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EFFECT OF SUBSURFACE NUTRIENT SUPPLIES ON THE VERTICAL MIGRATION OF *EUGLENA PROXIMA* (EUGLENOPHYTA)[1]

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***Euglena proxima* Dangeard inhabits intertidal sand flats and displays a tidal rhythm in vertical migration. During daytime low tides when the sand flat is emersed, millions of cells are visible on the sediment surface, but the population remains below the surface at all other times. An earlier study demonstrated that the extent of downward migration of *E. proxima* is reinforced by the presence of a subsurface layer of black sediment. The present study was designed to test the hypothesis that the higher availability of inorganic nutrients or organic substrates in or above the black layer is responsible for the enhancement of downward migration in *E. proxima*. This hypothesis was tested experimentally by manipulating the bottom water in 24 mesocosm containers in a tidal tank. Six replicates of each of the following nutrient treatments were tested: seawater control; deep porewater collected from 70 cm below the sediment surface; seawater enriched with ammonium, nitrate, and phosphate; and seawater enriched with acetate, glucose, and the preceding inorganic nutrients. Multivariate analysis of variance revealed that the chl *a* biomass and chl *a*-weighted mean depth of the population at high tide were significantly greater for replicates receiving inorganic nutrients. There was no difference between those receiving only inorganic nutrients and those enriched with inorganic nutrients, acetate, and glucose. These findings represent the first experimental evidence that subsurface nutrients are an important resource that reinforces the maintenance of vertical migration behavior in benthic microalgae.**

Key index words: **benthic microalgae; chl *a*; *Euglena proxima*; North Carolina; nutrients; nutricline; vertical migration**

Many species of microalgae exhibit a tidal vertical migration response in which the cells form large green and golden brown patches on the surface of intertidal sand and mud flats during low tide. Before tidal inundation, these microalgal populations burrow down into the sediment. Previous studies have considered the role of light (Aleem 1950, Perkins 1960, Palmer and Round 1965, Hopkins 1966a, Harper 1976, Pinckney and Zingmark 1991, Kingston 1999a), waves (Kingston 1999b), tides (Fauvel and Bohn 1907, Aleem 1950, Fauré-Fremiet 1951, Ganapati et al. 1959, Palmer and Round 1967, Happey-Wood and Jones 1988), currents (Heckman 1985), desiccation (Aleem 1950), submersion (Hopkins 1963, 1966b), temperature (Hopkins 1963, Palmer and Round 1965), rainfall (Bracher 1929), interstitial oxygen concentrations (Hopkins 1963), physical disturbance (Hopkins 1966b, Happey-Wood and Priddle 1984), meiofaunal predators (Buffan-Dubay and Carman 2000), and microfaunal predators (Fenchel 1968) in the maintenance of benthic microalgal vertical migration. Some authors have suggested that migration facilitates access to nutrients and dissolved organic compounds deeper in the sediments (Admiraal 1984, Happey-Wood and Priddle 1984, Happey-Wood and Jones 1988, Barranguet et al. 1998), but this hypothesis has never been experimentally tested.

The purpose of this study is to establish the role of high subsurface levels of dissolved inorganic nutrients and dissolved organic carbon in the maintenance of tidal vertical migration in *Euglena proxima* Dangeard. Previous research with this species revealed that downward migration during high tide periods was enhanced by the presence of a subsurface layer of black sediments (Kingston 1999b). This enhancement of downward migration may have been related to increased dissolved inorganic nutrients or dissolved organic carbon within or above these reduced sediments. Additionally, laboratory experiments revealed that the phototactic response of *E. proxima* was significantly affected by dissolved inorganic nutrient enrichment (Kingston 1990, 1996). The present study was undertaken to specifically investigate the effect of subsurface nutrient and dissolved organic compound reservoirs on the amplitude of vertical migration of *E. proxima*.

Recent studies have examined the standing stock and flux of dissolved inorganic nutrients and dissolved organic compounds on intertidal sand and mud flats. Langner Van Voorst and Hoepner (1996) reported that ammonia differs by a factor of 60 and phosphate by a factor of 750 in cm scale vertical transition zones within the sediments of intertidal sand flats. Ram and Zingde (2000) reported that the inorganic products of aerobic oxidation of organic matter (ammonium, nitrate, nitrite, and phosphate) accumulated in the 0- to 4-cm sediment layer. They found

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a maxima of ammonium and phosphate at intermediate depths in most sediment cores associated with low salinity interstitial porewater. Although tidal flushing of intertidal sediments causes one to two orders of magnitude higher losses in ammonium and phosphate than in subtidal sediments (Falcao and Vale 1995, Rocha 1998), the microphytobenthic community reduces the flux of ammonium, nitrate, and phosphate from the sediment to the water column during light periods in both habitats (Sundback et al. 1991, Feuillet et al. 1997, Kuwae et al. 1999). In addition to inorganic nutrients, organic compounds represent a potential resource for heterotrophic nutrition. Glucose and acetate, which are two important carbon sources for heterotrophic growth of *Euglena gracilis* Krebs (Cook 1968), represent rich resources in intertidal sediments. Glucose represents 80% of the colloidal monosaccharides and 37% of the refractory carbohydrate fraction on intertidal sand flats with associated diatom assemblages (Taylor et al. 1999). Holmer (1996) reported that acetate is an important component of the dissolved organic carbon pool of intertidal sand flats, especially at depths of 8–10 cm where it makes up 100% of the dissolved organic carbon.

In this study, I measured porewater nutrient concentrations at selected depths in intertidal areas where large patches of benthic microalgae form during low tide to verify that subsurface nutrient availability exceeds that on the sediment surface. I followed this field sampling with manipulative experiments conducted in mesocosm containers in a tidal tank to test the effect of four selected nutrient solutions on the chl *a* biomass and the mean depth of the population during high tide.

The general hypothesis tested in this study was that the mean depth of the *E. proxima* population and the abundance of cells in the population (as measured by chl *a*) would be greater in the presence of a subsurface inorganic nutrient reservoir. Because several strains of *E. gracilis* Krebs are capable of heterotrophic nutrition using glucose and/or acetate (Cook 1968, Levedahl 1968), these organic compounds were added to inorganic nutrients in one of the nutrient treatments. Because reduced compounds produced in the subsurface anoxic layer such as hydrogen sulfide affect the distribution of benthic microalgae (Admiraal and Peletier 1979), natural porewater containing hydrogen sulfide and other reduced compounds collected from deep within the anoxic layer was also included as one of the test solutions. The research design only permitted testing three *a priori* partial hypotheses: 1) porewater collected from within the anoxic layer would inhibit downward migration during high tide relative to a seawater control; 2) subsurface reservoirs of seawater enriched with dissolved inorganic nutrients (nitrate, phosphate, and ammonium) and dissolved organic compounds (glucose and acetate) would reinforce downward migration during high tide more strongly than reservoirs enriched with dissolved inorganic nutrients alone; and 3) enriched subsurface reservoirs (dissolved inorganic nutrient treatment and the dissolved inorganic nutrients plus dissolved organic compounds treatment) would result in deeper migration during high tide than unenriched reservoirs (seawater control and porewater treatments).

MATERIALS AND METHODS

In situ nutrient sampling. Benthic vertical nutrient gradients in the field were measured by collecting and analyzing porewater samples during June and July 1990. These field samples were collected on the intertidal sand flats adjacent to the Duke Marine Laboratory in Beaufort, North Carolina, which included sand flats around Pivers Island, Radio Island, and Town Marsh. The sampled sand flats were marked by visible patches of diatoms and euglenoids on the surface during daytime low tides occurring between 0.15 and 0.67 m above mean low water. Porewater samples (50 mL) were collected at depths of 1, 5, and 10 cm with a 12.5-cm long cannula (no. 16 Yale, Becton-Dickinson and Co., Parsippany, NJ, USA) attached to a 50-mL syringe during the day under emersed and submersed conditions. A 50-mL seawater sample was also collected with the syringe just above the surface of the tidal flat during submersed periods.

Porewater samples (50 mL) at depths of 30 and 70 cm were obtained using mini-piezometers installed 1 week before sampling. These samplers were constructed of polyethylene tubing (0.4 cm inner diameter [ID]) fitted into a perforated polyethylene cylinder (0.6 cm ID) covered with landscaping cloth. The design and use of mini-piezometers are described fully by Lee and Cherry (1978). An iron pipe with a loose stainless steel bolt cap was pounded in the sediment to a specified depth, the mini-piezometer threaded into the pipe, and the pipe removed, leaving the stainless steel bolt and mini-piezometer in place. A 50-mL aliquot of porewater was removed with a vacuum flask and pump. The first sample drawn from a mini-piezometer contained small particulates and organic matter, but subsequent samples were clear due to sediment packing around the piezometer head. The nutrient data reported in this study were collected more than 1 week after installation of the mini-piezometer.

After withdrawal, the porewater samples were placed in polyethylene bottles, stored on ice, and brought to the laboratory for analysis. All samples were filtered through A/E glass fiber filters (1 μm nominal pore size, Gelman Sciences, Ann Arbor, MI, USA) before analysis. Ammonium samples were processed immediately, whereas phosphorus and nitrate/nitrite were frozen at −5° C and processed within 2 weeks of collection. These samples were analyzed for ammonium (alternate method later in the article), phosphate, and nitrate/nitrite using the methods described in Parsons et al. (1984). Nitrate/nitrite is reported as one value because limited sample volumes required the entire sample to be run through the cadmium column. This methodology provided the concentration of total nitrogen in both forms but did not permit an assessment of the relative contribution of each.

Experimental design. Between 26 May and 2 June 1990, an experiment was conducted in a shallow outdoor tidal tank (1.3 m long, 0.8 m wide, and 0.1 m deep) holding 60 L of seawater and 24 plastic mesocosm containers. Ebb and flood tides were simulated by removing or replacing a standpipe over the drain in the tank. The timing of emersion and submersion was coincident with that in the area where the population of *E. proxima* for this experiment was collected. The seawater entering the tank was filtered through a filter bag (5 μm pore size; American Felt and Filter Co., New Windsor, NY, USA), which was cleaned on every tide change. The filter bag was replaced every 2 days. The seawater was supplied by gravity feed from the Duke Marine Lab seawater system settling tank at a flow rate of 4 $L \cdot min^{-1}$.

Two weeks before the experiment, 0.02 m^3 of sand was collected from the intertidal sand flat adjacent to the *E. proxima* patch and sifted to remove stones and shell fragments. The sand was brought to the laboratory and acid cleaned to remove all organic matter. The sand was first rinsed 10 times with

deionized water to remove most of the salt and small particulates. After decanting off the overlying water from the last rinse, 3.8 L of 37 N sulfuric acid was added to the sand. The sand–acid mixture was stirred for 30 min, and then 12 L of deionized water was added. After 2 h the diluted acid was decanted and the sediment was flushed with flowing deionized water for 2 h. Sodium hydroxide was used to neutralize the sand, and then it was rinsed three more times with deionized water. On the following day, the sand was spread on polyethylene sheets to dry in the sun for 5 h. After drying, the sand was stored in a polyethylene container in the laboratory.

Four nutrient treatments were tested in this experiment: seawater, seawater enriched with dissolved inorganic nutrients, seawater enriched with dissolved inorganic nutrients and dissolved organic compounds, and deep porewater from the intertidal zone. The seawater used in the first three nutrient treatments was drawn from the Newport River estuary at the Duke Marine Laboratory dock and filtered to a nominal pore size of 0.45 μm (type HA membrane filter; Millipore Corp., Bedford, MA, USA). The salinity of this seawater as measured with a hand-held refractometer (A. O. Scientific Instruments, Buffalo, NY, USA) was 33 psu. NH_4Cl, $NaNO_3$, and $NaH_2PO_4 \cdot H_2O$ were added to a 600-mL volume of seawater to result in final concentrations of 100 μm NH_4^+, 50 μm NO_3^-, and 25 μm PO_4^{-3}. This nutrient solution was split into two 300-mL volumes. One 300-mL volume of each nutrient solution was enriched with glucose and sodium acetate to result in a final concentration of 50 μm glucose and 100 μm acetate; the other volume received no organic supplements.

The concentrations of dissolved inorganic nutrients and dissolved organic compounds used in this experiment were higher than published standing stock concentrations of these nutrients in natural porewater. To control for the effects of extraneous variables, the organic component of the sand used in this experiment was removed. Consequently, there was no organic detritus or bacterial flora present at the initiation of this experiment to produce dissolved inorganic nutrients and dissolved organic compounds within the mesocosm containers. Because these compounds would not be continually replaced by natural processes, the mesocosm containers were spiked with dissolved inorganic nutrients and organic compounds at concentrations approximately equivalent to those supplied by natural intertidal sediment over a 7-day period. Flux rates from the literature indicate that a 100-cm^2 area of intertidal sand flat generates a mean flux of 609 μmol·wk^{-1} of nitrate, 14 μmol·wk^{-1} of nitrite, and 259 μmol·wk^{-1} of ammonium over an annual cycle (Mortimer et al. 1999). The natural mud flat biota within the 0- to 2-cm depth interval in a 100-cm^2 area consume a lower limit of 560 μmol·wk^{-1} of glucose (Sawyer and King 1993) and the bioavailable acetate turnover rates in estuarine sediment would generate 441 μmol·wk^{-1} of acetate (Wellsbury and Parkes 1995). Based on the literature, the dissolved inorganic and organic compound concentrations used in this experiment are well within projected 1-week generation rates in natural sediment.

Deep porewater (400 mL) was collected from the sand flat areas where large aggregations of *E. proxima* were found during low tide. The porewater was collected during high tide on the first day of the experiment using a hand-held vacuum pump and a mini-piezometer (Lee and Cherry 1978) that had been installed several weeks previously. The porewater was withdrawn from a depth of 70 cm below the surface of the sand flat to avoid contamination from the overlying water column. The salinity of the porewater as measured with a hand-held refractometer was 9 psu, reflecting the influence of subsurface groundwater flow. The porewater, which was not filtered before use in the experiment, contained small organic particulates and smelled of hydrogen sulfide. The inclusion of this treatment made it possible to evaluate the hypothesis that anoxic porewater from deeper, black, reduced sediment layers may serve as a barrier to downward migration for aerobic microalgae (Palmer and Round 1965, Hopkins 1966a).

On the morning of the first day of experimentation, the dried acid-cleaned sand was poured into 24 plastic mesocosm containers (10 × 10 cm, 5.5 cm deep) to form a layer of sand 2 cm deep. Four rectangular apertures (2 × 1 cm) had been cut into each side of each container and covered with Nitex screening (53 μm pore size, H. R. Williams Mill Supply, Kansas City, MO, USA). The 2-cm layer of sand was just below these windows. Fifty milliliters of the filtered seawater treatment solution was poured into the sand in six containers so that it just covered the 2-cm sand layer. Six separate containers received the seawater supplemented with inorganic nutrients, six others received the seawater plus inorganic/organic supplements, and the final six containers received the deep porewater collected earlier in the day. This interstitial water in the bottom of each container below the level of the windows will henceforth be referred to as "bottom water." After the bottom water was added, more dry sand was added to fill all the containers.

The mesocosm containers were systematically arranged in the outdoor tidal tank in a 4 × 6 arrangement with each treatment present only once in each row. The tidal tank was filled and drained once with the containers in place before inoculating each with *E. proxima*. The cells in the inoculum were collected from patch areas on the sand flat by scraping off the surface layer of sand. The cells were suspended in filtered seawater that was then decanted. The cell density in the supernatant was determined using a hemacytometer (A. O. Scientific Instruments, Buffalo, NY, USA). The cell suspension was inoculated into each container by inserting a thin-walled Lucite pipe (4.5 cm ID) into the sand in each container and pipetting the cell suspension into the top of this pipe. This protocol produced a uniform layer of 6.73 × 10^5 cells covering a 15.9-cm^2 circular area in the center of each container.

For the next 7 days the tank was maintained on a tidal cycle identical to that in the area where the inoculum cells were removed. During high tide periods, the mesocosm containers were covered by a 4-cm depth of seawater. During low tide periods, the water level in the tank was approximately half way up the windows in each mesocosm container so that evaporating water would be replaced. During the night and whenever it threatened to rain during the day, the tank was covered by a transparent polyethylene plastic sheet.

Data collection. It was essential to the design of this experiment that the nutrient concentration of the bottom water in the nutrient enrichment treatments remained substantially higher than that in the unenriched treatments. The ammonium concentration was used as an indicator of nutrient loss over time. Ammonium was chosen as the indicator because the assay is quick and only requires a small sample volume (5 mL). On the first and last day of the experiment, a 5-mL sample of the bottom water was removed from the bottom of three randomly selected containers, representing the seawater control, anoxic porewater, and enriched nutrient treatments, with a 5-mL syringe and cannula (no. 19 Yale, Becton-Dickinson and Co., Parsippany, NJ, USA). The cannula was inserted in the sediment away from the central area of the mesocosm container to prevent disturbance of the sediment in the central cell inoculation area. A sample of the seawater in the tidal tank overlying the mesocosm containers was also collected. These samples were analyzed immediately for ammonium with the alternative method of Parsons et al. (1984, p. 14) to evaluate bottom water dilution over the 1-wk course of the experiment.

During high tide of the last afternoon of the experiment, each container was sampled by extracting a core with a thin-walled plastic coring tube (1.05 cm ID). Before extracting each coring tube, a thin plastic square was inserted between the bottom of the coring tube and the bottom of the mesocosm container. After core removal, the overlying seawater was pipetted off and placed into a labeled 20-mL glass vial. After removal of the seawater, an aluminum plunger was inserted into the top of the coring tube and the sediment core extruded bottom first. The extruded core was sectioned with a razor blade and the sections placed into individual labeled 20-mL glass vials. Each core was sectioned into depth intervals of 0–10, 10–20, 20–25, 25–30, 30–35, 35–40, 40–45, and 45–50 mm. Ten milliliters of 90% acetone was added to each vial containing a section, and the overlying seawater samples were filtered onto a filter disk (type A/E glass fiber filter, 1 μm nominal pore size; Gelman Sciences, Ann Arbor, MI, USA) that was then placed into the seawater collection vial with 90% acetone. All samples were then

extracted for 48 h at −5° C. All vials were agitated twice per day to ensure complete extraction. The chl fluorescence of the seawater and sediment extracts was measured in a darkened room on a fluorometer (model 10-005B, Turner Designs, Sunnyvale, CA, USA). The chl *a* content of each sample was calculated using the equation for total chl (Parsons et al. 1984, p. 108).

The chl *a* biomass in each section of a core was used to calculate a mean depth for the population of cells in each mesocosm container. The proportion of chl *a* in each section was calculated by dividing the chl *a* biomass of each section by the sum of all sections. The proportion of chl *a* in each section was then multiplied by the mid-depth value of that section. These chl *a*-weighted depth values were summed to yield the chl *a*-weighted mean depth for each mesocosm container.

Cell counts were performed using 12 of the 45- to 50-mm sections of the extracted cores from the nutrient containers to confirm the value of chl *a* biomass as an estimate of cell density. This depth was chosen as the standard to compare all nutrient treatments because the mesocosm containers were cored during high tide conditions when the cells have reached their furthest limit of downward migration. The choice of this section ensured that both chl *a* and cell numbers would be at high enough levels to obtain precise measurements of cellular chl *a* content. Three of the six containers for each of the four nutrient treatments were randomly chosen for analysis. The vials containing the 45- to 50-mm section of each core were left opened under a fume hood until all the acetone had evaporated, leaving 0.5 mL of water over the sand in each vial. The cells were suspended in the overlying water and then loaded into a Palmer (0.1 mL, Wildlife Supply Co., Saginaw, MI, USA) counting chamber, and cell counts were performed at 100× magnification.

Statistical analysis. Linear regression was used to evaluate the relationship between cell density and chl *a* biomass using Sigma Plot statistical software (Sigma Plot version 3.1, Jandel Scientific, Corte Madera, CA, USA). These data were also used to calculate the cellular chl *a* content in the 12 randomly selected mesocosm containers. One-way analysis of variance (ANOVA) was used to evaluate the effect of the four nutrient treatment levels on cellular chl *a* concentration using the general linear models option in the SAS statistical software (SAS Institute, Cary, NC, USA).

Multivariate analysis of variance (MANOVA) procedure in the SAS statistical software was used to analyze the experimental results, with chl *a*-weighted mean depth and total core chl *a* serving as dependent variables and nutrient treatment as the independent variable. This statistical approach was chosen because two relevant variables were likely to be affected by the nutrient treatment in this study. First, the amplitude of vertical migration, as measured by the mean depth of the population during high tide, might be increased or decreased in response to nutrient concentration. Second, the number of cells in the population, as measured by the chl *a* biomass, might increase if the supplemented nutrients in the bottom water were available to vertically migrating cells. Using MANOVA, rather than performing separate univariate ANOVAs, protects against type I error and may reveal differences not shown in separate ANOVAs (Tabatchnick and Fidell 1983). The mean depth and total core chl *a* were $\log_{10}$ transformed before analysis to satisfy the requirements for normality and homogeneity of variance.

Aside from the MANOVA, which tests the complete null hypothesis of combined treatment effect, three *a priori* partial hypotheses were tested. These three partial hypotheses tested for differences between deep porewater and the filtered seawater control treatments, between the inorganic nutrient enriched bottom water and the bottom water enriched with both inorganic nutrients and organic compounds, and finally between the enriched bottom water treatments (inorganic enrichment and inorganic/organic enrichment) and the unenriched bottom water treatments (deep porewater and the filtered seawater control).

RESULTS

In July 1990 ambient levels of ammonium, phosphate, and nitrate/nitrite were low in the water column. The concentrations of these nutrients 1 cm below the surface of intertidal sand flats were substantially higher than in the overlying water column (Table 1). Of the three nutrients measured, ammonium showed the greatest change with depth; the mean ammonium concentration at 5 cm below the surface was over 15 times greater than that in the water column. Although the mean concentration of all three nutrients showed an increasing trend with depth, the variability at each depth was great. The range of ammonium concentrations measured at 5 cm below the surface was 6.8–46.6 μM for eight different intertidal sand flats.

Within the mesocosm containers, the rise and fall of the tide in the experimental tank was bound to subject the bottom water in each container to some degree of flushing. Biological uptake represents another pathway for nutrient depletion. The ammonium concentration in the bottom water of the nutrient enrichment containers remained high over the course of the experiment relative to the seawater control and deep porewater treatment containers (Table 2). The decrease in the initial ammonium level between the first and last day of the experiment was approximately 40% in the randomly selected inorganic enrichment treatment. Thus, the loss of nutrients did not deplete the bottom water below the range of ambient stand-

TABLE 1. Nutrient concentrations of seawater and sediment porewater at different depths on intertidal sand flats around the Duke University Marine Laboratory, Beaufort, North Carolina.

Depth	Ammonium	Phosphate	Nitrate/nitrite
Seawater	1.37 ± 0.74 (n = 3)	0.30 ± 0.37 (n = 3)	0.10 ± 0.01 (n = 3)
1 cm	16.6 ± 13.9 (n = 6)	2.86 ± 1.05 (n = 2)	0.295 (n = 1)
5 cm	21.6 ± 12.6 (n = 8)	—	—
10 cm	22.7 ± 17.8 (n = 5)	2.80 ± 3.05 (n = 2)	—
30 cm	29.3 ± 5.63 (n = 3)	5.76 ± 0.46 (n = 3)	0.37 ± 0.03 (n = 2)
70 cm	24.2 ± 10.5 (n = 20)	5.33 ± 4.19 (n = 20)	0.74 ± 1.35 (n = 15)

Concentrations are expressed as μmol·L^{-1}. Values are means ± SD. The number of replicates (n) used in the calculation of the mean appear within parentheses. The missing data for phosphate and nitrate/nitrite for depths 5 and 10 cm were due to the difficulty of obtaining a 50-mL sample before small particulates clogged the cannula opening

TABLE 2. Ammonium concentration expressed as μmol·L^{-1} in the bottom water of one randomly sampled mesocosm container for three of the four nutrient treatments at the start and end of the experiment.

Treatment	Day 1	Day 7
Seawater control	0.44	3.04
Deep porewater	5.68	4.91
Inorganic enrichment	95.5	57.0

ing stock concentrations *in situ* (Table 1). In fact, the ammonium concentration at 5 cm deep in a single randomly chosen inorganic enrichment container on the last day of the experiment was 57 μm (Table 2), which is dramatically higher than the mean of eight samples collected from the same depth *in situ* (Table 1). Although this indicates the ammonium concentration was much higher than average, it was not dramatically higher than the highest (46.6 μM) of the eight samples collected on the sand flats surrounding the Duke Marine Laboratory. The ammonium concentration of the overlying seawater in the tidal tank on the last day of the experiment was 1.0 μM, which was also similar to *in situ* measurements.

Linear regression analysis of the cell density and chl *a* content of the lowest 5 mm of sediment in 12 randomly selected mesocosm containers revealed a statistically significant correlation ($r^2 = 0.914$, $P < 0.0001$, Fig. 1). This result validates the approach of using chl *a* biomass to calculate the mean depth of the population. These samples were also used to calculate the cellular chl *a* content of the cells in the 12 randomly selected mesocosm containers. A one-way ANOVA was used to analyze the effect of nutrient treatment on the cellular chl *a* content of the cells at the bottom of each core. The results of this analysis indicate that the various nutrient treatments did not result in a significant difference in the cellular chl *a* content of *E. proxima* (Table 3). These results taken together indicate that the increase in chl *a* content of the nutrient enriched treatments resulted from an increase in the density of cells and not in cellular chl *a* levels.

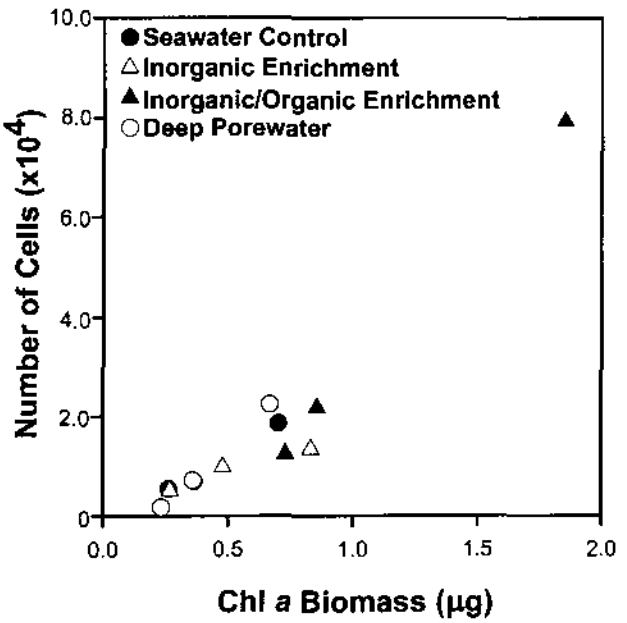

FIG. 1. Cell number as a function of chl *a* biomass in the bottom section (45–50 mm) of three randomly sampled cores from each nutrient treatment. The regression equation for these points is number of cells = 44354.1 × (chl *a*) −11214, $r^2 = 0.914$, $P < 0.0001$.

TABLE 3. One-way ANOVA results for the effect of nutrient treatment on chl *a* per cell (pg chl *a*·cell^{-1})

Source	*df*	MS	F	*P*
Nutrient	3	0.0202	0.54	0.6680
Error	8	0.0375		
Specific contrasts				
Seawater vs. deep porewater	1	0.0190	0.51	0.4967
Inorganic vs. inorganic/organic	1	0.0262	0.70	0.4276
Enriched vs. unenriched	1	0.0067	0.18	0.6838

MANOVA on the $\log_{10}$ transformed data resulted in a significant Wilks lambda value, indicating that the nutrient treatments produced a significant effect on both the mean depth and the chl *a* biomass of the *E. proxima* population (Table 4). Consequently, the null hypothesis that the nutrient content of the bottom water does not affect mean depth and chl *a* biomass was rejected. Of the three partial null hypotheses tested, the only one rejected was that there is no difference between the unenriched (seawater control and deep porewater) and enriched (inorganic enrichment and inorganic/organic enrichment) treatments. No significant difference was found in the mean depth or chl *a* biomass between the deep porewater and seawater control or between the inorganic enrichment and inorganic/organic enrichment treatments. The mean depth of the populations in the nutrient enriched treatments was 37.8 mm, whereas the mean depth in the unenriched treatments was 33.9 mm. The mean chl *a* concentration of the nutrient enriched cores was 0.275 mg·cm^{-3}, whereas that of the unenriched treatments was 0.179 mg·cm^{-3}.

The vertical profiles of the four nutrient treatments reveal that the increased chl *a* biomass in the enriched treatments was concentrated in the lowest

TABLE 4. MANOVA results testing for the overall effect of nutrient treatment on the $\log_{10}$ transformed chl *a* content and the $\log_{10}$ transformed mean depth of the *Euglena proxima* populations.

Source	*df*	F	*P*
Complete null hypothesis			
Wilks lambda	6,38	2.625	0.0315*
Partial null hypotheses			
Mean depth contrasts			
Seawater vs. deep porewater	1	0.13	0.7214
Inorganic vs. inorganic/organic	1	0.10	0.7529
Enriched vs. unenriched	1	8.27	0.0413*
Chl *a* contrasts			
Seawater vs. deep porewater	1	0.04	0.8478
Inorganic vs. inorganic/organic	1	1.70	0.2076
Enriched vs. unenriched	1	5.22	0.0333*

The significance of the nutrient treatment over the complete null hypothesis was calculated using the F approximation to Wilks lambda. The results of the *a priori* partial hypotheses are also shown.

*Significant effects ($P \leq 0.05$).

portion of the cores between 4 and 5 cm deep (Fig. 2). The average chl *a* biomass in the 4- to 5-cm layer was 0.916 mg·cm^{-3} in the enriched treatments (six inorganic enrichment replicates and six inorganic/organic enrichment replicates) and 0.279 mg·cm^{-3} in the unenriched treatments (six seawater control replicates and six porewater control replicates). The distribution of chl *a* biomass between 0 and 4 cm was strikingly similar in all four nutrient treatments.

DISCUSSION

The low concentrations of ammonium, phosphate, and nitrate/nitrite measured in the water column were typical for the Newport River estuary during summer. Typical summertime concentrations of ammonium vary between less than 0.5 μM in the afternoon to more than 2.0 μM after dawn, whereas nitrate/nitrite concentrations vary between 0.18 μm in the afternoon to 0.35 μM after dawn (Litaker 1986, Litaker et al. 1987, 1988). Phosphate levels average 0.5 μM throughout the day (Litaker 1986).

The subsurface concentrations of these three macronutrients within the sediment showed considerable variability but were substantially higher than in the overlying seawater. The ammonium levels reported here for intertidal sand flats are substantially lower than those reported in other intertidal habitats and subtidal habitats. Porewater samples withdrawn from the intertidal sediment of a salt marsh revealed ammonium concentrations of 20 μM in the top 2 cm and 100 μM at 20 cm (Howes et al. 1985). Henrichs and Farrington (1987) reported values for subtidal sand flats that exceeded 100 μM at the sediment surface and were 300 μM at 20 cm below the surface. Kenworthy et al. (1982) reported ammonium concentrations of 50 μM in the top 3 cm of sediment that increased to 300 μM at 20 cm depth in the bare zones outside seagrass beds. In each habitat the concentration of ammonium increases with depth, and ammonium also increases along a transect from intertidal to subtidal habitats. The lower ammonium levels reported in this study may be related to the high intertidal location of sampled areas (+0.15–0.67 m above mean low

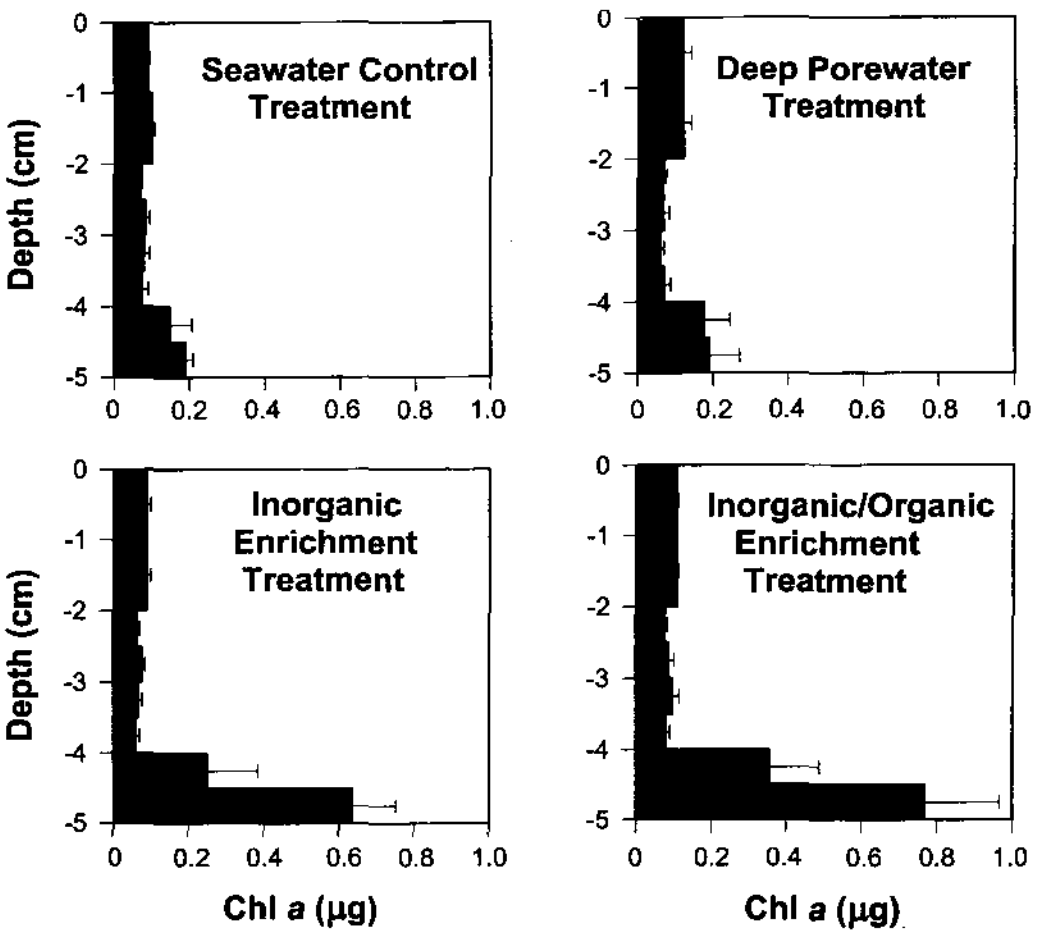

FIG. 2. Chl *a* vertical profiles in the four nutrient treatments. The histogram bars indicate the mean chl *a* content in each section of the cores (n = 6). The error bars illustrate the standard errors of the means for each section.

water). The disparity between the nutrient concentration of the overlying seawater and the 1 cm depth porewater sample collected indicates that subsurface nutrient reservoirs may represent a significant resource to vertically migrating microalgae.

The bottom water nutrient inocula used in this study were intentionally more concentrated than *in situ* levels to ensure that adequate nutrients would remain after continued tidal flushing during the 7-day course of the experiment. On the final day of the experiment, the ammonium concentration of the bottom water from a randomly chosen inorganic enrichment container suggests that about 40% of the dissolved inorganic nutrients were lost through tidal flushing and uptake by microalgae. The absolute ammonium concentration in the inorganic enrichment treatments at the end of the experiment was more than twice as high as the mean concentration of samples collected from nearby sand flats. However, one of the eight samples collected at a depth of 5 cm from nearby sand flats was also more than twice the mean. So, although the measured ammonium concentration at the end of this experiment was high relative to those observed on the average sand flat, it was not much beyond the range of ammonium concentrations observed in the field. Natural variability in sediment characteristics, the microbenthic community, tidal height, groundwater flow, and tidal flushing may affect the role that subsurface nutrient reservoirs play in maintaining the vertical migration behavior of microalgae on different sand flats.

Unlike ammonium, the concentration of nitrate and phosphate remained well above natural levels throughout the course of the experiment. If a 40% flushing/uptake rate is applied to these nutrients, then the estimate for nitrate and phosphate on the last day of the experiment would be 10 times and 18 times higher than measured *in situ*, respectively. Applying the same flushing/uptake rate to the inorganic/organic enrichment treatment, the final concentrations of acetate and glucose would have been 60 μM and 30 μM, respectively. Both estimates are higher than published values for porewater. Estimates of porewater glucose concentrations of 2 to 17 μM (Sawyer and King 1993) and porewater acetate concentrations of 3 to 20 μM (Wellsbury and Parkes 1995) have been measured on intertidal sand flats. The unusually high levels of glucose and acetate present at the end of this experiment did not appear to unduly affect the results because the difference between the inorganic enrichment and the inorganic/organic enrichment treatments was not statistically different.

MANOVA results indicate that the mean depth of the *E. proxima* population during high tide and its chl *a* biomass were significantly greater in the enriched treatments (inorganic enrichment and inorganic/organic enrichment treatments) than in the unenriched treatments (seawater control and deep porewater). This result supports the hypothesis that the population migrates to deeper depths during high tide when a subsurface nutrient reservoir is present. Access to higher nutrient levels was manifested as an increased chl *a* biomass of the population. The ANOVA results comparing the chl *a*·cell^{-1} values of the separate nutrient treatments indicate that the increased chl *a* biomass in the enriched treatments represented an increase in the number of cells and not the amount of chl *a* per cell.

Furthermore, the vertical profiles indicate that the nutrient-induced increase in chl *a* biomass seen in the enriched treatments was due to an increase in the proportion of the population found in the 4- to 5-cm depth layer during high tide. One interpretation of this finding is that the increase in chl *a* biomass reflects an increased reproductive rate in cells undergoing the full amplitude of migration from the surface to the subsurface nutrient reservoir between 4 and 5 cm. This interpretation is supported by the observation that the proportion of the population that underwent shorter vertical migrations, the cells between 0 and 4 cm during high tide, did not exhibit markedly different chl *a* concentrations in any of the four nutrient treatments.

The partial hypotheses of MANOVA comparing the seawater control with the deep porewater treatment and the inorganic enrichment treatment to the inorganic/organic enrichment treatment did not reveal significant differences in the mean depth at high tide or chl *a* biomass of the *E. proxima* populations. Apparently, the lower salinity (9 psu) and hydrogen sulfide in the anoxic porewater did not significantly inhibit downward migration as hypothesized. At the start of the experiment, the ammonium concentration in the porewater treatment was 5.24 μM greater than the seawater control treatment. By the end of the 7-day experiment, the difference between the ammonium concentration in the bottom water of a randomly chosen seawater control treatment and a porewater treatment was only 1.87 μM. If changes in ammonium reflect similar changes in the other macronutrients within the bottom water of the unenriched treatments, the lack of a significant difference may have been due to their similar nutrient profiles at the end of the experiment. The absence of a significant difference between the inorganic enrichment and the inorganic/organic enrichment treatment suggests that the change in mean depth at high tide and chl *a* biomass were not noticeably affected by acetate and glucose. Many strains of *E. gracilis* Krebs are capable of heterotrophic nutrition with these organic compounds, and some obligate photoautotrophic strains incorporate acetate in the light but not in the dark (Cook 1968). However, it appears that these organic compounds are not involved in the vertical migration response of *E. proxima* on intertidal sand flats. No published data are available on the heterotrophic ability of *E. proxima*.

Although the literature lacks observational or experimental studies demonstrating the role of subsur-

face nutrients in the vertical migration of benthic microalgae, this phenomenon has been well studied in large phytoplankton species inhabiting the pelagic realm. Mats of the diatom *Rhizosolenia* in the oligotrophic Pacific gyre undergo buoyancy-mediated vertical migrations between the nitrate-poor surface waters and nitrate-rich waters between 80 and 100 m deep (Villareal et al. 1993, 1999). A similar migration pattern was noted in another large diatom, *Ethmodiscus*, in the Sargasso Sea (Villareal and Carpenter 1994). Buoyancy-mediated vertical migration across a nutricline was also documented for the nonmotile dinoflagellate *Pyrocystis noctiluca* (Ballek and Swift 1986, Villareal and Lipshultz 1995) and the prasinophyte *Halosphaera* spp. (Villareal and Lipshultz 1995).

This study confirmed the role of subsurface inorganic nutrients in reinforcing the downward migration of *E. proxima*. A prior mesocosm study examining the effect of waves on the vertical migration of *E. proxima* indicated that a subsurface black layer of sediment possessed attractive properties that stimulated downward migration during high tide even under waveless conditions (Kingston 1999b). The attractive quality of the subsurface black layer measured in that study may have been related to higher nutrient concentrations above or within the black layer. The results reported here suggest that high subsurface nutrient concentrations entrain more of the population resulting in a greater proportion of cells migrating deeply, which increases the mean depth of the population during high tide. In the absence of these subsurface nutrient reservoirs, the downward migration response is weaker, resulting in a shallower mean depth of the population during high tide. These data suggest that subsurface nutrient reserves may play an important role in the maintenance of vertical migration of *E. proxima* on some sand flats, particularly those in lower wave energy environments. Such low energy environments might also be characterized by lower flushing rates and increased interstitial nutrient concentrations. In higher wave energy environments, the winnowing action of waves may be more important in reinforcing vertical migration behavior by stimulating deeper migration in all cells (Kingston 1999b) or by removing individual cells that fail to migrate deeply enough or at the proper time. The results reported here for *E. proxima* represent the first experimental evidence that subsurface nutrients are an important resource that reinforces the maintenance of vertical migration behavior in benthic microalgae.

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