

PHOTOSYNTHETIC AND HETEROTROPHIC IMPACTS OF NUTRIENT LOADING TO BLACKWATER STREAMS

MICHAEL A. MALLIN,¹ MATTHEW R. MCIVER, SCOTT H. ENSIGN, AND LAWRENCE B. CAHOON

Center for Marine Science, University of North Carolina at Wilmington, North Carolina 28409 USA

Abstract. Blackwater stream systems are the most abundant type of freshwater lotic system on the Coastal Plain of the eastern United States. Many of these ecosystems drain watersheds that receive large anthropogenic nutrient inputs, whereas some blackwater systems remain relatively pristine. A series of nutrient addition experiments was carried out over a four-year period to assess the roles of nitrogen and phosphorus loading on the phytoplankton, bacterioplankton, and respiration of two third-order and two fifth-order blackwater streams in southeastern North Carolina. Stream water was distributed into 4-L containers, amended with various nutrient addition treatments, and incubated in gently agitated outdoor pools over a six-day period. Chlorophyll *a* production, direct bacterial counts, ATP, and biochemical oxygen demand (BOD) were measured as response variables. Significant phytoplankton production over control occurred in most experiments involving nitrogen additions, regardless of whether it was in the form of ammonium, nitrate, or urea. Concentrations of nitrate or a urea–nitrate combination of 14.3 μM (0.2 mg N/L) or higher increased chlorophyll *a* production and significantly stimulated BOD. Organic or inorganic phosphorus additions did not stimulate phytoplankton production. Combined organic–inorganic phosphorus additions of 16.1–32.3 μM P (0.5–1.0 mg P/L) significantly stimulated bacterial abundance, ATP, and BOD on most occasions. Nitrogen inputs stimulate blackwater stream phytoplankton growth, which in turn dies and decomposes in deeper, higher order streams, becoming sources of BOD and lowering dissolved oxygen (DO). Phosphorus inputs directly stimulate bacterial growth, increasing BOD, and lowering stream DO. Thus, changes in nutrient loading stimulate two different biological pathways (photosynthetic and heterotrophic activity), which in turn reduce dissolved oxygen, potentially causing hypoxic conditions stressful to resident aquatic life. The low slope, slow summer flows, and naturally low summer DO conditions make Coastal Plain blackwater streams particularly susceptible to additional DO losses through BOD formation.

Key words: *bacteria; biochemical oxygen demand (BOD); blackwater; Coastal Plain; concentrated animal feeding operations (CAFOs); dissolved oxygen; hypoxia; nutrients; phytoplankton; streams.*

INTRODUCTION

Blackwater streams comprise the most abundant type of freshwater system in the coastal plain region of the southeastern United States (Meyer 1992, Smock and Gilinsky 1992). Pristine blackwater systems are characterized by low chlorophyll *a* and high dissolved organic carbon content; thus, it might be expected that planktonic metabolism in these systems is dominated by bacterial metabolism (Biddanda et al. 2001). Research has demonstrated that blackwater rivers are naturally high in bacterial abundance and associated protozoa, due to the inputs of dissolved organic material from surrounding wetlands (Edwards and Meyer 1987, Meyer 1990, Carlough 1994). Because of their naturally low dissolved oxygen content at certain times of the year, blackwater systems are vulnerable to organic loading and probably cannot tolerate as much anthro-

pogenic biochemical oxygen demand (BOD) as clearwater systems (Meyer 1992).

Inorganic nutrient concentrations of unimpacted blackwater streams are generally low in comparison with either upland streams or anthropogenically influenced blackwater systems (Meyer 1992, Smock and Gilinsky 1992, Wahl et al. 1997). However, the U.S. Coastal Plain is undergoing increasing urbanization with subsequent anthropogenic impacts to these streams (Wahl et al. 1997). The rural landscape, particularly in states such as Maryland, Virginia, and North Carolina, has also undergone a major transformation from traditional agriculture to industrial style animal production (Burkholder et al. 1997, Mallin 2000). These production facilities are called concentrated animal feeding operations (CAFOs), which raise thousands of swine or poultry in close quarters and store their concentrated wastes in outdoor lagoons (swine and some poultry) or spread it as dry litter on fields (poultry). Lagoon wastes are nutrient-rich (Cahoon et al. 1999) and are periodically sprayed on adjoining fields. Nearby streams and rivers have been

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¹ E-mail: mallinm@uncw.edu



PLATE 1. Mixed algal/floating aquatic macrophyte surface scum on a shallow blackwater tributary of the Northeast Cape Fear River, North Carolina. Photo credit: UNC Wilmington Aquatic Ecology Laboratory.

documented to receive heavy nutrient loads from CAFOs, chronically through surface and subsurface runoff (Stone et al. 1995, Gilliam et al. 1996) and acutely through lagoon breaches, leaks, and spills (Burkholder et al. 1997, Mallin 2000, Mallin et al. 2002). Acute incidents have also caused large BOD loads to impact nearby lotic systems and cause anoxic or hypoxic conditions (Mallin 2000, Mallin et al. 2002).

The influence of nutrient loading on bacterioplankton and other heterotrophs is likely to be critical in regulating stream metabolism. The most abundant constituents of the heterotrophic plankton community are the naturally occurring bacteria, which can utilize both inorganic and organic nutrients (Ford 1993, Bjorkman and Karl 1994). Fungi are also a part of the heterotrophic community (Padgett et al. 2000), as are grazers such as protozoans (Carlough and Meyer 1989, Carlough 1994). Since blackwater streams are vulnerable to organic loading, growth stimulation of bacteria and protozoans by nutrient inputs might reduce dissolved oxygen to levels that stress the benthic and fish communities. Recent experimentally derived evidence suggests that nutrient loading to blackwater rivers directly increases heterotrophic microbial biomass (Padgett et al. 2000, Mallin et al. 2001a), thus increasing BOD and leading to potentially decreased dissolved oxygen concentrations in these systems.

Phytoplankton biomass as chlorophyll *a* is normally low in unimpacted blackwater systems (Hurlong and Mallin 1985, Edwards and Meyer 1987, Meyer 1992). Studies documenting the effects of nutrient loading, particularly nonpoint source nutrient loading, on blackwater stream biota had been rare in the literature (Smock and Gilinsky 1992). However, recent studies show that anthropogenically impacted blackwater rivers and streams are capable of developing dense phytoplankton blooms (Burkholder et al. 1997, Mallin et al. 1997, Philips et al. 2000, Ensign and Mallin 2001).

In 1997–1998, we conducted 11 nutrient addition bioassay experiments on water from two fifth-order blackwater streams, the Black and Northeast Cape Fear rivers (Mallin et al. 2001a). These tests utilized ammonium, urea, orthophosphate, glycerophosphate, and an ammonium and orthophosphate combination as treatments, all at final-addition concentrations of 1 mg/L (71.4 μM N and/or 32.3 μM P). Strong biological responses to nutrient additions occurred in experiments run from spring through fall, with little biological response in winter. Nitrogen additions, either as ammonium or urea, often stimulated phytoplankton growth, whereas phosphorus treatments did not. However, glycerophosphate additions always yielded significant ATP increases, and orthophosphate additions occasionally did. Thus, these experiments demonstrated nitrogen limitation of the phytoplankton community and suggested that organic and inorganic phosphorus limited the growth of bacteria in these blackwater streams. This, along with research regarding salt marsh microbes and nutrients (Sundareshwar et al. 2003), demonstrated that ecosystem management requires consideration of nutrients that limit the heterotrophic community as well as the autotrophic community.

The potential for nitrogen inputs to cause blackwater algal blooms, plus the potential direct effects on heterotrophy by phosphorus loading, led us to test the hypothesis in the present research that the net result of nutrient loading in blackwater streams is a significant increase in BOD in the system. We were also interested in determining the concentration at which a given nutrient yielded significant photosynthetic and/or heterotrophic responses. Thus, our experiments were designed to test a range of potential nutrient concentrations, which were based on several years of monitoring these blackwater systems (Ensign and Mallin 2001, Mallin et al. 2001a, 2002) as well as other relevant literature (Meyer 1992). We chose to conduct these

TABLE 1. Background water chemistry of Colly Creek and Great Coharie Creek, January 1998–December 2000. Data are mean values (ranges) for 1998–2000, $n = 36$ mo.

Parameter	Colly Creek	Great Coharie Creek
Depth [†] (m)	0.7 (0.3–1.3)	0.9 (0.5–1.4)
Flow [†] (cm/s)	3.5 (0.1–41.0)	3.5 (0.1–30.0)
pH	3.8 (3.1–5.1)	6.1 (4.9–7.0)
Conductivity (μ S)	60 (40–90)	130 (60–200)
Nitrate-N (μ mol/L)	0.5 (0.4–6.4)	21.2 (9.3–66.4)
Ammonium-N (μ mol/L)	6.5 (0.7–38.6)	4.9 (0.7–12.1)
Total N (μ mol/L)	63.9 (41.4–125.0)	69.8 (48.6–12.9)
Orthophosphate-P (μ mol/L)	0.3 (0.2–0.5)	1.5 (0.4–4.6)
Total P (μ mol/L)	1.1 (0.2–4.5)	4.6 (1.3–18.4)
Total N/P (mol/L)	90	44
Inorganic N/P (mol/L)	31	33
Chlorophyll <i>a</i> (μ g/L)	1.4 (0.2–18.0)	1.3 (0.2–9.1)

[†] Depth and flow data are from May 2000–April 2001, $n = 12$ mo.

experiments on smaller streams within the same watersheds as our earlier experiments; one of which was relatively pristine, while the other was an anthropogenically impacted stream.

MATERIALS AND METHODS

Site description

Water for the experiments was collected on eight occasions in 1999 and 2000 from two blackwater streams located in the Cape Fear River watershed in southeastern North Carolina (see Plate 1). Colly Creek (34°27.900' N, 78°15.392' W) was chosen, as it represented a reasonably pristine blackwater system. There are two small sewage treatment plants (combined permitted discharge 1.60 ML/d) and six concentrated animal feeding operations (CAFOs) in the 326-km² watershed. In contrast, Great Coharie Creek (34°55.114' N, 78°23.324' W) is considered to be anthropogenically impacted, with two sewage treatment plants (combined permitted discharge 10.77 ML/d) and 95 CAFOs in the 554-km² watershed. Both are third-order streams with similar average depth and flow (Table 1).

Nutrient gradient response experiments

In this set of experiments we achieved three goals: (1) We verified the hypothesis that nitrogen loading to blackwater streams can cause spring and summer phytoplankton bloom formation. Furthermore, we tested the hypotheses that (2) phosphorus loading will stimulate high levels of heterotrophic activity through increased bacterial abundance, and (3) either nutrient, at a given concentration, will cause significant increases in stream biochemical oxygen demand. These hypotheses were tested by the use of nutrient gradient addition bioassays. The basis of these experiments is to add the experimental nutrient(s) covering a concentration gradient to replicated river water samples and determine if, and at what concentrations, the autotrophic and heterotrophic communities in the samples show a positive response (i.e., a chlorophyll and/or bacterial abundance increase) compared with unamended controls.

Water was collected on station in 20-L carboys, returned to the laboratory, and dispensed into 4-L containers called cubitainers (3 L per cubitainer). Our previous experiments (Mallin et al. 2001a) had established that both ammonium and urea readily stimulated phytoplankton growth in spring and summer experiments. In the current set of experiments, we tested for nitrate stimulation, since nitrate had caused the largest inorganic nitrogen peaks and most variability in the impacted creek (and the two fifth-order rivers; Mallin et al. 2001a) and is most subject to inputs from runoff pulses. We also included an organic component, since animal wastes are a mixture of both inorganic and organic nutrients (Burkholder et al. 1997, Mallin et al. 1997). Thus, nutrients tested were nitrate alone, total nitrogen (equimolar nitrate-N and urea-N), orthophosphate alone, and total phosphorus (equimolar orthophosphate-P and glycerophosphate-P). Each nutrient was tested on two separate occasions over a concentration range of no additions (control), 14.3, 35.7, 71.4, 143.0, and 357.0 μ mol N/L, and 0, 6.5, 16.1, 32.3, 64.5, and 161.3 μ mol P/L (= 0.2, 0.5, 1.0, 2.0, and 5.0 mg/L as N or P, expressed as final concentration), with each treatment run in triplicate. Nitrate was tested in August 1999 and July 2000; total nitrogen (TN) in September 1999 and August 2000; orthophosphate in July 1999 and May 2000; and total phosphorus (TP) in November 1999 and June 2000. Since our previous experiments had demonstrated that biologically significant responses did not occur during winter months (Mallin et al. 2001a), the experiments were conducted late spring through fall, the period of maximum photosynthesis and respiration (Fuss and Smock 1996).

Cubitainers were floated in flow-through pools near the laboratory at ambient water temperatures (ranging from 18°C in November 1999 to 27°C in July 1999). The pools were covered by two layers of neutral-density screening to allow solar irradiance penetration to the cubitainers of ~30% of that reaching the water surface, to prevent photostress to the phytoplankton (Mallin and Paerl 1992). The cubitainers were kept in

motion by constant circular agitation of the pool water using a submerged bilge pump (Mallin et al. 2001a). Incubations were run for six days, and the cubitainers were sampled on days 1, 3, and 6 for chlorophyll *a* and on day 6 for biochemical oxygen demand (BOD) and bacterial abundance.

Samples for chlorophyll *a* analysis were filtered onto Gelman A/E glass fiber filters (nominal pore size 1.0 μm ; Gelman, Ann Arbor, Michigan, USA) and analyzed with a Turner Model 10-AU fluorometer (Turner Designs, Sunnyvale, California, USA) following Welschmeyer (1994). The response of the heterotrophic community was measured by direct bacterial counts, following the acridine orange fluorescence microscopy technique pioneered by Francisco et al. (1973) as modified by Hobbie et al. (1977). Triplicate 2-mL samples were collected from each cubitainer on day 6 of the outdoor incubations and gravity filtered through polycarbonate black 0.2- μm filters with a 1.2- μm cellulose subfilter. Stained bacteria were enumerated at 1000 \times using a fluorescence-equipped Olympus BX50 compound microscope (Optical Elements, Dulles, Virginia, USA) equipped with the following filter combination: Olympus cube UM 566 (460–490 nm excitation filter, 500 nm beam splitter, and 5 nm 15-barrier filter). Type FF immersion oil was used. Sufficient random fields were counted to quantify at least 400 cells. Distilled water blanks were enumerated by counting the total number of bacteria in 40 random fields. Blanks were subtracted from counts to obtain final values (as number of bacteria per milliliter).

Five-day BOD (BOD5) analyses were performed according to standard methods (American Public Health Association [APHA] 1995) incubated in standard 300-mL BOD bottles in a darkened incubator at 20°C. Twenty-day BOD (BOD20) analyses were also conducted. As mentioned, the samples contained a mixture of phytoplankton, and other heterotrophs that could contribute to BOD. Biochemical oxygen demand analyses (in duplicate) were conducted on water, after being amended with nutrients, but before outdoor incubation. This was done to test for influences of nutrient loading on the nonphotosynthetic community, plus decay of the resident stream phytoplankton. These BOD analyses were also performed on the samples after the outdoor incubations on day 6 (to account for the influence of nutrient loading on both the photosynthetic and nonphotosynthetic community).

Statistical analyses of nutrient limitation test results were accomplished using the SAS procedure of Analysis of Variance (ANOVA). If a difference in response means ($P < 0.05$) existed among the treatments, the ANOVA test was followed by treatment ranking by the least significant difference (LSD) procedure (Day and Quinn 1989, Mallin et al. 2001a). Correlation analyses were conducted among the four response variables for each experiment individually and for all experiments combined using SAS (Schlotzhauer and Littell 1987).

While on site, water temperature, pH, dissolved oxygen, turbidity, and conductivity data were collected using a YSI 6920 multiparameter water quality instrument (YSI, Yellow Springs, Ohio, USA). The water used for the bioassays was assessed for a suite of nutrient parameters. Total Kjeldahl nitrogen (TKN), nitrate plus nitrite (referred to as nitrate), ammonium, and total phosphorus were measured using standard methods (APHA 1995). Total nitrogen was computed as TKN plus nitrate. Orthophosphate was measured by autoanalyzer using standard techniques (Parsons et al. 1984). Chlorophyll *a* was measured using the above fluorometric method (Welschmeyer 1994). During the period May 2000–April 2001, we collected monthly hydrological data at these two streams plus four others in the same region (Little Coharie Creek, Six Runs Creek, Hammond Creek, and Browns Creek). Depth was taken at three-meter intervals from bank to bank and averaged. Flow was measured in midchannel with a Marsh McBirney Flo-Mate Model 2000 meter held at middepth.

RESULTS

Blackwater stream water chemistry

Background water chemistry differed considerably between the pristine (Colly Creek) and anthropogenically impacted (Great Coharie Creek) streams (Table 1). The pH values of Colly Creek were low, indicating large contributions of organic-acid-rich swamp water, whereas the pH of Great Coharie Creek was considerably higher. The nutrient compositions of the two systems differed as well (Tables 1 and 2). Total nitrogen (TN) concentration was similar between creeks, but Great Coharie Creek had 38% of TN in the inorganic form (primarily nitrate), whereas only 11% of TN in Colly Creek was inorganic. Total phosphorus (TP) concentrations were approximately four times higher in Great Coharie Creek than in Colly Creek, as were orthophosphate concentrations (Table 1). Average total and inorganic N/P were well above the Redfield ratio for phytoplankton (16) in both Colly Creek and Great Coharie Creek for the 24-mo period (Table 1). However, with the exception of July 1999 in Colly Creek, inorganic N/P ratios for the spring and summer growing season were near or below the Redfield ratio (Table 2). Chlorophyll *a* was usually low in both creeks (Table 1). There were no substantial differences in mean depth and flow between the two systems (Table 1).

Nitrate addition experiments

Nitrate addition experiments were run in August 1999 and July 2000. Although there were differences between creeks and magnitude of responses, a similar pattern emerged from both experiments. In all cases, nitrate additions of $\geq 35.7 \mu\text{mol/L}$, and in most cases additions of $\geq 14.3 \mu\text{mol/L}$, led to significant chlorophyll *a* increases over control treatments (Table 3), with

TABLE 2. Ambient nutrient concentrations, and molar total (T) and inorganic (I) N/P ratios, in bioassay test waters before nutrient additions.

Date	Total N	NO ₃ ⁻ -N	NH ₄ ⁺ -N	Total P	PO ₄ ³⁻ -P	TN/TP	IN/IP
Colly Creek							
Jul 1999	70.7	0.7	12.1	1.6	0.3	43.8	39.9
Aug 1999	72.1	0.7	0.8	1.6	0.3	44.8	4.7
Sep 1999	73.6	0.7	5.7	0.7	0.3	114.0	19.9
Nov 1999	65.7	0.7	4.3	1.0	0.3	67.9	15.5
May 2000	60.0	0.7	4.3	1.0	0.7	62.0	7.8
Jun 2000	65.0	0.7	3.6	1.3	0.3	50.4	13.4
Jul 2000	72.9	0.7	10.7	1.6	1.0	45.2	11.8
Aug 2000	57.9	1.4	5.0	0.7	0.3	89.7	19.9
Great Coharie Creek							
Jul 1999	81.4	16.4	3.6	5.5	2.9	14.8	6.9
Aug 1999	65.7	5.7	8.6	7.4	3.2	8.8	4.4
Sep 1999	82.9	12.1	7.9	7.4	2.6	11.2	7.8
Nov 1999	52.1	8.6	3.6	3.9	1.6	13.5	7.5
May 2000	44.3	2.9	3.6	2.6	1.3	17.1	5.0
Jun 2000	51.4	1.4	4.3	5.8	1.9	8.9	2.9
Jul 2000	44.3	0.7	6.4	4.5	1.9	9.8	3.7
Aug 2000	60.0	0.7	7.9	6.8	2.3	8.9	3.8

Note: All concentrations are reported in micromoles per liter.

generally higher responses in the 2000 experiment than in the 1999 experiment (Fig. 1). In Great Coharie Creek, water the full magnitude of the nutrient additions was not realized until day 6; whereas, in Colly Creek, water the responses occurred somewhat sooner, but were smaller than in Great Coharie Creek (Fig. 2).

The chlorophyll *a* increases were reflected by significant postincubation BOD5 increases, beginning at either 14.3 or 35.7 $\mu\text{mol/L}$ (Table 4). The responses of BOD20 were similar to those of BOD5. It is notable that, besides the greater chlorophyll *a* responses, there were greater BOD responses during both experiments to water from Great Coharie Creek (the anthropogenically impacted system) than Colly Creek water (Figs. 1 and 3). The BOD analyses run on waters after nitrate additions but before outdoor incubations (i.e., preincubation) did not show significant increases in most cases (Table 5). For Great Coharie Creek in August

1999, nitrate additions of 35.7, 71.4, and 143.0 $\mu\text{mol/L}$ led to statistically significant preincubation increases in BOD5 over control treatments (Table 5), but these increases were $\leq 20\%$. There were no significant preincubation BOD20 responses to nitrate additions. Fluorescence microscopy indicated that postincubation bacterial counts were significantly greater than in controls for nitrate-N additions of either 71.4 or 143.0 $\mu\text{mol/L}$ and above for both creeks during both experiments (Table 6; Fig. 4).

Total nitrogen addition experiments

Total nitrogen (50% nitrate-N plus 50% urea-N) addition experiments were run in September 1999 and August 2000. There was no chlorophyll *a* response to the additions in Colly Creek in September 1999 (Fig. 1), but significant chlorophyll *a* increases in Great Coharie Creek water for the 71.4 and 357.0 $\mu\text{mol/L}$ treat-

TABLE 3. Nutrient addition levels at which statistically significant ($P < 0.05$) chlorophyll *a* responses occurred relative to control treatments.

Treatment	Colly Creek	Great Coharie Creek
Nitrate		
August 1999	143.0, 71.4, 35.7, 14.3	357.0, 143.0, 71.4, 35.7, 14.3
July 2000	357.0, 143.0, 71.4, 35.7, 14.3	357.0, 143.0, 71.4, 35.7
Nitrate plus urea		
September 1999	...	357.0, 71.4
August 2000	...	357.0, 143.0, 71.4, 35.7, 14.3
Orthophosphate		
July 1999	32.3	...
May 2000
Orthophosphate plus glycerophosphate		
November 1999
June 2000	...	32.3

Notes: All concentrations are reported in micromoles per liter. If no nutrient addition level is listed, no significant responses occurred for that experiment.

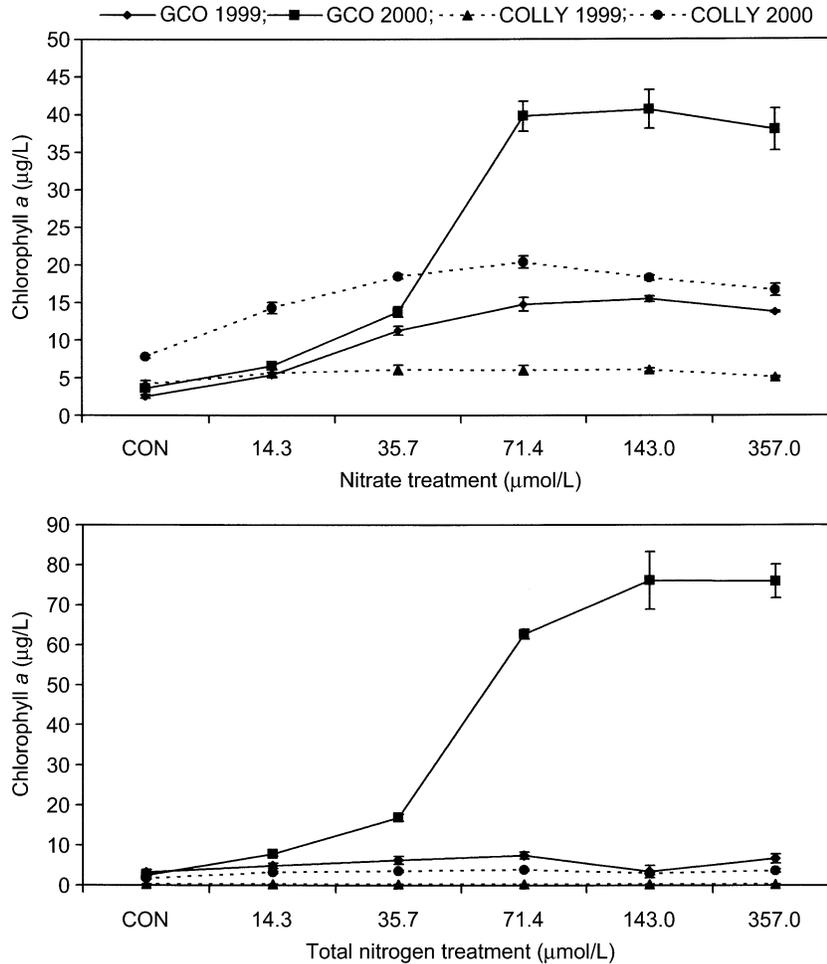


FIG. 1. Chlorophyll *a* response to nitrate-N and total nitrogen treatments for Great Coharie Creek (GCO) and Colly Creek (COLLY). Means are of three replicates \pm 1 SE; "CON" (control) indicates no nutrient additions.

ments (Table 3; Fig. 1). This experiment occurred after heavy rains from Hurricane Dennis influenced the watershed, so increased swamp water inputs into Colly Creek likely led to high water color but very low chlorophyll *a* concentrations (Fig. 1). As with the nitrate additions, the largest gains in chlorophyll *a* biomass were realized on day 6 for Great Coharie Creek water.

There were no significant postincubation BOD5 increases during September 1999 (Table 4; Fig. 3), but there was a significant BOD20 increase for Great Coharie Creek water at 357.0 $\mu\text{mol/L}$. The August 2000 experiment yielded high chlorophyll *a* production in the Great Coharie Creek water, with statistically significant increases in treatments of ≥ 14.3 $\mu\text{mol/L}$ (Fig. 1). This chlorophyll production was reflected in postincubation BOD5 and BOD20 increases, all of which were statistically significant at concentrations of ≥ 14.3 $\mu\text{mol/L}$ (Table 4; Fig. 3). Preincubation BOD5 increases were not significant for August 2000 (Table 5), indicating that postincubation BOD increases were probably driven through the photosynthetic pathway (i.e.,

through bacteria associated with phytoplankton growth and subsequent decay). There were significant preincubation BOD20 increases at the 142.9 and 357.0 $\mu\text{mol/L}$ levels for Great Coharie Creek in September 1999. There were no significant postincubation bacterial increases over control for either creek during the September 1999 experiment (Table 6). However, bacterial increases were significant at ≥ 14.3 $\mu\text{mol/L}$ for Great Coharie Creek and at 14.3 $\mu\text{mol/L}$ for Colly Creek in the August 2000 experiment (Table 6; Fig. 4).

Orthophosphate addition experiments

Orthophosphate addition experiments were run in July 1999 and May 2000. In almost all cases there were no significant chlorophyll *a* increases in response to inorganic phosphorus additions (Table 3). In Colly Creek in July 1999, the 32.3 $\mu\text{mol/L}$ treatment led to a significant chlorophyll *a* increase over control treatments, but as responses to all orthophosphate concentrations were < 3.0 $\mu\text{g/L}$ this was probably a spurious result. None of the postincubation BOD5 increases

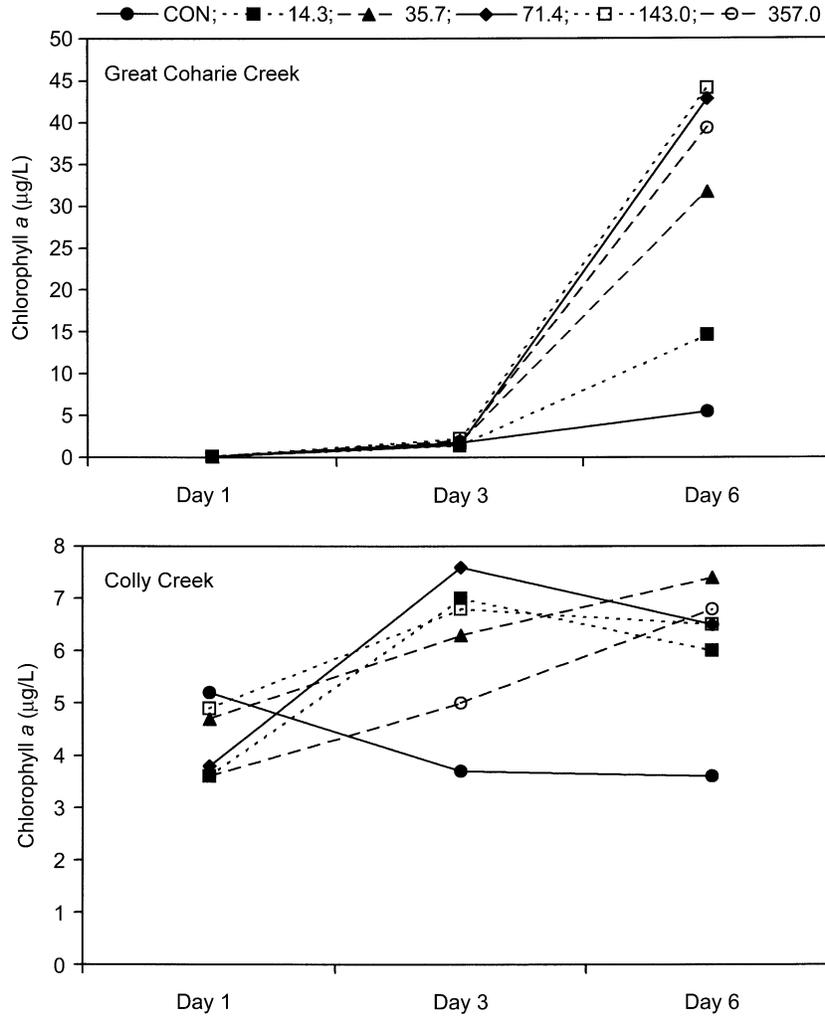


FIG. 2. Time course of chlorophyll *a* responses to nitrate inputs to Great Coharie Creek and Colly Creek water, August 1999. All concentrations in the key are micromoles N/L.

TABLE 4. Nutrient addition levels at which statistically significant ($P < 0.05$) BOD5 responses (postincubation) occurred relative to control treatments.

Treatment	Colly Creek	Great Coharie Creek
Nitrate		
August 1999	357.0, 143.0, 71.4, 35.7, 14.3	357.0, 143.0, 71.4, 35.7, 14.3
July 2000	357.0, 143.0, 71.4, 35.7, 14.3	357.0, 143.0, 71.4, 35.7
Nitrate plus Urea		
September 1999
August 2000	...	357.0, 143.0, 71.4, 35.7, 14.3
Orthophosphate		
July 1999
May 2000
Orthophosphate plus glycerophosphate		
November 1999	161.3, 64.5, 32.3	161.3
June 2000

Notes: All concentrations are reported in micromoles per liter. If no nutrient addition level is listed, no significant responses occurred for that experiment.

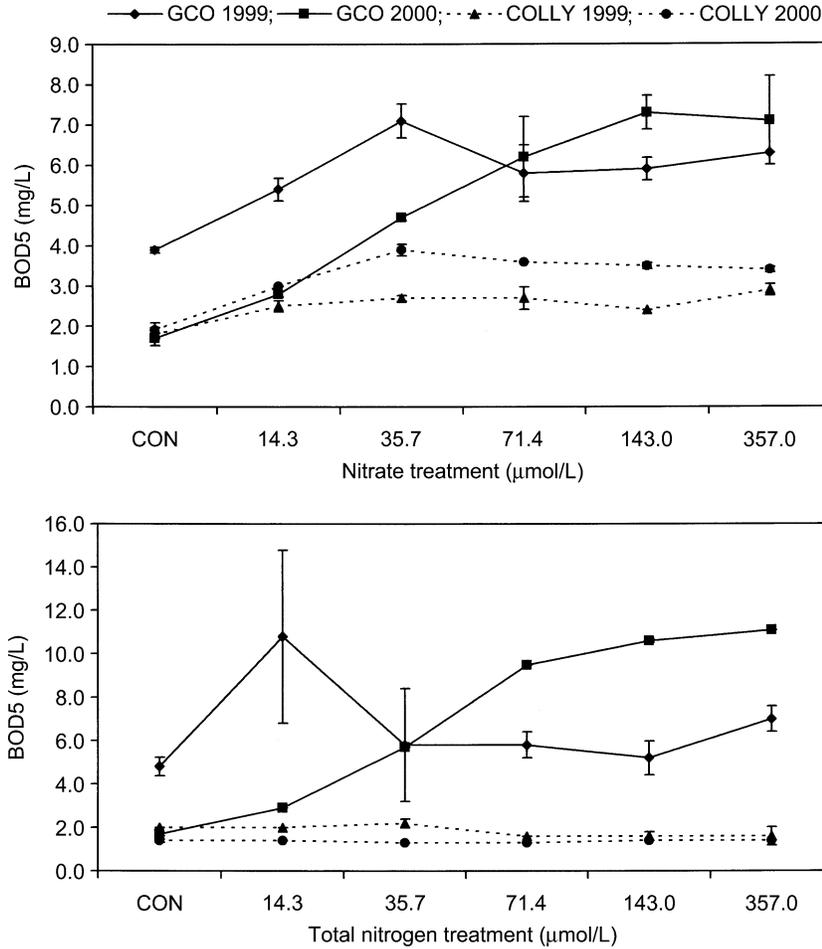


FIG. 3. Five-day biochemical oxygen demand (BOD5) response to nitrate-N and total nitrogen treatments for Great Coharie Creek (GCO) and Colly Creek (COLLY) following six-day outdoor incubations. Means are of three replicates \pm 1 SE; "CON" (control) indicates no nutrient additions.

TABLE 5. Nutrient addition levels at which statistically significant ($P < 0.05$) BOD5 responses (preincubation) occurred relative to control treatments.

Treatment	Colly Creek	Great Coharie Creek
Nitrate		
August 1999	...	143.0, 71.4, 35.7
July 2000
Nitrate plus Urea		
September 1999
August 2000
Orthophosphate		
July 1999
May 2000
Orthophosphate plus glycerophosphate		
November 1999	161.3, 64.5, 32.3, 16.1	161.3, 64.5, 32.3, 16.1
June 2000	161.3, 64.5, 32.3	161.3, 64.5

Notes: All concentrations are reported in micromoles per liter. If no nutrient addition level is listed, no significant responses occurred for that experiment.

TABLE 6. Nutrient addition levels at which statistically significant ($P < 0.05$) bacterial responses (postincubation) occurred relative to control treatments.

Treatment	Colly Creek	Great Coharie Creek
Nitrate		
August 1999	357.0, 143.0	357.0, 143.0, 71.4
July 2000	357.0, 143.0, 71.4	357.0, 143.0, 71.4
Nitrate plus Urea		
September 1999
August 2000	71.4	357.0, 143.0, 71.4
Orthophosphate		
July 1999
May 2000	161.3	64.5
Orthophosphate plus glycerophosphate		
November 1999	161.3, 64.5, 32.3, 16.1, 6.5	161.3
June 2000	...	161.3

Notes: All concentrations are reported in micromoles per liter. If no nutrient addition level is listed, no significant responses occurred for that experiment.

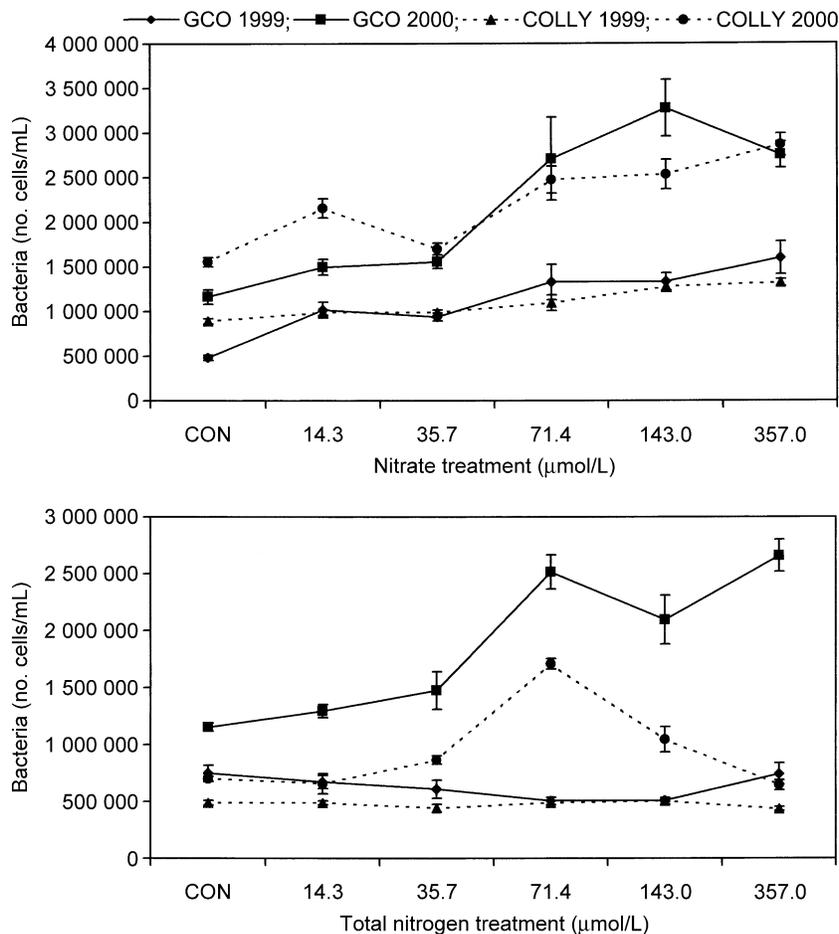


FIG. 4. Direct bacterial counts in response to nitrate-N and total nitrogen treatments for Great Coharie Creek (GCO) and Colly Creek (COLLY) following six-day outdoor incubations. Means are of three replicates \pm 1 SE; "CON" (control) indicates no nutrient additions.

were significant (Table 4); however, there were significant BOD₂₀ responses to orthophosphate at 6.5 and 32.3 $\mu\text{mol/L}$ in July 1999 for Colly Creek and at 161.3 $\mu\text{mol/L}$ in May 2000 for Great Coharie Creek (Fig. 5). None of the preincubation BOD₅ tests showed a significant increase over control treatments (Table 5), and equipment failure caused an inability to obtain BOD₂₀ data. There were no significant bacterial increases over controls for the July 1999 experiment (Table 6). The May 2000 experiment showed significant bacterial increases at 161.3 $\mu\text{mol P/L}$ for Colly Creek and at 64.5 $\mu\text{mol P/L}$ for Great Coharie Creek (Table 6; Fig. 6).

Total phosphorus addition experiments

Total phosphorus (50% orthophosphate-P plus 50% glycerophosphate-P) addition experiments were run in November 1999 and June 2000. None of the treatments yielded significant chlorophyll *a* increases except for the 32.3 $\mu\text{mol P/L}$ treatment in the June 2000 Great Coharie Creek experiment (Table 3). Again, this response was likely spurious as final chlorophyll *a* con-

centrations for all TP levels were $<3.0 \mu\text{g/L}$. In November 1999, the postincubation BOD₅ assays showed statistically significant increases over control treatments for 32.3, 64.5, and 161.3 $\mu\text{mol P/L}$ in Colly Creek, and for 161.3 $\mu\text{mol P/L}$ for Great Coharie Creek (Table 4). Postincubation BOD₅ increases in the June 2000 experiment were not significant. However, postincubation BOD₂₀ responses for June 2000 were significant for both creeks at either 64.5 or 161.3 $\mu\text{mol P/L}$ (Fig. 5). The preincubation BOD₅ and BOD₂₀ assays showed significant increases over control in November 1999 to all treatments $\geq 16.1 \mu\text{mol P/L}$ (Table 5, Fig. 7). In June 2000, there were significant preincubation BOD₅ and BOD₂₀ increases in Colly Creek water for $\geq 32.3 \mu\text{mol P/L}$ and in Great Coharie Creek water for treatments $\geq 64.5 \mu\text{mol P/L}$ (Table 5, Fig. 7). The BOD increases ranged from 15% increases at the low additions to more than doubling the BOD at the high-P additions. For the November 1999 experiment, bacterial counts were significantly greater than in controls at $\geq 6.5 \mu\text{mol P/L}$ for Colly Creek and at 161.3 μmol

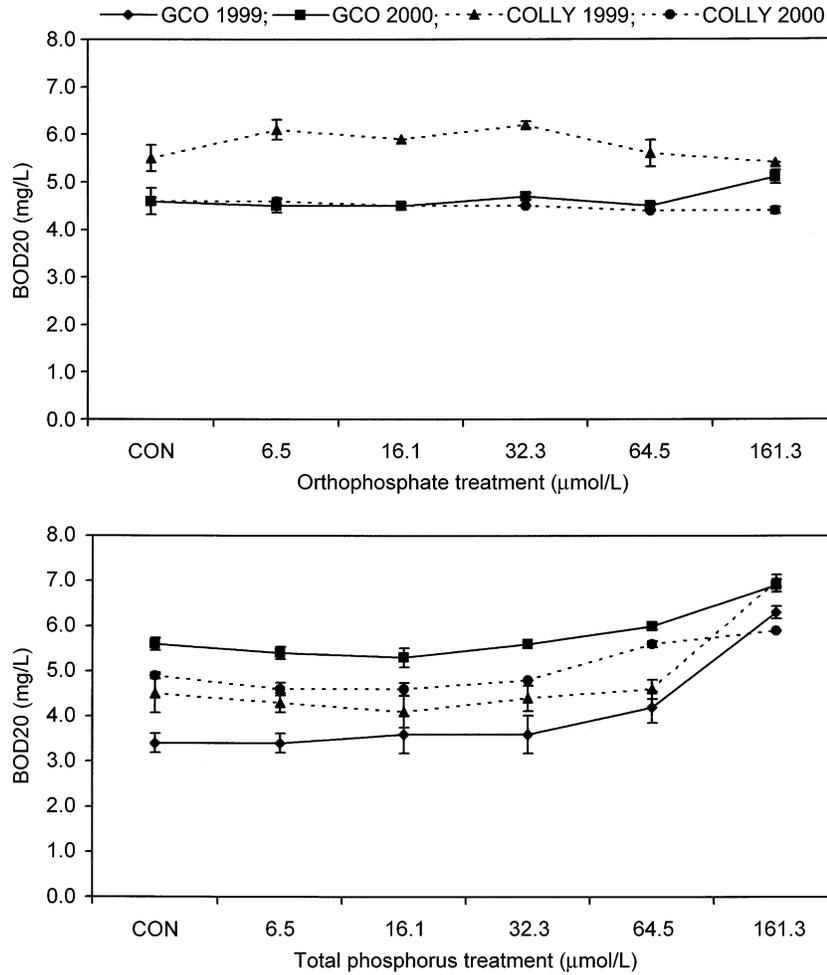


FIG. 5. Twenty-day biochemical oxygen demand (BOD20) response to orthophosphate-P and total phosphorus treatments for Great Coharie Creek (GCO) and Colly Creek (COLLY) following six-day outdoor incubations. Means are of three replicates \pm 1 SE; "CON" (control) indicates no nutrient additions.

P/L for Great Coharie Creek, and the June 2000 bacteria counts were significantly greater than controls at 161.3 μmol P/L additions (Table 6; Fig. 6).

Correlation analysis

Correlation analyses were run among all four response variables for outdoor postincubation data. For all experiments combined, bacterial counts were positively correlated with BOD5 ($r = 0.556$, $P < 0.001$), BOD20 ($r = 0.491$, $P < 0.001$), and chlorophyll *a* ($r = 0.605$, $P < 0.001$). Chlorophyll *a* was likewise positively correlated with BOD5 ($r = 0.752$, $P < 0.001$) and BOD20 ($r = 0.648$, $P < 0.001$). These correlations differed according to individual treatments (Table 7). For most nitrate experiments, chlorophyll *a* concentrations and bacterial counts were significantly correlated. Both chlorophyll *a* and bacterial counts were significantly correlated with BOD5 and BOD20 in most nitrate experiments, but more consistently in Great Coharie Creek water than in Colly Creek water (Table 7).

In the August 2000 TN experiment, there were strong correlations between both bacterial counts and chlorophyll *a* concentrations and the two BOD tests. There were no significant positive correlations between chlorophyll *a* and either bacterial counts or BOD for the inorganic phosphorus experiments (there was a considerable amount of variability in some of the bacterial counts; Fig. 6). However, bacterial counts were strongly correlated with BOD5 and BOD20 in the November 1999 TP experiment (Table 7). Additionally, in the June 2000 TP experiment, bacterial counts displayed near-significant correlations with BOD5 ($r = 0.565$, $P = 0.089$) and BOD20 ($r = 0.581$, $P = 0.078$).

DISCUSSION

Based on the collective results of 15 bioassay experiments over four years, the data clearly show nitrogen stimulation of phytoplankton growth in all four of the blackwater streams investigated. The previous experiments showed significant phytoplankton responses

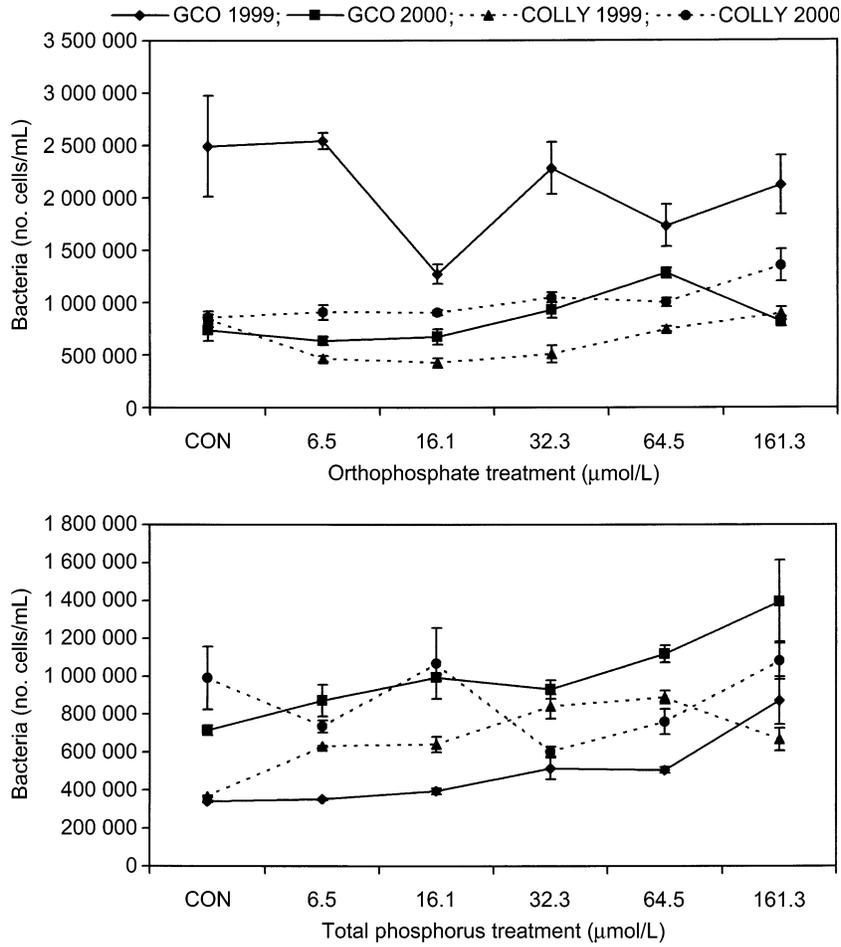


FIG. 6. Direct bacterial counts in response to orthophosphate-P and total phosphorus treatments for Great Coharrie Creek (GCO) and Colly Creek (COLLY) following six-day outdoor incubations. Means are of three replicates \pm 1 SE; "CON" (control) indicates no nutrient additions.

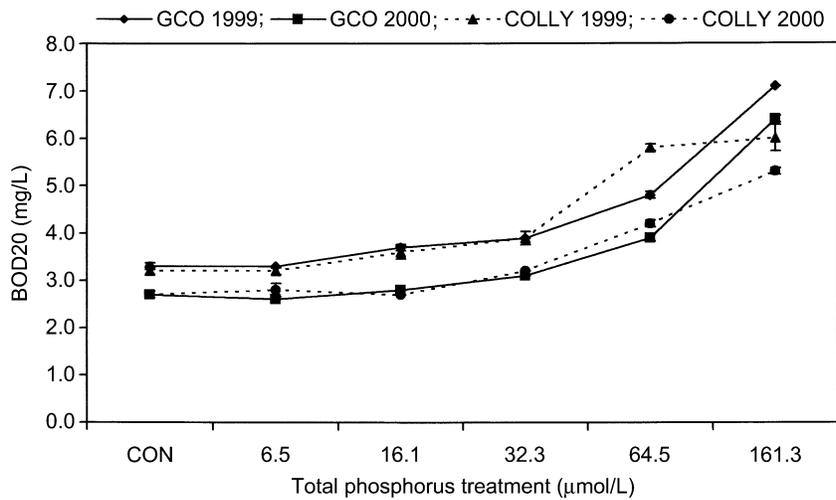


FIG. 7. Twenty-day biochemical oxygen demand response to total phosphorus treatments for Great Coharrie Creek (GCO) and Colly Creek (COLLY), conducted immediately following nutrient additions (means of two replicates \pm 1 SE); "CON" (control) indicates no nutrient additions.

TABLE 7. Positive correlations among postincubation variables for Colly and Great Coharrie Creeks by individual experiment.

Experiment	Colly Creek						Great Coharrie Creek					
	BOD5		BOD20		Chlor. <i>a</i>		BOD5		BOD20		Chlor. <i>a</i>	
	<i>r</i>	<i>P</i>	<i>r</i>	<i>P</i>	<i>r</i>	<i>P</i>	<i>r</i>	<i>P</i>	<i>r</i>	<i>P</i>	<i>r</i>	<i>P</i>
Nitrate												
Aug 1999												
Bacteria	0.631	0.001	0.657	0.001	0.587	0.045	NA	...	0.672	0.002
Chlor. <i>a</i>	0.654	0.021
Jul 2000												
Bacteria	0.493	0.044	0.762	0.001	0.656	0.021	0.757	0.001
Chlor. <i>a</i>	0.955	0.001	0.964	0.001	0.937	0.001	0.841	0.001
Total nitrogen												
Aug 2000												
Bacteria	0.889	0.001	0.835	0.001	0.758	0.001
Chlor. <i>a</i>	0.978	0.001	0.902	0.001
Nov 1999												
Bacteria	0.578	0.015	0.703	0.001
Jun 2000												
Bacteria	0.565	0.089	0.581	0.078

Note: Pearson correlation coefficients (*r*) are reported, along with *P* values.

to ammonium and urea in 6 out of 11 experiments in 1997 and 1998 (Mallin et al. 2001a). In the current set of experiments, there were positive responses to all nitrate addition experiments and positive responses to the TN additions in some experiments. Thus, ammonium, nitrate, and urea additions have all demonstrated positive chlorophyll responses in these blackwater streams. The use of urea as a nutrient source by numerous phytoplankton species has been documented elsewhere (see Antia et al. 1991). Our experiments showed that bacterial counts taken following the outdoor incubations were positively correlated with chlorophyll *a* yield, and both of these parameters were correlated with biochemical oxygen demand (BOD; Table 7). We assume that much of the increased bacterial abundance occurred as a consequence of senescing phytoplankton biomass. Thus, nitrogen additions to blackwater systems have the potential to increase BOD (and subsequently decrease dissolved oxygen). This can occur through the indirect autotrophic pathway of algal bloom formation, algal bloom death and decay, bacterial increase, and increased BOD. Optimal conditions for algal bloom formation are nitrogen inputs into shallow, slow-moving streams during spring and summer, with sufficient light availability (i.e., relatively open forest canopy). Blooms in shallow streams will eventually enter larger and deeper light-limited blackwater rivers where bloom death will occur, with consequent BOD increases.

Canopy shading of lower order streams is certainly a limiting factor to phytoplankton growth in forested regions. However, the modern mid-Atlantic and southeastern Coastal Plain (Maryland, Virginia, North Carolina, and increasingly South Carolina) has been transformed by logging and agricultural activities, both traditional crop and large-scale concentrated animal feed-

ing operations (CAFOs), which have decimated much of the forest cover. Also, CAFO-rich watersheds such as the Cape Fear River system have no required vegetated buffer zones, either for agriculture or forestry operations. Thus, most lower order streams pass through a landscape mosaic consisting of farm fields, CAFO spray or poultry litter fields, canopied forests, and numerous road crossings (clearly visible from the air), allowing opportunity for significant solar irradiance to impact stream waters.

Water color is a major factor attenuating light in deep, higher order blackwater streams and rivers. Because light is rapidly attenuated in blackwater rivers, algal blooms in shallow streams that enter deeper, well-mixed rivers will be largely entrained below the euphotic zone, die from light limitation, and become BOD. It had previously been believed that water column light attenuation restricted algal growth in blackwater streams as well as rivers (Smock and Gilinsky 1992). However, an analysis of the characteristics of lower order blackwater stream irradiance indicates that this is not the case. Based on extensive stream monitoring data, the median summertime (May–August, years 2000 and 2001) depth *z* of the aforementioned selection of six second- to third-order streams was 0.9 m (*n* = 47). The median summer light attenuation coefficient *k* for the nearby Black River was 3.66 m⁻¹. Considering the median summer surface irradiance (*I*₀) of 1515 μmol photons·m⁻²·s⁻¹ for the Black River, determination of the mean irradiance *I*_{*x*} for a 0.9-m water column by the equation $I_x = I_0[1 - e^{-kz}]/kz$ yields 443 μmol photons·m⁻²·s⁻¹, which is approximately the 30% irradiance level that yields maximum photosynthesis, *P*_{max}, in estuarine waters (Mallin and Paerl 1992). Even the bottom would receive approximately 56 μmol photons·m⁻²·s⁻¹, still well within the euphotic zone. First-

order streams are even shallower; thus, under nonpristine conditions, the summer irradiance field of lower order blackwater streams can and does provide hospitable conditions for blooms (see Plate 1).

Recent publications (Burkholder et al. 1997, Phlips et al. 2000) have documented phytoplankton bloom formation in anthropogenically impacted blackwater systems. In the Cape Fear River watershed, Mallin et al. (1997) described a massive nutrient load from a 4-ML CAFO leak into a set of first- to third-order largely canopied blackwater streams that led to chlorophyll *a* blooms up to 120 $\mu\text{g/L}$ for over two weeks. In the nearby blackwater Goshen Swamp, Ensign and Mallin (2001) documented nutrient loading following forest clear-cutting operations in which phytoplankton blooms $>160 \mu\text{g/L}$ as chlorophyll *a* occurred as a response. Formation of these dense algal blooms alternated with periods of severe hypoxia and anoxia, as decaying algal blooms became large sources of BOD that led to loss of dissolved oxygen.

In several of our experiments, nitrogen inputs as low as 14.3 $\mu\text{mol/L}$ were sufficient to produce significant chlorophyll *a* and BOD increases over controls. However, the 35.7–71.4 $\mu\text{mol N/L}$ range appeared to produce notably greater responses that would be indicative of water quality degradation in terms of algal blooms, bacterial increases, and/or BOD loading (Figs. 1–4). Our previous investigations utilized 71.4 $\mu\text{mol N/L}$ as our test concentrations and found large and statistically significant increases in chlorophyll *a* over controls (Mallin et al. 2001a).

Based on various types of experiments, phosphorus is generally considered to be the principal nutrient that limits the growth of phytoplankton in freshwaters (Hecky and Kilham 1988). Some reasons given for freshwater P limitation include the high N/P ratios in river water, the high N/P ratio in water draining fertilized agricultural catchments, the larger amounts of nitrogen in rainfall relative to phosphorus, and the often abundant quantities of nitrogen-fixing blue-green algae in lakes (Paerl 1982, Hecky and Kilham 1988). However, during 15 experiments over four years, we have found significant phosphorus stimulation of phytoplankton growth on only one occasion. In contrast, we have demonstrated nitrogen limitation of phytoplankton growth in two fifth-order rivers (Mallin et al. 2001a) and two third-order streams in southeastern North Carolina. There are some potential reasons for this nitrogen limitation. The inorganic N/P ratio in these streams during the growing season is generally near or below the Redfield ratio (Table 2). The agricultural landscape is dominated by industrialized animal production facilities. Swine waste lagoon liquid has an average TN concentration of 43 900 $\mu\text{mol N/L}$ (615 mg/L) and an average TP concentration of 3230 $\mu\text{mol P/L}$ (100 mg/L; Westerman et al. 1990). Ammonium comprises 80% of the TN and orthophosphate 80% of the TP sprayed onto adjoining fields (Wester-

man et al. 1990, Mallin et al. 1997). Animal waste sprayed on the landscape thus has a generally lower N/P ratio compared with ratios used for row crop fertilization schemes (Cahoon et al. 1999, Arbuckle and Downing 2001). Thus, the sources of nutrients to impacted waters might be instrumental in forcing the nitrogen limitation of phytoplankton. Also, phosphorus is mobilized from anoxic and hypoxic sediments during summer, likely decreasing inorganic N/P ratios. It is notable that the TN/TP ratio in Colly Creek is high, in contrast to the inorganic N/P ratio (Table 2). However, the majority of total nitrogen in pristine blackwater systems is organic and swamp-derived (Meyer 1992, Clark et al. 2000). Thus, as in other environments (Jackson and Williams 1985), much of the nitrogen in Colly Creek might be refractory and unavailable to phytoplankton.

The phytoplankton community of these two streams was not taxonomically assessed during this research effort. However, during research on two blackwater rivers in this region, it was determined that chlorophytes, diatoms, and cryptomonads responded positively to ammonium and urea inputs, whereas dinoflagellates and euglenoids were minor parts of the community and displayed little response to the treatments (Mallin et al. 2001a). Blue-green algal (cyanobacterial) blooms have occurred in disturbed blackwater streams in this region (Ensign and Mallin 2001), but normally blue-green algae comprise only a minor component of the phytoplankton community in these systems (Mallin et al. 2001a). Thus, nitrogen fixation is probably not a significant nutrient source to these waters. Additionally, denitrification likely removes some biologically available nitrogen in the extensive wetlands in the Colly Creek watershed (Aelion and Shaw 2000).

Phosphorus inputs did not increase chlorophyll *a* biomass in these third-order streams. This finding verified our previous experiments using water from the Black and Northeast Cape Fear rivers (Mallin et al. 2001a). However, phosphorus loading was found to lead directly to significant increases in biochemical oxygen demand. This occurred in BOD tests run using water that had been amended with total phosphorus but not incubated outdoors. Direct bacterial counts conducted after outdoor incubations showed significant increases without corresponding increases in chlorophyll *a* (Tables 3 and 6). Thus, the combined inorganic-plus-organic phosphorus treatments caused direct bacterial growth stimulation that was sufficient to significantly increase BOD as well. Although previous work in the blackwater rivers showed ATP stimulation by orthophosphate alone (Mallin et al. 2001a), in the current experiments the inorganic phosphorus alone caused few significant BOD or bacterial increases. It required organic phosphorus inputs to do so consistently. The previous work also showed that organic phosphorus inputs almost always led to significant ATP increases (Mallin et al. 2001a). In our experiments, addition of

inorganic phosphorus alone led to increases in bacterial growth in some situations (Fig. 6), but only a few of these were statistically significant (Table 6). We also note that on two occasions some BOD was apparently also generated by nitrogen through nonphotosynthetic stimulation (Table 5).

The TP addition experiments showed significant BOD increases occurring either at the 16.1 or 32.3 $\mu\text{mol/L}$ concentrations (Table 5). Our previous work utilized 32.3 $\mu\text{mol/L}$ for the inorganic and organic P treatments, which both yielded significant ATP increases (Mallin et al. 2001a). Other experiments have also shown notable BOD increases at the 32.3 $\mu\text{mol/L}$ P level (Mallin 2000). Collectively these experiments indicate phosphorus limitation of bacterial growth in these blackwater streams and rivers, despite the rather low growing season N/P ratios. We note here that although orthophosphate and TP concentrations in Great Coharie Creek were four times that of pristine Colly Creek, the Great Coharie Creek phosphorus concentrations were not unusually high in comparison with a variety of other nonpristine Coastal Plain blackwater streams (Currie 1990, Meyer 1992, Ensign and Mallin 2001, Mallin et al. 2001a, 2002). Phosphorus has been determined to limit or stimulate bacterial growth in a variety of other freshwater, marine, and wetland situations as well (Elwood et al. 1981, Morris and Lewis 1992, Bjorkman and Karl 1994, Chrzanowski et al. 1995, Thingstad et al. 1998, Cotner et al. 2000, Sundareswar et al. 2003). Bacteria might be limited at lower N/P and C/P ratios than conventional Redfield ratios for primary producers indicate. This might in part be due to the phosphorus in membrane phospholipids and nucleic acids and to high ATP turnover rates in bacteria; thus, bacteria have a higher P requirement than do phytoplankton (Vadstein et al. 1988, Kirchman 1994). Bacteria have molar N/P ratios approaching 5:1, lower than standard Redfield ratios of 16:1 for phytoplankton (see Gaedke et al. 2002), so P limitation of bacteria is quite likely even in an anthropogenically impacted system with P loading.

The data from our experiments showed similar general patterns between the impacted stream (Great Coharie Creek) and the pristine system (Colly Creek). However, Great Coharie Creek clearly showed stronger responses to nutrient loading in terms of chlorophyll *a* (Table 3; Figs. 1 and 2), as well as stronger coupling among chlorophyll, bacteria, and BOD (Table 7). This might reflect the presence of a plankton community that is adapted to utilize the periodic pulses of nutrients that Great Coharie Creek receives during rainfall-driven runoff events. Great Coharie Creek has 12% watershed wetlands coverage and 95 CAFOs, compared with 55% wetlands coverage and 6 CAFOs for the Colly Creek drainage basin (Mallin et al. 2001b), thus elevated inorganic and labile organic nutrients are more likely to enter receiving streams after storm events in the Great Coharie Creek watershed (Table 2; Mallin

2000). In research comparing 11 Coastal Plain watersheds in this region, Mallin et al. (2001b) found that in watersheds with <13% wetlands coverage (including Great Coharie Creek) there were strong correlations between rainfall and stream fecal coliform and turbidity concentrations. However, when watershed wetlands coverage exceeded 13% (including Colly Creek), this relationship became nonsignificant, demonstrating that wetlands do buffer the impacts of certain nonpoint source pollutants. These data indicate a need for further studies elucidating how anthropogenically impacted watersheds alter both blackwater stream habitat and how stream biota respond to further inputs.

Our experiments demonstrate the ecosystem-level impacts that nutrient loading can have on blackwater streams. Changes in nutrient loading stimulate both photosynthetic and heterotrophic activity, which in turn reduce dissolved oxygen. The physical nature of Coastal Plain blackwater streams make them particularly susceptible to reduced dissolved oxygen through BOD formation. On the Coastal Plain, the slope of headwaters streams averages $\sim 0.1\%$ (or a one-meter drop per kilometer), and the slope of middle-order streams averages a mere 0.02% (Smock and Gilinsky 1992), making for slow-flowing waters. Since gas exchange is a function of current speed, little reaeration occurs under these conditions. In the aforementioned set of six blackwater streams, we documented median summer current speeds of 0.21 cm/s ($n = 47$), with a range from 41 cm/s down to 0 cm/s, or stagnant water. Thus reaeration is low in summer, when biological growth is most active in these systems. Additionally, the substrata of these streams are principally well-sorted sands, with scattered patches of coarse and fine particulate organic matter. Thus, there are no falls or cascades, and very few riffles to encourage physical reaeration through relief. Reaeration should therefore rarely be a significant factor alleviating the effects of a BOD increase during summer on the Coastal Plain.

Coastal Plain streams are under increasing pressure from current and potential nutrient loading. We have demonstrated that phytoplankton production can be limited by nitrogen at the same time that the heterotrophic bacterioplankton growth can be limited by phosphorus. Thus, blackwater systems can present an additional dimension to nutrient control efforts. Recent research in a salt marsh ecosystem has also demonstrated that it is essential to manage nutrients to control heterotrophs as well as autotrophs (Sundareswar et al. 2003). Without increased attention paid to controlling both nutrients, deterioration of water quality can be expected in the extensive blackwater systems characterizing the U.S. Coastal Plain, and likely other ecosystems as well.

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