

## Direct uptake of nitrogen by *Pfiesteria piscicida* and *Pfiesteria shumwayae*, and nitrogen nutritional preferences

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Received 5 December 2005; received in revised form 10 April 2006; accepted 28 April 2006

### Abstract

The rates of uptake of a range of forms of nitrogenous nutrients were measured in cultures of *Pfiesteria piscicida* and *Pfiesteria shumwayae* maintained at varying physiological states. The measured rates of dissolved N uptake under some conditions approached the rates of N uptake that are achieved through phagotrophy. Rates of dissolved N uptake by *P. piscicida* contributed <10% of the cellular N of flagellated cells feeding on algae, but were equal to or greater than phagotrophic N acquisition in cells recently removed from fish cultures. Specific N uptake rates ( $V, \text{h}^{-1}$ ) were higher for cells that were maintained on algal prey for long periods (months) than those that were grown with live fish. However, rates of N uptake on a cellular basis for cells grown on or recently removed from fish were comparable to those maintained on algal prey, likely reflecting differences in the sizes of cells of different physiological condition. Preferences for form of N generally followed a decreasing trend of amino acids > urea >  $\text{NH}_4^+$  >  $\text{NO}_3^-$ . Nitrate consistently was not a preferred form of N. Although *Pfiesteria* spp. are often found in eutrophic environments, the relationship between *Pfiesteria* spp. and nutrient availability is likely to be primarily indirect, mediated through the production of various prey on which *Pfiesteria* spp. feed. These findings also confirm, however, that when dissolved N concentrations are elevated, they can contribute to the supplemental nutrition of these cells, and thus may provide a significant source of N to *Pfiesteria* spp. in nature.

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**Keywords:** *Pfiesteria*; Nitrogen nutrition; Heterotrophy; Toxicogenic dinoflagellates

### 1. Introduction

*Pfiesteria piscicida* and *Pfiesteria shumwayae* (Marshall et al., in press) are heterotrophic dinoflagellates that feed phagotrophically on fish tissue, microalgae and other particles (Burkholder and Glasgow, 1997; Burkholder et al., 1998; Lewitus et al., 1999a,b). Nutrient-enriched conditions have been correlated with *Pfiesteria* abundance (e.g. Burkholder and Glasgow, 1997; Lewitus

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et al., 1999b; Glibert et al., 2001, 2004), but the causes of this relationship are not fully understood. Unless *Pfiesteria* has the ability to use dissolved nutrients directly, the correlation with high nutrient loading would have to be mediated by the production of other organisms such as algal prey (Burkholder et al., 2001a,b). Although primarily phagotrophic, *P. piscicida* previously has been reported to take up some dissolved substrates, such as amino acids and protein hydrolysate (Burkholder and Glasgow, 1997). Moreover, Lewitus et al. (1999b) reported that *P. piscicida* has the ability to take up dissolved inorganic nitrogen (N) compounds, and that rates under nutrient-rich conditions may approach those of N acquisition through phagotrophy. Lewitus et al. (1999b) suggested that two pathways linking *Pfiesteria* with nutrient enrichment might occur: during spring, *Pfiesteria* may depend on phagotrophy to a greater extent when chlorophyll abundance is high, whereas during the summer when pathways of recycling dominant the flows of nitrogen, a direct pathway of N uptake may be more significant. Glasgow et al. (2001a) expanded this conceptual framework to include the role of fish prey.

It is now well recognized that *Pfiesteria* spp., among many other autotrophic and heterotrophic dinoflagellate species, display considerable differences among species, strains, and even within the same strain when grown under different growth conditions (Burkholder et al., 2001a,b, 2005). For *Pfiesteria* spp. three operational terms have been used to describe different functional types (toxicity status) among the same strain or species. These have been identified as TOX-A, cells that are grown in the presence of live fish and can actively kill fish with toxin involvement; TOX-B, cells that had previously been grown under fish-killing conditions, then were removed from fish and grown on algal prey, still retaining the ability to kill fish with toxin when re-exposed; and non-inducible (NON-IND), cells that have been grown for extended periods on algal prey and have apparently lost the ability to kill fish with toxin upon re-exposure (Turgeon et al., 2001). NON-IND strains are the most common types collected from estuaries and maintained in culture, especially when grown for extended periods on algal prey (Burkholder et al., 2001a,b). Many differences in physiology and behavior of these functional types have previously been described, such as in response to fish, algal prey and inorganic nutrients (Burkholder et al., 2001a). Furthermore, Stoecker et al. (2002) and Lewitus et al. (in press) demonstrated that grazing by microzooplankton on TOX-A *P. piscicida* was significantly less than that of TOX-B and NON-IND functional types of the same strain.

In this study we address the uptake of dissolved nitrogenous compounds by *P. piscicida* and *P. shumwayae* in several functional states. We also estimate the contribution of these forms of N to the potential growth of these species relative to heterotrophic consumption under nutrient-rich conditions.

## 2. Methods

Two types of experiments were conducted. The first experiment (27–28 June 2000) was designed to investigate the range of N uptake rates by one clone of *P. piscicida* under differing growth conditions. The second experiment (12–13 December 2002) was designed to compare the rates of N uptake by *P. piscicida* and *P. shumwayae* grown under a range of nutritional and toxic conditions, and to assess the relationship between N uptake and substrate concentration in more detail. All cultures were clonal but not bacteria free.

### 2.1. Experiment 1

*Pfiesteria piscicida* (clone CAAE 416T) was originally collected from Middle River, Maryland (27 August 1999), and isolated using flow cytometric procedures (Burkholder et al., 2001a). Clonal status was cross-confirmed by the heteroduplex mobility assay (Oldach et al., 2000). Cultures were maintained at 23 °C on a 12-h:12-h L:D cycle at 80  $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ . The general protocol for culturing *Pfiesteria* with fish in standardized bioassays was described by Burkholder et al. (2001b). Toxic cultures were fed 2–3 juvenile tilapia (*Oreochromis mossambicus*, length 5–7 cm) daily up to the time they were removed for the experiment.

Three sub-cultures were used in this experiment. A TOX-A sub-culture was removed from fish cultures on the morning before the experiment. A TOX-B sub-culture was removed from fish cultures 2 weeks before the start of the experiment, re-cloned, and maintained on cryptophyte prey (*Rhodomonas* sp. CCMP757, Bigelow Laboratory Culture Collection). A NON-IND sub-culture was removed from fish cultures 10 months before the experiment and maintained on the same cryptophyte prey. Toxicity for all cultures was tested using the standardized bioassays of Burkholder et al. (2001b) and confirmed in the TOX-A and -B cultures by toxin analysis (Moeller et al., 2001; Burkholder et al., 2005).

Twelve 100 ml aliquots from each culture type were dispensed into clean tissue culture flasks. Half of these

sub-cultures were given *Rhodomonas* sp. as prey, while the other sub-cultures were not fed during the experimental period. The abundances of *Rhodomonas*, their change with time, and rate of grazing by *Pfiesteria* on this prey is the subject of a separate study (Lewitus et al., in press). Cell density for each sub-culture was estimated by light microscopy (400–600 $\times$ ) using Lugol's-preserved subsamples and a Palmer–Maloney counting chamber (Wetzel and Likens, 1991).

The 12 flasks were then further divided so that equal numbers of flasks with and without prey were incubated under 560  $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$  (hereafter referred to as “high light”), and 12  $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$  (hereafter referred to as “low light”). All data reported here are the treatments without prey only. The treatments with prey were used in grazing studies (Lewitus et al., in press).

Rates of uptake of various N substrates were then assessed for the treatments without prey on Day 1, and again 24 h later (Day 2). The rate of uptake of N by the control cultures of *Rhodomonas* sp. was assessed on Day 2. Rates of N uptake were determined by transferring 50 ml from each flask to a clean tissue culture flask and enriching with  $^{15}\text{N}$  substrates, either  $\text{NH}_4^+$ ,  $\text{NO}_3^-$  or urea, at a final concentration of 40  $\mu\text{mol N L}^{-1}$ . All  $^{15}\text{N}$  substrates were 97–99% enriched. The concentration of  $^{15}\text{N}$  added, 40  $\mu\text{mol N L}^{-1}$ , represented from  $\sim 5$ –70 at.% enrichment of ambient. As described below, data were rejected from further analysis when the initial enrichment was <10%. Tissue culture flasks were returned to their incubation light intensity for a period of  $\sim 1.25$  h, and then filtered onto precombusted GF/F filters (0.7  $\mu\text{m}$  pore size). Filters and filtrates were reserved for later analysis. Filtrates were immediately frozen, and the filters were dried at 50  $^\circ\text{C}$  for about 24 h. An initial sample from each culture condition was also filtered onto a precombusted GF/F filter for particulate nitrogen (PN) analysis and the filtrate retained for nutrient analysis. The  $^{15}\text{N}$  samples were all later analyzed by mass spectrometry on a Nuclide mass spectrometer, and the PN samples on a Control Equipment CHN analyzer. The filtrates were analyzed for concentrations of  $\text{NH}_4^+$ ,  $\text{NO}_3^-$  or urea by standard autoanalyzer techniques for the inorganic substrates and by the urease method for urea (Parsons et al., 1984).

## 2.2. Experiment 2

*Pfiesteria piscicida* (clone 1332-AC2; CCMP 2361) and *P. shumwayae* (clone 1024C; CCMP2359) were grown as described above for Experiment 1 to yield

cultures in various nutritional states. For *P. piscicida* these included a NON-IND culture, and for *P. shumwayae*, a TOX-A, as well as four different TOX-B cultures, two which were within 2 weeks of removal from fish, and two which were removed from fish several months before the experiment. The latter two are referred to herein as “long-term” (LT), as their toxicity status was not confirmed. A true NON-IND strain of this *P. shumwayae* clone was not available. All TOX-B cultures were maintained on algal prey (cryptophyte *Storeatula major* HP9101). TOX-A cultures were freshly removed from live fish cultures. In order to yield a range of nutritional states in the TOX-B cultures, the algal prey was grown at two nutrient levels (f/20 and f/200 media) representing nutrient-replete (NR) and nutrient-deplete (ND). All algal prey were depleted (<5 cells  $\text{ml}^{-1}$ ) at the time the experiments were initiated. In all, experiments were conducted on two *P. piscicida* cultures of varying nutritional state, and five cultures of *P. shumwayae* of varying functional type and/or nutritional state.

For each culture type, samples were divided into 25 tissue culture flasks, each of 50 ml volume. In sets of five, the samples were enriched with  $^{15}\text{N}$  substrates at concentrations ranging from 0.5 to 24  $\mu\text{mol N L}^{-1}$ . Each set was enriched with a different N substrate,  $\text{NO}_3^-$ ,  $\text{NH}_4^+$ , urea, glutamic acid or glycine. The number of manipulations precluded full replication of the  $^{15}\text{N}$  experiments. However, 25% of the samples were replicated throughout all phases of the experiment and analysis. The initial at.% enrichment ranged from <1 to 98%; data from experiments in which at.% enrichment was <10% were rejected from further analysis.

All incubations were conducted at 23  $^\circ\text{C}$  and 80  $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$ , the same conditions as those of the maintenance cultures. Incubations were  $\sim 1$  h in duration, and were terminated by filtration onto precombusted GF/D filters (2.7  $\mu\text{m}$  pore size). The use of GF/D filters, rather than GF/F as used in Experiment 1, was to reduce the bacterial contamination on the filtered sample: the larger pore size of the GF/D filters retained the dinoflagellate cells but allowed more unattached bacteria to pass. Ambient PN concentrations and nutrients were determined by filtering samples at the beginning of the experiment for each sub-culture type. Analyses of PN and dissolved inorganic nitrogen and urea nutrients were as described for Experiment 1. Total dissolved free amino acids were determined by fluorescence (Parsons et al., 1984); individual amino acid determination was not conducted. All values were <0.5  $\mu\text{mol N L}^{-1}$  (data not shown); the availability of

glycine and glutamic acid were conservatively estimated at  $0.2 \mu\text{mol N L}^{-1}$  for the purpose of rate calculations. If the individual amino acid concentrations were less than estimated, the corresponding rates of uptake would be higher. The  $^{15}\text{N}$  samples from this experiment were analyzed on a Europa mass spectrometer.

### 2.3. Data analysis

Available culture volume precluded full replication; however, 35 samples were replicated throughout all phases of  $^{15}\text{N}$  addition and analysis. The mean variability in  $^{15}\text{N}$  isotope enrichment for these replicates was  $<0.5\%$ . This variability was assumed to be the same for the non-replicated samples. Rates are not reported for those samples for which the initial at.% enrichment was  $<10\%$ , as artificially elevated rates are estimated at these low levels (Glibert et al., in preparation). Inasmuch as nutrient samples were taken but the data were not immediately available at the beginning of the experiment when the initial additions were made, it was impossible *a priori* to avoid experimental conditions of at.% enrichments  $<10\%$ .

Nitrogen-specific uptake rates ( $V$ ,  $\text{h}^{-1}$ ) were calculated according to the equation of Dugdale and Goering (1967) and are used as the primary form of data presentation. These rates can be interpreted as N taken up per cell N. Absolute uptake rates per  $\text{L}^{-1}$ , ( $\rho$ ,  $\mu\text{mol N L}^{-1} \text{h}^{-1}$ ), were calculated by multiplying  $V$  by the initial PN. Concentrations of PN did not change significantly over the short duration of these experiments. Data were also transformed to absolute cellular uptake rates ( $\rho_{\text{cell}}$ ,  $\text{fmol N cell}^{-1} \text{h}^{-1}$ ) by dividing  $\rho$  by the cell density.

To assess the relative rate of uptake of a particular form of N relative to its availability, the relative preference index (RPI; McCarthy et al., 1977) was calculated. The RPI was calculated for each substrate used in Experiment 1. Using  $\text{NO}_3^-$  as an example:

$$\text{RPI}_{\text{NO}_3^-} = \left[ \frac{(V_{\text{NO}_3^-})}{(V_{\text{NO}_3^-} + V_{\text{NH}_4^+} + V_{\text{urea}})} \right] / \left[ \frac{[\text{NO}_3^-]}{([\text{NO}_3^-] + [\text{NH}_4^+] + \text{urea}]} \right] \quad (1)$$

in which  $V_{\text{NO}_3^-}$ ,  $V_{\text{NH}_4^+}$  and  $V_{\text{urea}}$  are the ambient specific uptake rates of each of the designated substrates, and  $\text{NO}_3^-$ ,  $\text{NH}_4^+$  and urea are the ambient concentrations of each substrate. A value of unity for a particular N form indicates uptake equitable with availability, a value in excess of unity reflects preference and a value less than unity reflects rejection of that substrate relative to its availability. For Experiment 2, the RPI was calculated

with all substrates (including amino acids), then recalculated without inclusion of the amino acid uptake rates to make the values comparable to those derived from Experiment 1.

Experiment 2 was designed for the calculation of parameters for nitrogen uptake kinetics based on the Michaelis–Menten equation:

$$V = (V_{\text{max}} \times S) / (K_s + S) \quad (2)$$

where  $V$  is the specific uptake rate ( $\text{h}^{-1}$ ),  $V_{\text{max}}$  is the maximal specific uptake rate,  $S$  is the substrate concentration ( $\mu\text{mol N L}^{-1}$ ), and  $K_s$  is the half-saturation constant ( $\mu\text{mol N L}^{-1}$ ). However, the elevated ambient nutrient concentrations for several of the substrates violated one of the basic assumptions of the kinetic model, that the nutrient of interest is depleted at the cell surface. Most of the experiments were best related to nutrient concentration by a linear model. Where the Michaelis–Menten model did fit the data, the kinetic constants,  $V_{\text{max}}$  and  $K_s$ , were derived from a linear transformation of the data, following the Lineweaver–Burke equation:

$$1/V = ((K_s/V_{\text{max}}) \times (1/[S])) + 1/V_{\text{max}} \quad (3)$$

## 3. Results

### 3.1. Experiment 1

Cell densities on Day 1 ranged between  $2 \times 10^4$  and  $3 \times 10^4$  cells  $\text{mL}^{-1}$  for all cultures. At the initiation of the experiment, ambient nutrient concentrations in each of the treatment conditions were high (Table 1), and were similarly enriched. Thus, in all cases the physiological state of the cells was nutrient replete. Uptake of all of the provided N forms was detected in all cultures (Fig. 1A–C). In all cases, however, the rates of uptake were significantly less than the rates of the cryptophyte control cultures. Significantly lower specific uptake rates were found for all forms of N for TOX-A cultures relative to the other functional types and the cryptophyte controls. Yet, when normalized per cell, TOX-A cultures had rates of uptake that were comparable to those of the other functional types (Fig. 1D–F).

For TOX-B and NON-IND cultures, the highest specific uptake rates were observed for  $\text{NO}_3^-$  and the lowest rates were found for urea (Fig. 1). No differences were discerned for any of the cultures in rates of N uptake in the high light treatment compared to the low light treatment (Fig. 1). For each N substrate, the specific uptake rates determined on Day 2 (in the

Table 1  
Ambient dissolved nutrient concentrations at the initiation of each experiment

Experiment and culture type	Urea ( $\mu\text{mol N L}^{-1}$ )	$\text{NH}_4^+$ ( $\mu\text{mol N L}^{-1}$ )	$\text{NO}_3^-$ ( $\mu\text{mol N L}^{-1}$ )
Experiment 1			
P pisc TOX-A	88.8	17.80	830
P pisc TOX-B	91.1	31.40	750
P pisc NON-IND	93.6	20.70	730
Experiment 2			
P pisc NON-IND ND	0.34	5.49	5.85
P pisc NON-IND NR	4.26	15.00	6.61
P shum TOX-A	2.92	188.0	n.d.
P shum TOX-B ND	0.27	7.97	24.10
P shum TOX-B NR	0.47	16.80	15.70
P shum LT ND	0.60	8.05	38.00
P shum LT NR	0.74	26.30	25.90

P shum and P pisc represent *P. shumwayae* and *P. piscicida* cultures, respectively. For each culture type, various functional types were investigated, including TOX-A, TOX-B, NON-IND and long-term (LT) non-inducible. Furthermore, for each TOX-B and LT non-inducible cultures, the algal food was either nutrient deplete (ND) or nutrient replete (NR).

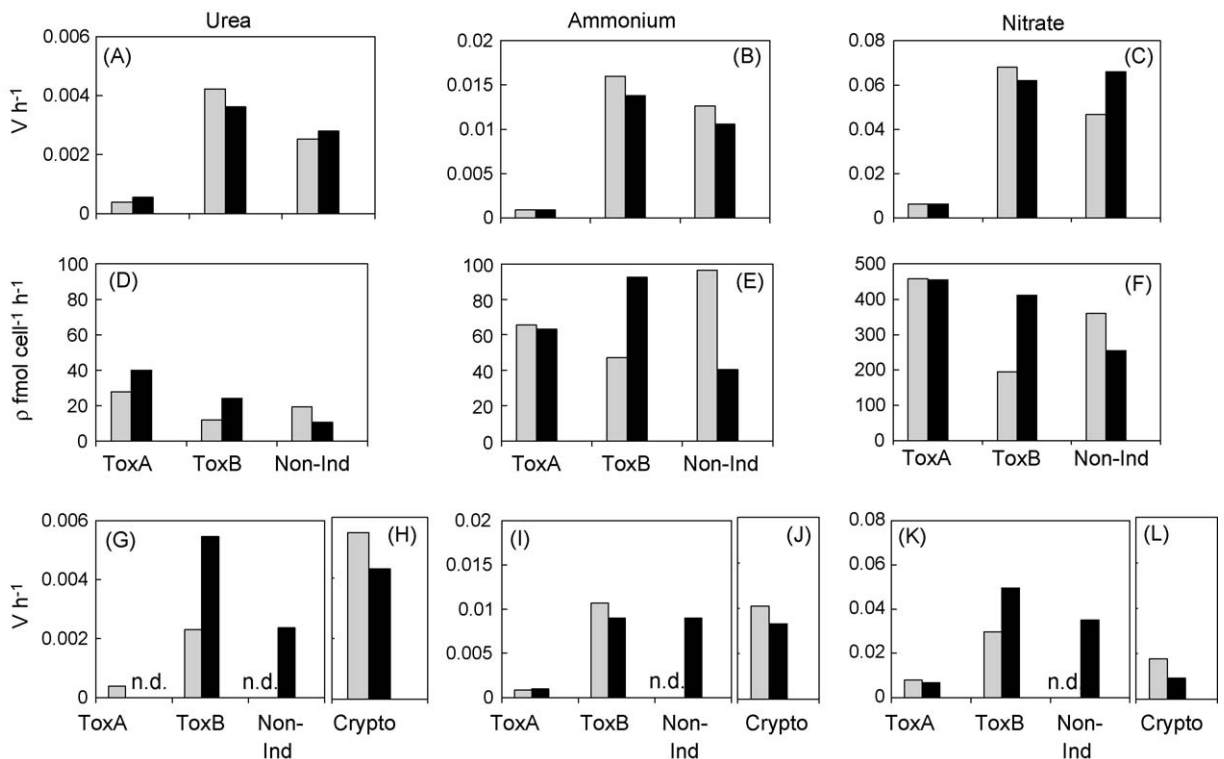


Fig. 1. Rates of nitrogen uptake by *Pfiesteria piscicida* and the cryptophyte (*Rhodomonas* sp.) control cultures from Experiment 1. Panels A–F represent data derived from measurements made on Day 1, and panels G–L represent Day 2. Data are expressed both as specific uptake rate ( $V$ ,  $\text{h}^{-1}$ , panels A–C and G–L) and as absolute uptake rate per cell ( $\rho$   $\text{fmol N cell}^{-1} \text{h}^{-1}$ , Day 1 data only, panels D–F) for the N substrate indicated on the top of the panels. For each culture type, the light bars represent the “high light” cultures, whereas the dark bars represent the “low light” cultures. For Day 2, no data (n.d.) are available for TOX-A urea uptake high light only, and for all nitrogen forms for the NON-IND low light cultures. Note scale changes between panels. All values for the cryptophyte cultures (panels H, J, L) are divided by 10 in order to display on the same axes.

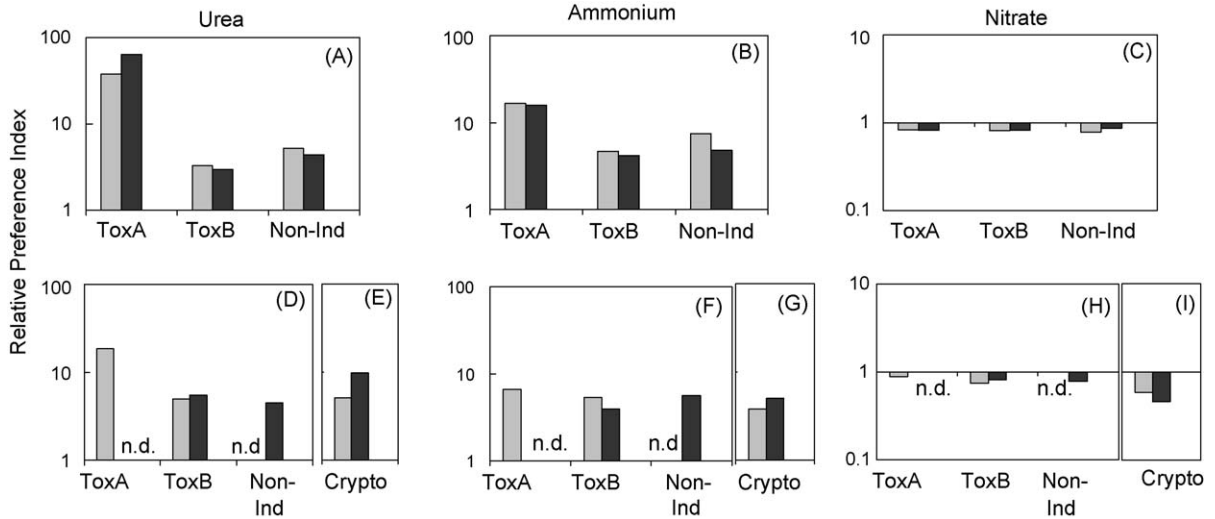


Fig. 2. Relative preference indices (RPI) for the different forms of nitrogen indicated for cultures from Experiment 1. Panels A–C are derived from measurements made on Day 1, and D–I are derived from Day 2. For each culture type, the light bars represent the “high light” cultures, whereas the dark bars represent the “low light” cultures. For Day 2, no data (n.d.) are available for TOX-A urea uptake high light only, and for all nitrogen forms for the NON-IND low light cultures; thus RPI for these treatments cannot be calculated. Note scale changes between panels.

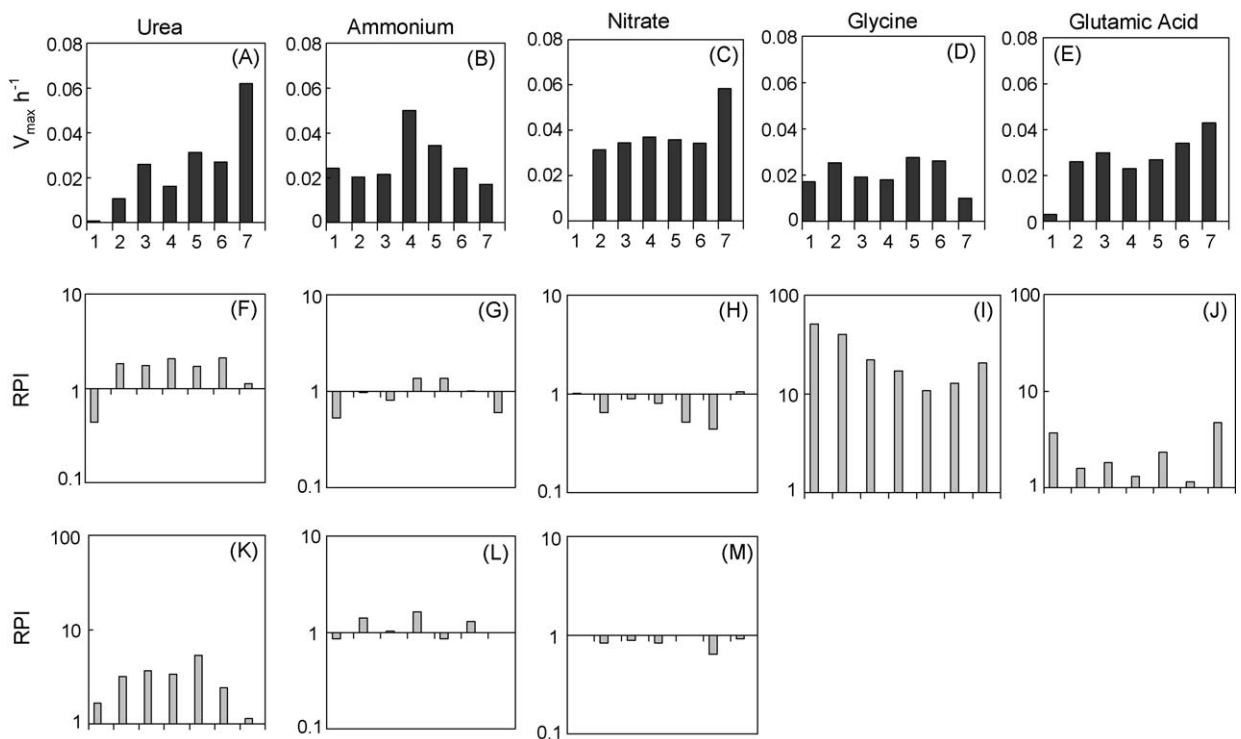


Fig. 3. Rates of nitrogen uptake by *Pfiesteria piscicida* and *P. shumwayae* cultures from Experiment 2. Data are given as specific uptake rate ( $V_{max}$ ,  $h^{-1}$ ) in panels A–E, and the relative preference index (RPI) for each culture is given in panels F–J, calculated for saturating substrate levels only. In panels K–M the RPI values are calculated using only the substrates nitrate, ammonium and urea, and using rates determined from ambient substrate levels. The latter calculation is analogous to the calculation of RPI from Experiment 1. The various cultures are (1) *P. shumwayae* TOX-A; (2) *P. shumwayae* TOX-B nutrient deplete (ND); (3) *P. shumwayae* TOX-B nutrient replete (NR); (4) *P. shumwayae* long term (LT) ND; (5) *P. shumwayae* LT NR; (6) *P. piscicida* NON-IND ND; and (7) *P. piscicida* NON-IND NR. See text for full description of the differences in the functional types. The data in panels A–J represent the mean of two replicates and the average variation in replicated  $^{15}N$  analyses was  $<0.5\%$ . Note scale changes between panels.

Table 2

Relationships between uptake velocity ( $V$ ,  $\text{h}^{-1}$ ) and substrate concentration ( $S$ ,  $\mu\text{mol N L}^{-1}$ ) for each of the substrates and culture types given, based on laboratory culture experiments as described in text

Substrate	Culture type	$N$	Linear equation	$R^2$	$\alpha$	Logarithmic equation	$R^2$	$\alpha$	$K_s$ ( $\mu\text{mol N}$ )	$V_{\text{max}}$ ( $\text{h}^{-1}$ )
$\text{NO}_3^-$	P shum TOX-A	–								
	P shum TOX-B ND	5	$V = 0.0003S + 0.0165$	0.640	0.1					
	P shum TOX-B NR	4	$V = 0.0005S + 0.0095$	0.666						
	P shum LT ND	–								
	P shum LT NR	4	$V = 0.0007S + 0.0008$	0.906						
	P pisc NON-IND ND	4	$V = 0.0006S + 0.0042$	0.968						
	P pisc NON-IND NR	4	$V = 0.0011S + 0.0033$	0.972						
$\text{NH}_4^+$	P shum TOX-A	–								
	P shum TOX-B ND	5	$V = 0.0003S + 0.0093$	0.943	0.05					
	P shum TOX-B NR	4	$V = 0.0001S + 0.0152$	0.365						
	P shum LT ND	4	$V = 0.0002S + 0.0114$	0.967						
	P shum LT NR	4	$V = 4E-05S + 0.048$	0.097						
	P pisc NON-IND ND	4	$V = 0.0004S + 0.0144$	0.824						
	P pisc NON-IND NR	3	$V = 0.0003S + 0.0093$	0.670						
Urea	P shum TOX-A	5	$V = 1E-05S + 0.0001$	0.943	0.01					
	P shum TOX-B ND	5	$V = 0.0002S + 0.0007$	0.921	0.01					
	P shum TOX-B NR	5	$V = 0.0005S + 0.0008$	0.881	0.05					
	P shum LT ND	5	$V = 0.0003S + 0.0013$	0.811	0.05					
	P shum LT NR	–	$V = 0.0006S + 0.0013$	0.998	0.01					
	P pisc NON-IND ND	5	$V = 0.0005S + 0.002$	0.922	0.01					
	P pisc NON-IND NR	4	$V = 0.0012S + 0.0022$	0.961						
Glutamic acid	P shum TOX-A	5	$V = 7E-05S + 0.0008$	0.834	0.05	$Y = 0.0005 \ln x + 0.0008$	0.999	0.01	2.64	0.003
	P shum TOX-B ND	5	$V = 0.0008S + 0.0127$	0.833	0.05	$Y = 0.0058 \ln x + 0.0124$	0.897	0.01	1.07	0.026
	P shum TOX-B NR	5	$V = 0.0006S + 0.0123$	0.838	0.05	$Y = 0.0045 \ln x + 0.012$	0.907	0.01	1.30	0.030
	P shum LT ND	5	$V = 0.0006S + 0.0099$	0.865	0.05	$Y = 0.0045 \ln x + 0.0095$	0.973	0.01	1.46	0.023
	P shum LT NR	–	$V = 0.0006S + 0.0146$	0.842	0.05	$Y = 0.0046 \ln x + 0.0141$	0.957	0.01	0.82	0.027
	P pisc NON-IND ND	5	$V = 0.0007S + 0.018$	0.766	0.05	$Y = 0.0055 \ln x + 0.0173$	0.905	0.01	0.98	0.034
	P pisc NON-IND NR	5	$V = 0.0012S + 0.0232$	0.935	0.01	$Y = 0.0081 \ln x + 0.0234$	0.904	0.01	0.58	0.043
Glycine	P shum TOX-A	5	$V = 2E-05S + 0.0003$	0.818	0.05	$Y = 0.0002 \ln x + 0.0003$	0.982	0.01	4.61	0.001
	P shum TOX-B ND	5	$V = 0.00001S + 0.0121$	0.628						
	P shum TOX-B NR	5	$V = 0.0004S + 0.0054$	0.973	0.01					
	P shum LT ND	5	$V = 0.0003S + 0.0041$	0.990	0.01					
	P shum LT NR	–	$V = 0.0003S + 0.0029$	0.980	0.01					
	P pisc NON-IND ND	5	$V = 0.0004S + 0.0076$	0.948	0.05					
	P pisc NON-IND NR	4	$V = 0.0004S + 0.0062$	0.975						

P shum and P pisc represent *P. shumwayae* and *P. piscicida* cultures, respectively. For each culture type, various functional types were investigated, including TOX- A, TOX-B, NON-IND, and long-term (LT) maintenance on algae for which toxicity status was uncertain. Furthermore, for each TOX-B and LT or NON-IND culture, the algal food was either nutrient deplete (ND) or nutrient replete (NR).  $N$  represents the number of substrate concentrations that were used in each calculation. Most of the data were best fit by a linear relationship, but those for glutamic acid and glycine, TOX-A only, fit a saturating hyperbolic relationship. It is only for these hyperbolic relationships that a  $K_s$  and  $V_{\text{max}}$  can be calculated. The  $R^2$  and significance level for each fit are also given. Significance is not reported for relationships based on 4 or fewer substrate levels.

absence of prey) were of the same order of magnitude as those measured on Day 1, although in general the rates on Day 2 were lower than those on Day 1 (Fig. 1G–L). Although the cultures contained bacteria, the very low specific N rates in the TOX-A cultures suggest that bacteria contributed minimally to these measured rates of N uptake.

All cultures, including the controls, displayed a preference for urea and  $\text{NH}_4^+$  over  $\text{NO}_3^-$ , based on the calculation of RPI (Fig. 2). The preference for urea was two- to three-fold that for  $\text{NH}_4^+$ . The preference for urea and  $\text{NH}_4^+$  was most apparent in the TOX-A cultures, where RPIs exceeded 40 for urea and 15 for  $\text{NH}_4^+$ . In all cases, the RPI for  $\text{NO}_3^-$  was  $<1$ .

### 3.2. Experiment 2

The broader range of manipulations in Experiment 2 compared to Experiment 1 permitted us to assess not only the rates of uptake and RPI for each culture, but also the relationships between uptake and substrate availability for some substrates.

Initial cell density averaged  $\sim 5 \times 10^3$  cell  $\text{mL}^{-1}$ . Ambient N concentrations were considerably lower than in Experiment 1, particularly  $\text{NO}_3^-$  and urea (Table 1). The concentrations of  $\text{NH}_4^+$ , while in the same range of those in Experiment 1, were consistently about a factor of 2 lower in the ND (grown on nutrient-

deplete prey) cultures relative to the NR (grown on nutrient-replete prey) cultures.

Comparing the maximal specific velocities of uptake for these cultures ( $V_{\text{max}}$ ,  $\text{h}^{-1}$ ; Fig. 3A–E), several patterns emerge. First, for TOX-A cultures of *P. shumwayae*, the specific uptake rate of N was very low for urea,  $\text{NO}_3^-$  and glutamic acid, but higher rates of uptake were found for  $\text{NH}_4^+$  and glycine. Specific uptake rates for  $\text{NH}_4^+$  and urea were higher than those measured during Experiment 1, and this was of particular note for *P. piscicida*. When RPI values were calculated for each substrate, the overwhelming preference was for glycine, followed by glutamic acid and urea (Fig. 3F–J). When RPI values were recalculated using only the substrates urea,  $\text{NH}_4^+$  and  $\text{NO}_3^-$  at ambient levels, comparable to the calculation for Experiment 1, the preference for urea was modestly strengthened, but the general patterns were unchanged (Fig. 3K–M). The uptake of  $\text{NH}_4^+$  was roughly proportional to its availability, and preference was modestly increased when the amino acids were excluded from the calculation. As in Experiment 1, no culture displayed a preference for  $\text{NO}_3^-$  (Fig. 3H and M).

The relationship between ambient uptake rate and substrate concentration was best described by a linear fit for urea,  $\text{NH}_4^+$ ,  $\text{NO}_3^-$  and glycine (except for *P. shumwayae* TOX-A; Table 2). Only the glutamic acid

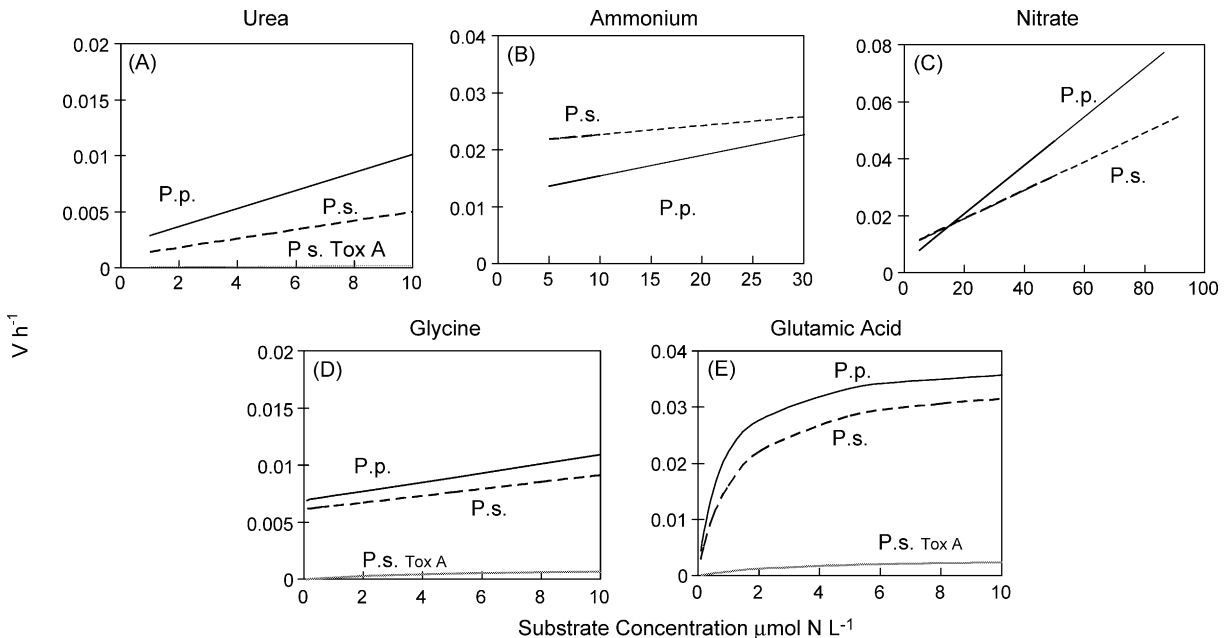


Fig. 4. Generalized relationships between specific uptake rate  $V$ ,  $\text{h}^{-1}$  and substrate concentration for each nitrogen nutrient given at the top of each panel. These relationships are graphic depictions of the equations given in Table 2. In each panel, P.p. represents *Pfesteria piscicida*, P.s. represents *P. shumwayae*, and P.s. Tox-A represents *P. shumwayae* TOX-A relationships.

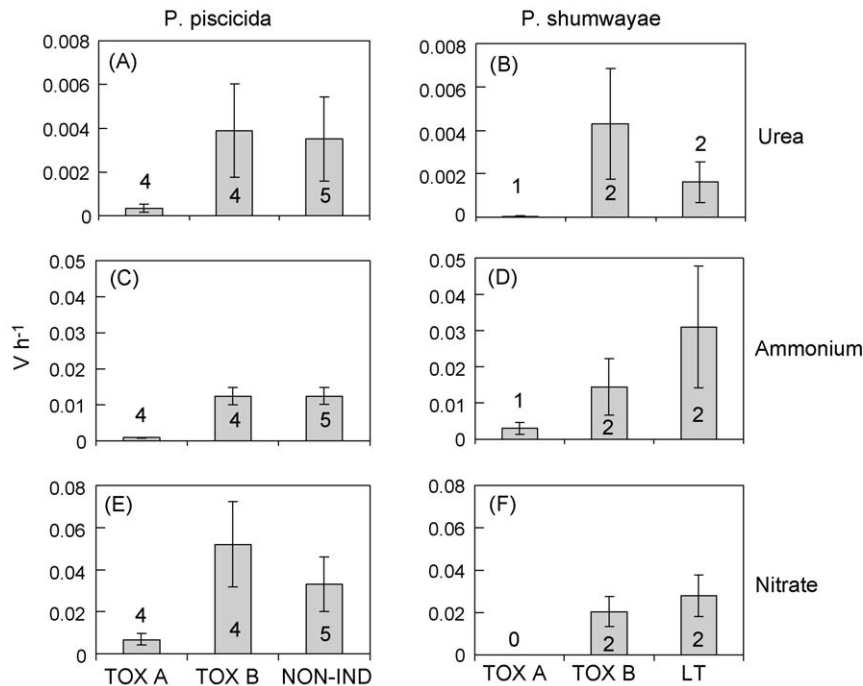


Fig. 5. Comparison of rates of nitrogen uptake (as  $V, h^{-1}$ ) for urea, ammonium and nitrate for *P. piscicida* (panels A, C, E) and *P. shumwayae* (panels B, D, F). Data shown are from both Experiments 1 and 2 for the various functional types given. The numbers at the top of, or inside, the individual bars represent the number of individual measurements used in the average. Error bars represent average standard deviations for each substrate.

uptake rates for all *P. piscicida* cultures and the *P. shumwayae* TOX-A cultures could be fit to a Michaelis–Menten relationship (Table 2). The generalized relationships between uptake rates and substrate concentration (Fig. 4) reveal several differences between substrates and between culture types. First, for urea,  $NO_3^-$  and glycine, the linear relationship and positive slope suggest that diffusion of substrate as well as mediated transport into the cells may have been the mechanisms by which these nutrients were taken up. For  $NH_4^+$ , the relationship was linear, but without a positive slope, suggesting that these rates were all within the saturated portion of a hyperbolic curve, but the high concentrations in the culture did not permit resolution of the initial slope of the curve. Rates of glutamic acid followed the classic saturating response. For urea, glycine and glutamic acid, the rates of uptake at typical ambient concentration levels were consistently higher for *P. piscicida* than for *P. shumwayae*, while for  $NH_4^+$  *P. shumwayae* displayed higher rates (Fig. 4). For  $NO_3^-$ , *P. shumwayae* had higher uptake rates at concentrations  $< ca. 10 \mu mol N L^{-1}$ , but *P. piscicida* had higher uptake rates at more elevated concentrations (Fig. 4C).

### 3.3. Comparisons across experiments

The comparison of all data from both experiments shows that for both species, functional type was the major factor regulating rates of N uptake (Fig. 5). For both species, specific rates of N uptake by TOX-A subcultures were lower than for the other functional types. For the other functional types, no significant differences were found between species for each N form. The higher variability in the specific uptake rates for *P. shumwayae*, relative to *P. piscicida*, may reflect the smaller number of measurements, but also the use of cultures that fed on algae of differing nutritional states.

## 4. Discussion

The results reported herein confirm that direct uptake of dissolved N does occur by *P. piscicida*, and this conclusion can now also be extended to *P. shumwayae*. These results also indicate that direct N uptake is regulated differently in cells of different functional type and nutrient status. Furthermore, in conjunction with microzooplankton grazing experiments conducted simultaneously on the same clones used for the experiments reported herein (Stoecker et al., 2002;

Lewitus et al., in press), estimates of the contribution of direct uptake to the cells relative to other consumption of N through grazing can be made.

#### 4.1. Nutrient uptake by- or stimulation of – *Pfiesteria* spp.

Specific N uptake rates ( $V, h^{-1}$ ) were higher for cells that were maintained on algal prey for long periods (months) than for those that were grown with live fish, but when normalized by cell number, rates of N uptake for cells grown on (TOX-A), or recently removed from (TOX-B), fish were comparable to those maintained for long periods on algal prey. Cell size in *P. piscicida* has been previously shown to vary by as much as a factor of 4 depending on nutritional and toxicity history (Burkholder et al., 2001a). Cell size was not determined in this set of experiments, but cells feeding on fish tend to be significantly larger than cells feeding on algae (Burkholder and Glasgow, 1997; Burkholder et al., 2001a). If the flagellate cells of the TOX-A cultures were larger in size than those of the other functional types, rates of uptake by TOX-A cultures on a per-cell basis would have been expected to increase compared to the N-based rates. There are few reported direct measurements of N uptake by *Pfiesteria*, only one of which also employed stable isotopic tracer techniques (Lewitus et al., 1999b). Rates of  $NH_4^+$  compared well across all studies, on both a volumetric and per-cell basis (Table 3). Concentrations of  $NH_4^+$  in the Lewitus et al. study were generally higher

than in either Experiment 1 or 2, but rates were not as variable as for the other substrates, consistent with the suggestion that uptake rates for  $NH_4^+$  do saturate and that all experiments were conducted at or near the maximal uptake rate (Fig. 4). Rates of  $NO_3^-$  uptake, both volumetric and cell-based, determined in the Lewitus et al. study compared favorably with the results of Experiment 1, but not Experiment 2 (Table 3). The concentrations in Lewitus et al. (1999b) were comparable to those in Experiment 1, but not Experiment 2, and thus the lower  $NO_3^-$  uptake rates from Experiment 2 were likely a function of substrate concentration. For urea, the results of Experiments 1 and 2 yielded comparable rates, but those of the Lewitus et al. study were much lower, as the concentrations they used were considerably lower than in either of our experiments, again likely reflecting substrate regulation. Glutamic acid rates were also lower in this study than in Lewitus et al. (1999b), although the rates herein are likely conservative as they were based on a conservatively estimated glutamic acid concentration. The very large differences between substrate concentration and prior nutritional state are all likely factors contributing to the differences observed between experiments. Burkholder and Glasgow (1997) and Glasgow et al. (1998) reported from track autoradiography observations that *P. piscicida* had the ability to take up amino acids and protein hydrolysate, and the effect of nutrient enrichment on *Pfiesteria* spp. production in batch cultures with algal prey has been well demonstrated. Burkholder et al. (2001a) reported on the growth of *Pfiesteria* functional

Table 3

Comparison of absolute uptake rates (as  $\mu\text{mol N h}^{-1}$  and as  $\text{fmol N cell}^{-1} \text{h}^{-1}$ ) of *P. piscicida* between this study and those reported by Lewitus et al. (1999b)

Treatment	$NO_3^-$	$NH_4^+$ ( $\mu\text{mol N h}^{-1}$ )	Urea	Glutamic acid	Reference
High light	$1.5 \pm 0.40$	$0.13 \pm 0.04$	$0.0079 \pm 0.003$	$0.95 \pm 0.40$	Lewitus et al.
	$3.0 \pm 1.41$	$0.60 \pm 0.10$	$0.18 \pm 0.09$	n.d.	This study—Experiment 1
Low light	$2.90 \pm 0.20$	$0.13 \pm 0.03$	$0.0020 \pm 0.001$	$1.00 \pm 0.37$	Lewitus et al.
	$3.19 \pm 1.17$	$0.53 \pm 1.12$	$0.22 \pm 0.16$	n.d.	This study—Experiment 1
	$0.17 \pm .001$	$0.16 \pm 0.03$	$0.14 \pm 0.05$	$0.14 \pm 0.01$	This study—Experiment 2
Treatment	$NO_3^-$	$NH_4^+$ ( $\text{fmol N cell}^{-1} \text{h}^{-1}$ )	Urea	Glutamic Acid	Reference
High light	$120 \pm 30$	$12 \pm 3$	$0.54 \pm 0.21$	$72 \pm 38$	Lewitus et al.
	$100 \pm 48$	$20 \pm 3$	$6.1 \pm 3.0$	n.d.	This study—Experiment 1
Low light	$200 \pm 70$	$12 \pm 3$	$0.54 \pm 0.21$	$65 \pm 29$	Lewitus et al.
	$109 \pm 40$	$18 \pm 38$	$7.5 \pm 5.4$	n.d.	This study—Experiment 1
	$34 \pm 2$	$32 \pm 6$	$28 \pm 1$	$28 \pm 0.5$	This study—Experiment 2

Both studies included a high light and a low light treatment. The high light treatment in the Lewitus study was  $360 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ , and the low light was  $70 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ . In this study the high light treatment was  $560 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ , and the low light treatment was  $12 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ . Although the light levels for Experiment 2 were not measured, the incubation conditions were the same as low light Experiment 1. The results for Experiment 1 (Exp 1) are the average of the uptake rates for all assay treatments (TOX-A, TOX-B, and NON-IND), measured on Day 1 only. The results for Experiment 2 (Exp 2) are the average of NON-IND (NR and ND) *P. piscicida* only. Only Experiment 2 included a glutamic acid treatment; thus no data (n.d.) are available for this substrate for Experiment 1.

types in the presence and absence of inorganic N and P. In their study, increases in cell number were significant for TOX-B and NON-IND strains, in the treatments with inorganic nutrients and cryptophytes, but not in the TOX-A strains, consistent with the findings here that direct uptake by TOX-A is low on an N-specific basis. For all functional types, cell production was negligible when cryptophytes were excluded from the experiments; in fact, without cryptophytes most cells encysted (Burkholder et al., 2001a).

Glasgow et al. (2001b) observed species differences in response to N (as  $\text{NO}_3^-$ ) and phosphorus (P, as  $\text{PO}_4^{3-}$ ) enrichments. For the strains used, *P. shumwayae* had higher cell production than *P. piscicida* in 4-day trials under N enrichment, but *P. piscicida* displayed a higher cell production upon enrichment with P. This pattern was reproduced over three enrichment levels for each nutrient source. Burkholder et al. (2001a) also showed that without further additions of algal prey, cultures of *P. piscicida* TOX-B and NON-IND cultures increased 10-fold within a period of days when exposed to inorganic N and  $\text{PO}_4^{3-}$ , but TOX-A cultures did not.

Chemosensory experiments (Burkholder and Glasgow, 1997; Burkholder et al., 2001a; Cancellieri et al., 2001) have demonstrated a strong attraction of toxic *Pfiesteria* spp. strains to fresh fish tissue, mucus or excreta. Most significantly, while all functional types of cells displayed an attraction to sterile fish mucus and excreta in short-term exposures in microcapillary tubes, attraction of the TOX-A cultures was far greater than that of the other functional types (Burkholder et al., 2001a; Cancellieri et al., 2001). Although specific uptake rates by all TOX-A sub-cultures from the experiments in the present study were very low, all displayed either a strong preference for urea or for glycine or glutamic acid. Whether this preference for organic nutrients by TOX-A cultures is related to the chemosensory attraction previously reported will require further investigation.

#### 4.2. N uptake versus phagotrophy

There is no question that *Pfiesteria* spp. has nutritional strategies that range from uptake of dissolved compounds, as demonstrated directly herein and shown by Lewitus et al. (1999b), to phagotrophy, which has been well described (reviewed by Burkholder and Glasgow, 1997). Lewitus et al. (1999b) roughly calculated that N uptake rates by kleptoplastic *Pfiesteria* might approach, or possibly exceed, those obtained through phagotrophy. The rates of grazing determined for Experiment 1 cultures (reported in Lewitus et al., in press) permitted us to assess the relative contribution of N uptake and heterotrophy under these experimental conditions. When *Rhodomonas* prey were added to cultures of each functional type, both TOX-B and NON-IND types grew exponentially, but those of TOX-A did not; indeed, their cell numbers were reduced with time (Lewitus et al., in press). However, all cultures, including those of TOX-A, showed evidence of chlorophyll retention within several hours, suggestive of some degree of grazing. The TOX-A cultures may have grazed, but not retained, the *Rhodomonas* pigments to the extent of the other cultures (Lewitus et al., in press). To compare the grazing rates with N uptake rates, ingestion rates were derived from Table 1 of Lewitus et al. (in press), and converted to N units, based on the assumption that a *Rhodomonas* cell contains 1 pmol N cell<sup>-1</sup> (Lewitus and Caron, 1990). On Day 1, N uptake contributed <10% of the N obtained by ingestion for all functional types (Table 4). From Day 1 to Day 2, this general relationship was maintained for both TOX-B and NON-IND functional types, although both N uptake rates and N ingestion per cell decreased sharply. Interestingly, N uptake per cell in TOX-A cultures increased, so that by the end of Day 2, N uptake contributed more to total cell

Table 4

Comparison of the total (as the sum of urea, ammonium, and nitrate only) nitrogen uptake rate per cell (fmol N cell<sup>-1</sup> h<sup>-1</sup>) and the ingestion rate, converted from units of prey predator<sup>-1</sup> h<sup>-1</sup> (from Lewitus et al., in press; Table 1) to units of fmol N cell<sup>-1</sup> h<sup>-1</sup>, for cultures of *Pfiesteria piscicida* of varying functional type from Experiment 1

Functional type	N uptake rate day 1 (fmol N cell <sup>-1</sup> h <sup>-1</sup> )	Ingestion rate $I_{[0-3 \text{ h}]}$ (fmol N cell <sup>-1</sup> h <sup>-1</sup> )	N uptake rate Day 2 (fmol N cell <sup>-1</sup> h <sup>-1</sup> )	Ingestion rate $I_{[3-23 \text{ h}]}$ (fmol N cell <sup>-1</sup> h <sup>-1</sup> )
TOX-A	185 ± 2	2050 ± 900	352 ± 226	155 ± 52
TOX-B	130 ± 65	2600 ± 405	16 ± 10	500 ± 40
NON-IND	130 ± 40	2700 ± 750	34 ± 13	380 ± 105

Data are the averages of both high light and low light treatments. The conversion was based on a *Rhodomonas* cell N content of 1 pmol N cell<sup>-1</sup> (from Lewitus and Caron, 1990). Comparisons are based on Day 1 uptake values with ingestion rates calculated from 0–3 h, and Day 2 uptake rates with ingestion rates calculated from the 3–23 h period (as described in Lewitus et al., in press).

nutrition than did grazing (Table 4). Thus, these calculations are consistent with those of Lewitus et al. (1999b), but only for the TOX-A cells.

Parrow et al. (2001) compared ingestion rates and estimated percent mixotrophy rates for varying functional types of both *P. piscicida* and *P. shumwayae* and found that in TOX-A cells the percentage of auto-fluorescent pigmented inclusions (potential kleptoplasts) was very small relative to the other functional types. They concluded that photosynthetic rates of TOX-A cells would be expected to be low compared to the other cell types. These findings are consistent with those reported here, as the proportion of potential kleptochloroplasts was higher in our TOX-A cultures relative to theirs. These comparisons demonstrate that the potential for dissolved N uptake is important in the nutrition of these heterotrophic cells, but also confirm the high degree of variability in the proportion by which it may be used.

#### 4.3. Eutrophication and *Pfiesteria* spp.

Links between nutrient enrichment and *Pfiesteria* have previously been made (e.g. Burkholder and Glasgow, 1997; Burkholder et al., 1999; Magnien, 2001; Samet et al., 2001; Glibert and Magnien, 2004; Glibert et al., 2001, 2004), although many of these links have emphasized the potentially high P demands of these cells, rather the N demands. Toxic *Pfiesteria* outbreaks generally occur in waters that are shallow, poorly flushed, and enriched with nutrients. The geographic distribution of both *Pfiesteria* spp. in the U.S. appears to be confined to the East and Gulf of Mexico coasts (e.g. Rublee et al., 2001, 2004, in press), and its abundance has been positively related to eutrophication levels (e.g. Bricker et al., 1999; Lewitus et al., 2002). Not only have higher abundances *P. piscicida* and *Pfiesteria*-like dinoflagellates been observed near wastewater discharge sites, compared to control sites in the New River Estuary, North Carolina (Burkholder and Glasgow, 1997), but in Chesapeake Bay, the highest abundances of *P. piscicida* enumerated in sediment samples were also found in those sites for which urea concentrations were highest among 27 sites surveyed over 5 years (Glibert et al., 2004, 2005). *Pfiesteria* spp. are typically found in Chesapeake Bay on the lower eastern shore, a region of intensive agriculture operations and chicken production, and near Baltimore, a nutrient-polluted region (Glibert et al., 2005; Bowers et al., in press). In the lower eastern shore, the majority of nutrients are delivered via non-point

source runoff, from application of chicken manure and organic fertilizers and from sewage input from septic systems. Highest annual mean concentrations as well as highest individual measurements (exceeding  $20 \mu\text{mol N L}^{-1}$ ) have been observed for those Chesapeake Bay tributaries with the most intensive agriculture and poultry operations (Glibert et al., 2005). Burkholder et al. (1997) reported stimulation of *Pfiesteria* spp. by a swine effluent waste spill in a North Carolina estuary.

The association with eutrophication is, however, complex (e.g. Anderson et al., 2002; Glibert and Burkholder, in press), and does not necessarily imply direct cause and effect. Indeed, the most likely pathway by which nutrients stimulate *Pfiesteria* is indirect. As previously emphasized, nutrient enrichment may lead to the proliferation of the microalgae on which *Pfiesteria* feeds (Burkholder and Glasgow, 1997); in fact, chlorophyll *a* concentration has been found to be a good predictor of *Pfiesteria* abundance in several field sites, such as the Chesapeake Bay tributary which had the most significant *Pfiesteria* outbreak in 1997 (Glibert and Magnien, 2004). The comparison of the rates of uptake given here, for the cryptophyte prey and the *Pfiesteria* cultures used in these experiments, underscores the premise that nutrient enrichment may act more indirectly than directly in stimulating *Pfiesteria*. Uptake rates by the cryptophyte were roughly 10-fold higher for all forms of N than by *Pfiesteria* TOX-B or NON-IND cultures. Furthermore, as demonstrated by Lewitus et al. (in press) for the same cultures, within the 24 h experimental period, the TOX-B and NON-IND cultures nearly depleted the cryptophyte prey, suggesting that phagotrophy was the primary mechanism by which nutrition was derived. For nontoxic or weakly toxic cells, the ability to take up N directly in dissolved form may either confer an adaptive advantage when sufficient food is unavailable, or may simply be a consequence of diffusion into cells that thrive in high-nutrient environments. Highly toxic cells may have a higher dependency on dissolved substrates, and appear to prefer organic substrates when fish tissue or other preferred foods are not available.

Based on these finding, the conceptual model of Lewitus et al. (1999b) on the potential role of nutrients can now be extended. In their model, an indirect stimulation of *Pfiesteria* spp. growth during spring, following spring runoff, was suggested, but a more direct nutrient stimulation was suggested in summer (in the absence of fish prey; see Glasgow et al., 2001a)

when phytoplankton biomass, and therefore food for grazing, is generally lower. More likely, direct nutrient stimulation is episodic and ephemeral. Glasgow et al. (2001a) extended Lewitus et al.'s (1999) model to include varying presence of fish prey, and supported the premise that stimulation of *Pfiesteria* spp. by dissolved nutrients, alone, is likely episodic and ephemeral. Consistent with these models is the fact that regenerated and organic nutrients, the preferred N forms for *Pfiesteria* spp. are more likely to be in abundance during the summer months. Even so, direct nutrient uptake is unlikely to be seasonally sustained.

## 5. Conclusions

These studies have shown that (1) direct uptake of N occurred by all functional types of these strains of *P. piscicida* and *P. shumwayae*; (2) specific N uptake rates are higher for the functional types TOX-B and NON-IND than for TOX-A, but uptake per cell for TOX-A cells is comparable to that of the other functional types, likely reflecting the smaller size of the TOX-B and NON-IND cells under these experimental conditions; (4) N preferences generally follow a decreasing trend of amino acids > urea >  $\text{NH}_4^+$  >  $\text{NO}_3^-$  and  $\text{NO}_3^-$  is never a preferred form of N; (5) uptake rates are related to N availability, although kinetic relationships could not be established for all N forms; (6) N uptake by *P. piscicida* contributes <10% of the cellular N of actively feeding TOX-B and NON-IND types, but may be equal to or greater than phagotrophic N acquisition in TOX-A cells (when prey is available), on a per-cell basis. Although *Pfiesteria* spp. is often found in eutrophic environments, the relationship between *Pfiesteria* spp. and nutrient availability is likely to be primarily indirect, mediated through the production of suitable prey cells on which *Pfiesteria* spp. feeds. However, when N concentrations are elevated, they can contribute to the supplemental nutrition of these cells, and may even provide a significant source to cells that are highly toxic. The diversity of functional types and nutritional modes displayed by these *Pfiesteria* spp. are large, and there is much yet to be understood regarding the environmental or biochemical factors that regulate the processes by which these cells switch from heterotrophy to mixotrophy to acquire their nutrients. Mechanistic models, such as those of Zhang et al. (2004) and Hood et al. (in press), in which the effects of various physical, physiological and trophic interactions may be explored for different functional types, are helping to yield insights about these complex dynamics.

## Acknowledgements

We thank H.B. Glasgow Jr., N. Deamer-Melia, E. Hannon, J. Springer and C. Zhang for assistance with experiments conducted at NCSU Center for Applied Aquatic Ecology, and J. Alexander, E. Haramoto, C. Shoemaker and L. Lane for assistance with analysis at the Horn Point Laboratory. This research was supported by the ECOHAB Program (NOAA/NSF/EPA/NASA/ONR) grant NA860PO493. This is Contribution No. 186 from the ECOHAB Program and 3965 from the University of Maryland Center for Environmental Science.

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