supported by the strong correlation between primulin fluorescence area and nonstructural carbohydrate content in cells of the cryptophyte, Gymatosoma sp. (Fig. 4). The correlation coefficient between the primulin-stained area per cell and the carbohydrate content was 0.92 or 0.87 when carbohydrates were measured in the pelleted or total extract, respectively.

Particulate nitrogen decreased after incubation at low or high light by an amount (64% or 55%, respectively; Fig. 5A) that corresponded roughly to the decrease in zoospore cell abundance (61% or 59%, respectively, Fig. 1). The dissolved nitrogen pool was predominantly composed of NO₃ throughout the experiment (Fig. 5B), but the mean ratio of NH₄⁺, urea, and DFAA to NO₃ concentration increased over time by 61%, 164%, and 62%, respectively, at low light, and 40%, 183%, and 90%, respectively, at high light. These patterns suggest the net biological removal of NO₃ and regeneration of NH₄⁺, urea, and DFAA as a result of the process of the experiment.

Uptake of all four ¹⁵N-labeled substrates was detected (Table 1). Not surprisingly, given the relatively high NO₃ concentrations in the dissolved N pool, NO₃ was taken up at the greatest rate, followed by glutamate, NH₄⁺, and urea. Cells grown at high light took up NH₄⁺ or urea at significantly greater rates than low-light-grown cells, but NO₃ or glu-
ticate uptake did not increase with growth irradiance. Because these were mixed cultures (P. plicatula and Rhodomonas), it is impossible to determine absolute uptake rates in either group. However, given the relatively low abundance of cryptophytes (ca. 1% of zoospore abundance), the contribution of Rhodomonas to overall uptake rates was likely to be very minor. In fact, applying Stolte and Riegman's (1996) maximum NO3 or NH4 uptake rate per cell surface area of 8.5 × 10^{-11} \text{mol} \cdot \text{cell}^{-1} \cdot \text{h}^{-1}, estimates of Rhodomonas population (mean area = 45 \mu m^2) uptake were approximately 0.7 ng at N \text{L}^{-1} \cdot \text{h}^{-1}, or roughly 0.02% to 0.04% (for NO3) or 0.5% (for NH4) of the measured absolute uptake rates (Table 1). Even in the case of urcha, where relatively low uptake rates were measured, theoretical estimates of Rhodomonas population uptake (using a maximum uptake rate of 5 mmol N-cell^{-1} \cdot \text{h}^{-1}; Syrett et al. 1986; Price and Harrison 1988) only accounted for 28% or 8% of low or high light estimates, respectively. Therefore, the measured N uptake rates can be accounted for predominantly by P. plicatula uptake.

**DISCUSSION**

In Stoecker's (1998) review of mixotrophic models, kleptoplastidic protists were considered to acquire nutrients predominantly through phagotrophy, and photosynthesis was thought to be important as a survival mechanism for maintaining respiratory requirements during prey limitation. An inference of this model is that photosynthetically driven cell growth (and therefore associated saprophytic nutrient uptake) is slow relative to that supported by phagotrophy. The potential contribution of photosynthesis to P. plicatula's growth was not addressed in this study, and remains to be determined as a critical test of the model. However, the present findings suggest that saprophytic uptake can play an important role in P. plicatula's nutrition. The results not only establish that the dinoflagellate can acquire N-nutrients directly when kleptoplastidic, but, based on the following arguments, suggest that nutrient uptake rates were comparable to those estimated for phytoplankton, and rivaled N uptake through phagotrophy in P. plicatula.

When normalized per cell (based on the entire P. plicatula population; i.e. with and without ingested plastid present), uptake rates of NO3, NH4, and glutamate (Table 1) were within the range of those reported for phytoplankton (Syrett et al. 1986, Antia et al. 1991, Lomas and Gilbert 1999A). Hypothetically, direct nutrient uptake may have been predominated by, or even confined to, kleptoplastidic P. plicatula. Lewitus et al. (1999) presented evidence for greater survival of kleptoplastidic cells over apoplastidic cells in phagotrophically active P. plicatula cultures. The speculation that a fraction of the dinoflagellate population was responsible for the bulk of nutrient uptake would suggest that cellular uptake rates can be potentially higher than those reported here.

Based on previous experiments on P. plicatula grazing properties (Glasgow et al. 1998, unpublished data), comparisons can be made between potential nutrient acquisition via phagotrophy and kleptoplastidic ingestion rates by P. plicatula zoospores on Cryptomonas sp. LP757, a cryptophyte resembling Rhodomonas in size and morphology, averaged 1.3 cryptophyte cells per zoospore per day over a 16-h period. Using this estimate for ingestion of cryptophytes, assuming that all of the prey contents were ingested per encounter (a false assumption, given the myzocystic mode of feeding), and using an estimate of cryptophyte N content of 1 pmol-cell^{-1} (Lewitus and Caron 1990), an N uptake rate can be estimated at 54 mmol N per P. plicatula zoospore per hour. Based on a population density equivalent to those measured in this study, phagotrophic N uptake would be approximately 0.8 \mu g at N \text{L}^{-1} \cdot \text{h}^{-1}.

Though rough, this estimate suggests that rates of N acquisition by kleptoplastidic P. plicatula (Table 1) may approach or even exceed that obtained through grazing.

The disproportional distribution of ambient nutrients in experimental cultures precludes meaningful comparisons of substrate-specific uptake rates. It is likely that the relatively high NO3 uptake rates were related to high ambient NO3 concentrations (Lomas and Gilbert 1999, and references therein). Also, it is possible that the relatively low urcha uptake rates were a function of caustolic enzyme inhibition (e.g. urease) by the relatively high ambient NH4 concentrations (Fynn and Butler 1986, Antia et al. 1991, Berg et al. 1997). Perhaps a more relevant comparison is the effect of growth irradiance on uptake rates for specific substrates. Only urcha (absolute and cellular rates) or NH4 (cellular rates) were taken up at greater rates at the higher irradiance level. Research is needed to determine whether this effect of growth irradiance reflects N substrate preference in kleptoplastidic P. plicatula.

From microscopic observations of natural popu-
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As pointed out, the physiological ecology of kleptoplasticic P. piscicida may bear importantly on the mechanism by which nutrients may regulate their growth. In laboratory experiments (Burkhoder et al. 1995, 1998, Burkhoder and Glasgow 1997, Glasgow et al. 1998), nutrient (N or P) stimulation of P. piscicida’s growth has been demonstrated, either in response to elevated phytoplankton prey supply (the so-called “indirect stimulation” by nutrients) or through direct acquisition of substrates, as shown with 14C-labeled protein hydrolysate (“direct stimulation”). Based on these laboratory results and direct correlations between “presumptive Pfiesteria” abundance, phytoplankton abundance, and nutrient concentrations in natural waters (Burkhoder et al. 1997, Burkhoder and Glasgow 1997), a working hypothesis depicting seasonal changes in mechanisms of nutrient stimulation in a temperate estuary such as the Neuse River is presented in Figure 6. Most of the fish kills involving P. piscicida occur in summer. However, a presumably important factor determining the extent and magnitude of toxic activity is the abundance of nontoxic zoospores, the direct precursors of toxic zoospores. Nontoxic zoospore abundance in the Neuse River has been shown to co-occur with chlorophyll during spring phytoplankton bloom periods (Burkhoder and Glasgow 1997), with the implication that nutrient regulation of phytoplankton biomass also will indirectly control nontoxic P. piscicida abundance (“indirect nutrient stimulation”). Also, bloom parameters (magnitude, composition) may affect the proportion of nontoxic zoospores that become kleptoplasticic. We hypothesize that the maintenance (i.e. as zoospores rather than other cell forms such as cysts or amoebae) and/or growth of these kleptoplasticic populations are dependent on the quantity and quality of available nutrients (“direct stimulation”). In this respect, P. piscicida’s potential to cause fish kills could depend on the supply of nutrients available to support seed populations of nontoxic kleptoplasticic zoospores that “fuel” toxic outbreaks. Whether or how nutrients stimulate P. piscicida is a critical issue in predicting the dinoflagellate’s potential impact on estuarine fish populations. The organism is widespread (from Delaware Bay to Mobile Bay, Alabama), but toxic outbreaks have been documented in a relatively narrow range of its latitudinal distribution; that is, in various North Carolina estuaries from 1991–1998, and in the Poconos River in summer, 1997 (Burkhoder et al. 1995, Lewitus et al. 1995, Burkhoder and Glasgow 1997, Maryland Department of Natural Resources 1997, University of Maryland Center for Environmental Science 1997). On comparing regions affected by P. piscicida toxicity with those where P. piscicida is found but not known to cause problems, certain general distinctions in estuarine properties are suggested, including tidal flushing characteristics and fish population dynamics, and also nutrient concentrations (Burkhoder and Glasgow 1997). For example, using fish mortality bioassays and scanning electron microscopic confirmation, P. piscicida was recently discovered in the pristine North Inlet estuary, South Carolina (Lewitus, Willis, Glasgow, and Burkhoder, unpublished data). In contrast to sites of known P. piscicida toxic events, North Inlet is characterized not only by higher flushing rates, but also by a lack of anthropogenic influence and relatively low inorganic nutrient concentrations (e.g. seasonal maxima in dissolved inorganic nitrogen at some sites rarely exceed 5 μM; Lewitus et al. 1998). Although the North Inlet P. piscicida populations became toxic in fish aquariums, this potential toxicity, to our knowledge, has not been exhibited in a natural habitat. The hy-
POthesized link between high nutrient concentrations and *P. piscicida* toxic activity suggests that continued coastal eutrophication may lead to an increase in the magnitude and geographic range of *P. piscicida* toxic events.

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