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AN INSECT-BACTERIA BIOINDICATOR FOR ASSESSING DETRIMENTAL NUTRIENT ENRICHMENT IN WETLANDS

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Abstract: Field and laboratory studies were conducted to evaluate the use of bacterial growth on aquatic insects as a metric for determining the existence of nutrient impacts in wetlands. Results from field investigations indicated that elevated concentrations of nitrate and phosphate were associated with growth of filamentous bacteria on insect body surfaces and that there were significantly fewer mayflies (Ephemeroptera) in the nutrient-enriched wetland. Laboratory investigations confirmed a strong linkage between bacterial growth and reduced survival of mayflies. Survival was examined for individuals with bacterial infestation ranging from 0% to 60% body coverage. A threshold for catastrophic mortality was present at about the 25% level of coverage; there were very few survivors above that level. Based on these findings, the diagnostic endpoint for the bioindicator is 25% body coverage by bacterial growth, a level that signifies major differences in insect populations in the field and is also easy to detect visually. This study provides evidence that the insect-bacteria bioindicator is a reliable tool for assessing nutrient impacts on wetland macroinvertebrate communities. The bioindicator could be useful in the development of a Wetland Bioassessment Protocol.

Key Words: bioindicator, nutrient pollution, eutrophication, macroinvertebrates, aquatic bacteria, nitrogen, phosphorus, Ephemeroptera, wetlands

INTRODUCTION

Nutrient enrichment is a long-standing problem that threatens to disrupt the ecological balance of many important wetlands in the USA and seriously alter the benefits they provide to society. In the Southeast, for example, the cumulative effect of excess nutrients has resulted in eutrophication of important Atlantic Coast wetland-estuarine systems such as Chesapeake Bay in Virginia and the Albemarle-Pamlico system in North Carolina. Recent outbreaks of a toxic estuarine dinoflagellate (*Pfiesteria piscicida* Steidinger and Burkholder), which caused massive kills of recreationally and economically important fish and affected human health, have been attributed to nutrient enrichment of up-gradient streams and wetlands (Glasgow et al. 1995). The Florida Everglades have undergone extensive biological changes in response to nutrient inputs from agricultural activities (Davis 1994). Freshwater shrimp (*Palaemonetes paludosus* Gibbes), a key food item for many species of birds and fish, have been nearly extirpated from nutrient-enriched areas of this important hemispheric reserve for wildlife (Rader and Richardson 1992, 1994).

While the end result of excess nutrients can be fairly easily described and documented, predicting or detecting impacts at a stage when management intervention can prevent negative impacts from occurring is more difficult. Wetland managers need to precisely evaluate nutrient enrichment with the aid of early warning tools—bioindicators—for two reasons: (1) to gauge impending effects on wetland biota long before a catastrophic threshold is reached and (2) to monitor the success of efforts to reduce nutrient impacts at locations where the threshold has been exceeded (i.e., to determine if best management practices result in measurable improvements).

Our research evaluates whether growth of filamen-

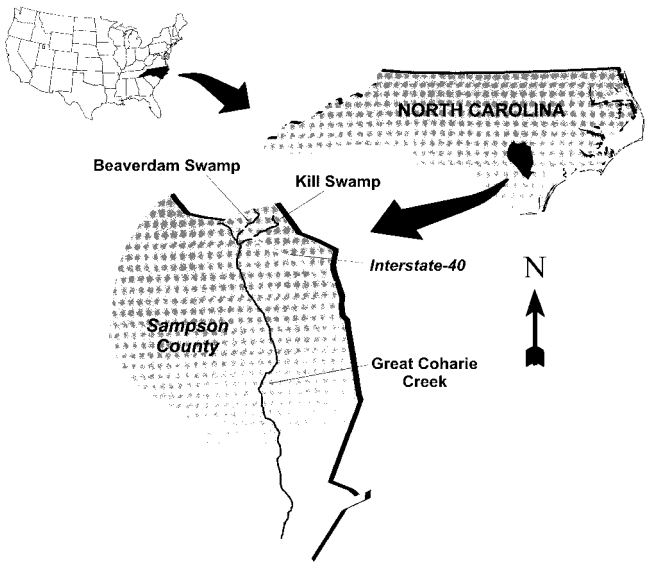

Figure 1. Location of the study wetlands in the coastal plain region of North Carolina, USA.

tous bacteria on immature aquatic insects can be a useful early-warning bioindicator of detrimental nutrient enrichment in wetlands. This technique is an extension of the method devised by Lemly (1998) for application to streams. Growth of *Sphaerotilus* sp. and *Leptothrix* sp. on stream insects has proven to be a useful addition to the USEPA Rapid Bioassessment Protocol for Macroinvertebrates (Plafkin et al. 1989) because it reveals a specific cause-effect linkage between nutrient enrichment and impaired insect communities (Lemly 1998, in press). Practical application of this method is quick, simple, and provides for rapid screening of insects in the field.

The basic premise of the bioindicator (i.e., that bacterial growth reflects nutrient enrichment sufficient to impair insect populations and thus threaten ecosystem integrity) should be equally true for wetlands and streams. A combination field and laboratory study was undertaken to investigate this question and determine if the bioindicator can be applied to wetlands.

METHODS

Field Investigations

Study Area. The study wetlands, Beaverdam and Kill Swamps, are third-order watersheds located along Interstate-40 in Sampson County, North Carolina, USA (35° 14′ N, 78° 21′ W; Figure 1). Both are low-flowing, cypress-gum wetlands that are part of the Cape Fear River watershed and within the Middle Atlantic Coastal Plain Ecoregion (Omernik 1987). These sites were selected because both (1) are bisected by fill-culvert type bridges, where fill dirt rather than pilings is used to support the road over the floodplain, (2) have similar watershed areas upstream from the crossings (24.1 and 18.4 km^2 for Beaverdam and Kill Swamps, respectively), (3) are on Bibb-Johnston association soils (hydric), and (4) have similar proportions of area among land uses within their watersheds, with the exception that hog-rearing facilities (a major nutrient source) are present only in the Kill Swamp watershed. Highway crossings, which were constructed in 1989, are separated by 1.8 km. Width of permanently flooded wetland habitat at the highway crossings is 200 m and 180 m for Beaverdam and Kill Swamps, respectively. We delineated areas of study as 200 m upstream and downstream from the highway crossings.

Trees of the study wetlands were primarily bald cypress (*Taxodium distichum* (L.)) and swamp tupelo (*Nyssa sylvatica* var. *biflora* Marshall). Macrophyte assemblages were dominated by the invasive Asian spiderwort (*Murdannia keisak* (Hassk.) Hand.-Mazz.) and rice cutgrass (*Leersia oryzoides* (L.) Swartz). Duckweed (*Spirodela polyrrhiza* (L.) Schlied.) was also abundant in open-canopy areas near the highway crossings. These sites were typical of bottomland forested wetlands found throughout the southeast USA (Clark and Benforado 1981, Rheinhardt et al. 1998).

Sampling Mayfly Abundance. Mayflies were selected for evaluation as a bioindicator because previous stream studies had indicated that this taxon was typically the most heavily colonized by bacteria and experienced greater impacts (reduction in numbers) than other insect groups (e.g., Plecoptera, Trichoptera; Lemly 1998, in press). The validity of this initial choice was verified for Beaverdam and Kill Swamps by examining three samples of the insect community (one collected in October 1995, one in April 1996, and one in October 1996). Results of this analysis indicated that Ephemeroptera had a greater prevalence and intensity of bacterial infestation than other insect groups. Thus, we chose mayflies for use in laboratory survival experiments as well as for abundance estimates in the field.

Transects were used to select plots for sampling rather than randomly scattering plots across the entire wetlands because other studies of these swamps had shown that mayflies were generally more numerous near the highway crossing (Richardson et al. 1997). Thus, to make valid comparisons between swamps, it was necessary to use data that were normalized for distance. The transect approach addressed that concern but still only provided pseudoreplicates since nutrients and other factors differed between swamps. Transects were marked parallel to the highway crossings across the full width of the wetlands. Transects were placed

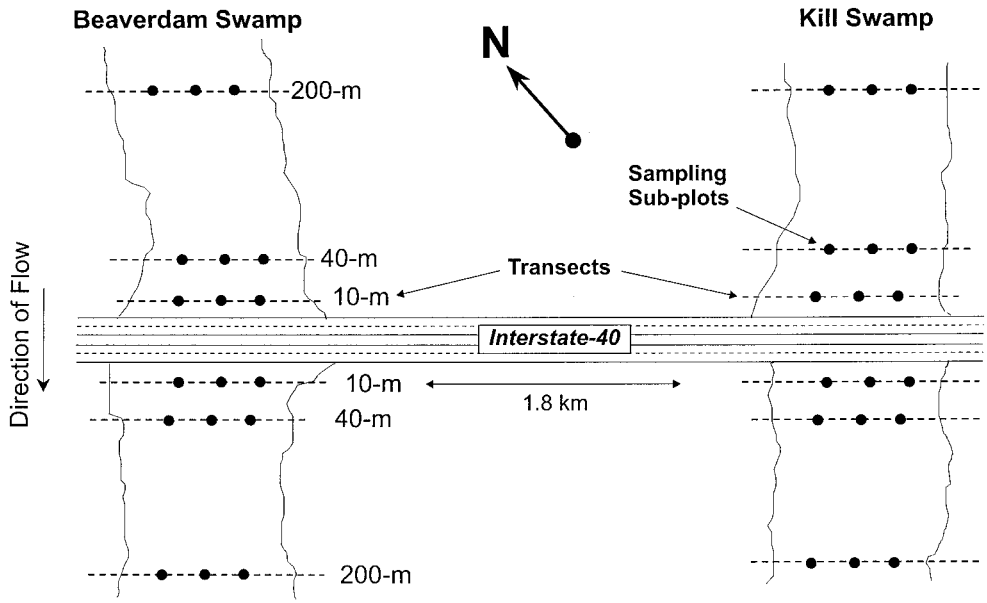

Figure 2. Schematic view of transects and sampling sub-plots in Beaverdam and Kill Swamps in relation to the fill-culvert highway crossings on Interstate-40. Transect distances correspond to distance from highway crossings.

at 10, 40, and 200 m distances on both sides of the crossing at both wetlands (Figure 2), and three 5 m radius sub-plots spaced 40 m apart were placed along each transect. The median sub-plot was placed at the lowest elevation adjacent to, but not within, the main channel. The remaining two plots flanked the median plot to standardize comparisons among transects. All plots were inundated with >10 cm of surface water at the time of sampling.

On March 26–27, 1997, mayfly abundance was sampled using protocols developed by the North Carolina Division of Water Quality (NCDWQ 1997) and the Mid-Atlantic Coastal Streams Workgroup (USEPA 1997) for low-gradient coastal plain streams. A composite sample was produced by taking subsamples from multiple habitats within a site. We identified four sub-habitats common in all areas of the wetlands: (1) herbaceous macrophytes, (2) bald cypress and swamp tupelo trunks, (3) sediments, and (4) submerged woody debris (snags). A D-frame aquatic sweep net (0.3 m wide, 595 μm mesh) was used to collect samples. D-frame sweep nets are the most commonly used sampling tool in stream assessment protocols (e.g., FDEP 1996, NCDWQ 1997, USEPA 1997) and are useful for estimating community composition in wetlands (Cheal et al. 1993, Turner and Trexler 1997). Habitat-specific samples were collected by ''jabbing'' a D-frame sweep net into the target area for a distance of 0.5 m. One sample was collected from each habitat nearest to each sub-plot centroid. Samples were taken from all three sub-plots along each transect (12 samples per transect). Net contents were placed in a sieve bucket (600 μm mesh), washed, and preserved with 95% ethanol for laboratory processing. All mayflies were enumerated to genus (no subsampling).

Assessing Bacterial Growth. Insects were examined for bacterial growth using a dissection microscope (10–200× magnification). Some individuals of each mayfly genus were prepared and viewed with scanning electron microscopy (SEM) using a Philips Model 501 instrument. Filamentous bacteria were identified to genus (400–1000× magnification using a compound microscope with phase-contrast optics and fiber optic light sources) with identification keys that use external morphological features of the sheaths (e.g., Buchanan and Gibbons 1974). When present in mature stages, which was the case for bacteria examined in this study, sheath-forming bacteria are easy to identify using simple characteristics such as the presence or absence of iron or manganese oxide crusts on sheaths and the presence or absence of swollen tips on sheaths. Even preserved material can be used, eliminating the need for culturing or staining.

The extent of bacterial growth on individual insects was quantified using a block-grid recording technique. An outline sketch of a generalized representative from each order (an enlargement of a line-drawing from a taxonomic key) was copied onto quad-ruled engineering paper (25 squares/cm^2; each insect ~240 mm long, one insect per page) and used as a data sheet for recording bacterial growth. An insect was viewed under the microscope, and bacterial growth was recorded by shading the corresponding body part on the sketch

with a highlighter pen. A dorsal view and a ventral view were sketched for each individual. The highlighted squares in both views were counted and compared to the total number of squares within the outline of the insect to calculate the percent of the body covered by bacteria.

Water Quality. Concentrations of dissolved nutrients (nitrate+nitrite, total N, orthophosphate, total phosphate, in 0.45 μm filtered samples, 4 replicates) were measured when insects were sampled in October 1995, April 1996, October 1996, and April 1997 using methods approved by USEPA for *in-situ* analysis (USEPA 1992). Grab samples were taken upstream and downstream from the highway culvert in each wetland (four upstream, four downstream), filtered in the field, and immediately refrigerated for transport to the laboratory. Nitrate-nitrite concentrations were determined by copper-cadmium reduction. Total nitrogen concentrations were determined by hydrazine reduction following a persulfate digestion. Nitrogen samples were analyzed on a Traacs 800 spectrophotometer. Orthophosphate and total P concentrations were determined by the Murphy-Riley phospho-molybdate blue complex reaction. Total P concentrations were determined after persulfate digestion. Phosphorus samples were measured using a Beckman DU-64 spectrophotometer.

Laboratory Tests

Ephemerella sp. and *Drunella* sp. were selected for study because (1) during April and October (the months during which live mayflies were collected), they were the numerically dominant taxa in both swamps and combining genera ensured that enough individuals were available for the experiments; (2) their herbivorous feeding mode made them amenable to long-term laboratory studies; and (3) field collections showed that they were heavily colonized by bacteria. In April and October 1996 and again in April and October 1997, live *Ephemerella* and *Drunella* from each swamp were placed into aerated, polypropylene jars and transported (in a water bath at 15°C to prevent thermal stress on the insects) to Virginia Tech University for survival studies. The experiments were structured to answer two questions: (1) does bacterial growth influence survival and (2) if survival is affected, what levels of infestation are necessary (i.e., what is the threshold) for significant impacts.

Three Plexiglas® aquaria with recirculating, aerated, and temperature-controlled water supplies were used for these experiments (Figure 3). Each aquarium held five 1.5-L chambers (containing several 3–5 cm cobbles) into which insects were placed. The sides and bottoms of the chambers had holes large enough to allow water to circulate freely but small enough to prevent insects from escaping. Chambers were submerged to a depth of 10 cm.

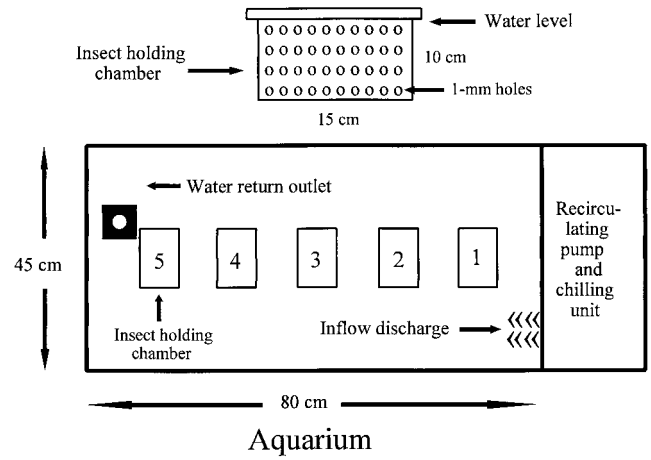

Figure 3. Schematic top view of aquarium containing five chambers used to hold mayflies in the survival experiments, and a side view of a single chamber. A total of 3 aquaria and 15 chambers was used.

Temperature and pH in aquaria were checked daily and, when necessary, adjusted to maintain conditions similar to the natural swamp (range 14–16°C; pH 5.3–5.7). A 12 h:12 h light:dark regime was maintained throughout each 30-d experiment. Dissolved nutrient concentrations (nitrate+nitrite, orthophosphate) were measured on day 1, 10, 20, and 30.

During experiments, mayflies fed on algae, diatoms, and associated microorganisms that grew as a biofilm on the stones in the experimental chambers. Dogwood (*Cornus florida* L.) leaves were placed among the cobbles to supplement mayfly diets and stimulate the growth of the biofilm. Leaves were conditioned by incubating them in the chambers for 30 d prior to introducing the insects. Mayflies were recovered and enumerated at the end of each experiment and percent mortality was determined. Surviving individuals were examined for bacterial growth under a dissection microscope.

Mayflies were divided into test groups based on the degree of bacterial infestation. Gross visual estimates, rather than the quantitative block-grid procedure used for preserved insects, were used to determine the degree of bacterial infestation. The experience gained from quantifying bacterial growth on insects from previous studies (Lemly 1998) made it possible to efficiently sort mayflies into groups. The four survival experiments tested the following levels of infestation: Experiment (1) two groups—0% and >50% body coverage (18 April to 20 May 1996); Experiment (2) three groups—0%, 10–25%, and 25–50% coverage (9 October to 12 November 1996); Experiment (3) three

Table 1. Degree of bacterial growth on the insect community of Beaverdam Swamp and Kill Swamp, Sampson County, NC.

Sampling Date and Insect Order	# Examined (# with Bacteria, %) (# Heavily Infested*, %) Beaverdam Swamp	Kill Swamp
October 1995		
Ephemeroptera	21 (0) (0)	32 (26,81) (5,16)
Trichoptera	13 (0) (0)	20 (3,15) (0)
Odonata		
dragonflies	44 (0) (0)	19 (4,21) (1,5)
damselflies	19 (0) (0)	25 (21,84) (2,8)
Diptera	36 (0) (0)	60 (9,15) (0)
Hemiptera	19 (0) (0)	27 (18,67) (0)
Coleoptera	22 (0) (0)	39 (5,13) (1,3)
April 1996		
Ephemeroptera	63 (0) (0)	49 (41,84) (23,47)
Trichoptera	17 (0) (0)	16 (4,25) (0)
Odonata		
dragonflies	52 (0) (0)	66 (22,33) (7,11)
damselflies	50 (0) (0)	104 (63,61) (38,37)
Diptera	50 (0) (0)	75 (31,41) (5,7)
Hemiptera	50 (0) (0)	44 (25,57) (2,5)
Coleoptera	50 (0) (0)	50 (18,36) (0)
October 1996		
Ephemeroptera	49 (0) (0)	50 (41,82) (12,24)
Trichoptera	10 (0) (0)	7 (0) (0)
Odonata		
dragonflies	41 (0) (0)	24 (6,25) (0)
damselflies	29 (0) (0)	39 (22,56) (5,13)
Diptera	50 (0) (0)	30 (4,13) (0)
Hemiptera	25 (0) (0)	25 (12,48) (0)
Coleoptera	25 (0) (0)	22 (3,14) (0)

* ≥25% of body colonized.

Table 2. Experimental design for survival tests with mayflies.

Experiment 1 (18 April–20 May 1996)
Treatments: 0 = no bacterial growth, X = >50% body coverage, — = empty chamber

	Chamber (5 Mayflies in Each)				
Aquarium #	1	2	3	4	5
1	X	0	X	0	X
2	0	X	0	0	X
3	—	0	X	X	0

Experiment 2 (9 October–12 November 1996)
Treatments: 0 = no bacterial growth, X = 10–25% body coverage, + = 25–50% body coverage

	Chamber (7 Mayflies in Each)				
Aquarium #	1	2	3	4	5
1	+	0	X	X	0
2	+	X	+	0	X
3	X	+	0	+	0

Experiment 3 (9 April–9 May 1997)
Treatments: 0 = <10% body coverage, X = 10–20% body coverage, + = 20–30% body coverage

	Chamber (5 Mayflies in Each)				
Aquarium #	1	2	3	4	5
1	X	+	0	0	+
2	+	0	X	X	0
3	0	X	+	+	X

Experiment 4 (16 October–17 November 1997)
Treatments: 0 = 10–20% body coverage, X = 20–30% body coverage, + = 30–40% body coverage

	Chamber (7 Mayflies in Each)				
Aquarium #	1	2	3	4	5
1	X	0	+	X	0
2	+	X	0	0	X
3	0	+	X	+	+

groups—<10%, 10–20%, and 20–30%, coverage (9 April to 9 May 1997); Experiment (4) three groups—10–20%, 20–30%, and 30–40% coverage (16 October to 17 November 1997). Each testing chamber received 5 individuals (April experiments) or 7 individuals (October experiments) from one of the groups, and each chamber was randomly assigned to one of the three aquaria (Sokal and Rohlf 1981, Table 2). The test groups for the May 1996 experiment had 7 replicates; the other three experiments had 5 replicates of each group.

Data Analysis

The mayfly numbers per transect in the two swamps were compared using one-way analysis of variance (ANOVA). Transects represented pseudoreplicates since nutrients and other factors varied between the swamps. To reduce variances and meet assumptions of normality required for parametric tests, these data were transformed (log[x + 1]) prior to analysis. Comparisons of nutrient concentrations between the two swamps were made for each sampling date using *t*-tests.

RESULTS

Field Investigations

Bacterial Growth. Bacterial assemblages were composed of *Sphaerotilus* sp. and *Leptothrix* sp. Bacterial

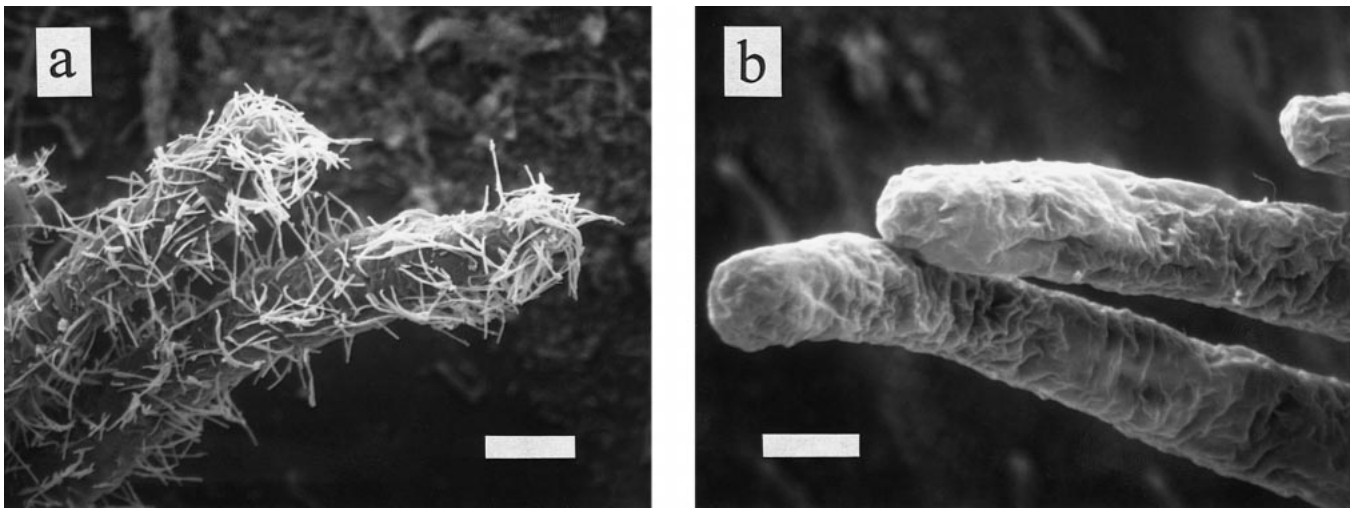

Figure 4. Scanning electron micrographs of *Ephemerella* sp. illustrating (a) gills heavily infested (>25% covered) with the filamentous bacteria *Sphaerotilus* sp. and *Leptothrix* sp. and (b) uncolonized gills. Infestation of the degree shown in plate a was associated with 100% mortality in laboratory survival studies and reduced numbers of mayflies in the field. Scale bars = 250 μm.

growth was especially luxuriant on insect gills, but bacterial colonies with similar sheath density occurred on all insect body surfaces. Scanning electron microscopy in Figure 4 demonstrates the extent to which these filamentous bacteria could colonize individual gill filaments.

Under low magnification of the dissection scope, the bodies of infested insects appeared fuzzy, supporting a light-colored film. Bacterial growth on heavily infested (>25% covered) individuals was easily detected with just a hand lens (10–20×) when insects were immersed in water or preservative. Caudal cerci of Ephemeroptera proved to be particularly good for rapidly screening individuals to assess the degree of bacterial growth, both in the lab and field (Figure 5).

A total of six orders of insects were examined for filamentous bacteria (Table 1). All orders from Beaverdam Swamp were free of bacterial growth. In contrast, in Kill Swamp, all orders were colonized by filamentous *Sphaerotilus* sp. and *Leptothrix* sp. Ephemeroptera were consistently the most heavily infested, with up to 47% of individuals having >25% of their bodies colonized. Zygoptera also had a relatively high percentage of heavily colonized individuals. Infestation was lowest on Trichoptera, Coleoptera, and Diptera. The prevalence and intensity of bacterial infestation was consistent across the three sampling dates.

Water Quality. Concentrations of dissolved nutrients were consistently greater in Kill Swamp than in Beaverdam Swamp (Table 3). In particular, the concentrations of nitrate+nitrite and orthophosphate, which are nutrients responsible for many bacterial and algal blooms in aquatic systems, were about 5 times greater in Kill Swamp in spring (April) and about 10 times greater in fall (October).

Mayfly Abundance. Four genera of mayflies were collected from each swamp (Table 4). *Ephemerella* were most abundant, followed by *Drunella, Caenis,* and *Callibaetis.* In both swamps, mayfly abundance was greater along transects near the highway crossing (10 m or 40 m) than along the most distant transect (200 m). The abundance of all four genera was significantly lower in Kill Swamp (Table 4). *Caenis* sp. and *Callibaetis* sp. were almost nonexistent in Kill Swamp but were frequently encountered in samples from Beaverdam Swamp. Even among the two numerically dominant taxa, there were dramatic differences in abundance, with 71% fewer *Ephemerella* sp. and 75% fewer *Drunella* sp. in Kill Swamp. Within transects, there were no significant upstream-downstream differences in abundance in either swamp.

Laboratory Tests

In all 4 experiments, mayflies that supported heavy bacterial growth (>25% body coverage) suffered nearly 100% mortality within the 30-d experimental run. In contrast, mean survivorship among uninfested mayflies was 86.5% (±2.1 SE), and these individuals appeared healthy (some had grown enough to develop wing pads). None of the surviving mayflies that were uninfested at the start of the tests became colonized by bacteria, indicating that there was no chamber-to-chamber growth of bacteria. Concentrations of dissolved nutrients (orthophosphate, nitrate+nitrite) in the aquaria remained below 10 μg/L throughout all of

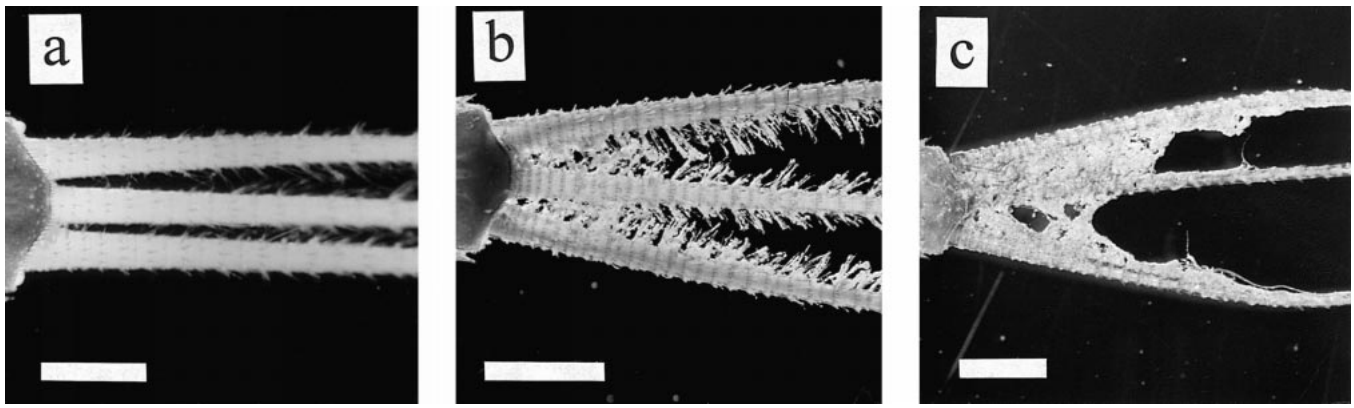

Figure 5. Characteristic appearance of bacterial growth on caudal cerci (tail filaments) of *Ephemerella* sp. immersed in 80% ethanol. Plate a (20× magnification) shows uninfested cerci with delicate, hair-like setae visible. Plate b (20× magnification) illustrates the appearance of heavy bacterial growth (>25% of body covered). Bacterial sheaths nearly fill the space between cerci and obscure the delicate setae. Plate c (15× magnification) shows an advanced stage of colonization in which bacterial filaments have become matted and partially covered by silt particles. Infestation of the degree shown in plates b and c was associated with 100% mortality in laboratory survival studies and reduced numbers of mayflies in the field. The condition can be easily diagnosed in the field using a hand lens with 10–20× magnification. Scale bars = 0.5 mm.

Table 3. Mean concentrations of dissolved nutrients (μg/L) in Kill Swamp and Beaverdam Swamp, Sampson County, NC during 1995–97, $n = 4$. *t*-probabilities (Kill–Beaverdam comparison): *** = $p < 0.001$; — = parameter not measured.

Month, Year, and Nutrient	Kill Swamp	Beaver-dam Swamp	Ratio of Kill : Beaver-dam	*t*-probability
October 1995				
Nitrate + nitrite	864.7	80.4	10.6	***
Total nitrogen	1926.9	715.0	2.7	***
Orthophosphate	119.2	11.1	10.7	***
Total phosphate	—	—	—	—
April 1996				
Nitrate + nitrite	—	—	—	—
Total nitrogen	2639.2	780.0	3.4	***
Orthophosphate	164.8	34.4	4.8	***
Total phosphate	243.4	96.8	2.5	***
October 1996				
Nitrate + nitrite	—	—	—	—
Total nitrogen	2162.7	1274.7	1.7	***
Orthophosphate	276.0	24.7	11.2	***
Total phosphate	620.4	198.1	3.1	***
April 1997				
Nitrate + nitrite	—	—	—	—
Total nitrogen	3888.9	1967.7	2.0	***
Orthophosphate	100.3	17.5	5.7	***
Total phosphate	168.8	54.2	3.1	***

the tests. A plot of the relationship between severity of bacterial infestation and survival of *Ephemerella* sp. and *Drunella* sp. revealed that a threshold for catastrophic mortality exists at about the 25% level of body coverage (Figure 6). Almost all individuals with >25% body coverage died, but many of those with 10–25% coverage survived and appeared to be healthy.

DISCUSSION

Diagnostic Capability

The findings of this study parallel those of Lemly (1998), who concluded that the occurrence of epizoic bacterial colonization of aquatic insects can be a useful, quick indicator of detrimental point- or non-point-source nutrient enrichment in streams. Our study supports that conclusion in a wetland. The degree of bacterial growth associated with the mortality threshold can be used as a diagnostic endpoint. When mortality data from the laboratory experiments are examined in combination with relationships between mayfly abundance and bacterial infestation in the field, 20–30% body coverage emerges as a range in which a diagnostic endpoint for the bioindicator can be identified (Figure 6). Survival of insects with 10–25% coverage can be good, but beyond 30%, survival is unlikely. Thus, the metric hereafter designated to signify harmful impacts of nutrients on wetland mayfly populations is 25% body coverage by filamentous bacteria.

As with Lemly's earlier findings, results of this study seemed to indicate a cause-effect linkage between nutrient concentrations, bacterial growth, and

Table 4. Abundance (number collected per transect, refer to Figure 2) of mayflies in Kill Swamp (K) and Beaverdam Swamp (B), Sampson County, NC in March 1997. ** = $P < 0.01$ ($F_{(1,2)}$ for totals, $F_{(1,5)}$ for grand total), Kill–Beaverdam comparison.

	Number of Individuals (ANOVA Result)							
	Ephemerella		*Drunella*		*Caenis*		*Callibaetis*	
Transect	K	B	K	B	K	B	K	B
Upstream								
10-m	6	21	7	29	0	9	0	7
40-m	11	37	4	15	0	5	0	2
200-m	4	13	0	8	0	1	0	0
Total	21	71**	11	52**	0	15**	0	9**
Downstream								
10-m	9	33	6	25	0	11	1	7
40-m	7	20	5	18	2	6	0	5
200-m	3	12	3	7	0	0	0	1
Total	19	65**	14	50**	2	17**	1	13**
Grand Total	40	136**	25	102**	2	32**	1	22**

insect mortality. However, since the laboratory studies used field-infested mayflies, it is not possible to know if the bacteria alone were responsible for death, or if death was due to a combination of bacteria and other stressors to which the insects were exposed in the field (e.g., turbidity, flow, dissolved oxygen fluctuations, etc.). Also, nutrient concentrations in the ''clean'' wetland were sometimes as high (at their peak) as in the enriched wetland (at their low). A bloom stage growth of *Sphaerotilus* and *Leptothrix* can be created when nutrient/temperature relationships reach some critical point, but that point is not well defined, even in controlled outdoor channels (Phaup and Gannon 1967). However, up to the critical point, nutrients can be elevated and not cause bacterial blooms (Curtis 1969). Thus, although there was an overlap of nutrient levels in the two study wetlands, concentrations must have stayed below the critical point in Beaverdam Swamp but exceeded that threshold in Kill Swamp. This finding actually strengthens the diagnostic power of the bioindicator—it is only evident when detrimental enrichment occurs.

We believe that the evidence for a cause-effect link between nutrients and bacterial growth is strong. Nutrients were significantly and consistently higher in Kill Swamp, which was the only location where bacterial growth on insects occurred. Results of the survival studies, in combination with evidence from the field surveys, indicate that bacterial growth can have a major influence on wetland insect populations. For example, mayflies from the field samples were often heavily colonized by bacteria (e.g., up to 47% of Ephemeroptera, Table 1). In the laboratory experiments, <10% of the heavily infested mayflies survived, whereas >85% of those without bacterial growth survived and appeared to be healthy. The abundance of mayflies in the study wetlands was significantly lower where nutrients were elevated and bacterial growth occurred (Tables 1, 4). Our results show that the insect-bacteria bioindicator can correctly diagnose nutrient enrichment as a cause for impaired mayfly populations.

Reliability and Simplicity

This is now the third study to confirm experimentally that bacterial infestation of insects has practical application as a bioindicator of detrimental nutrient enrichment in a field setting. Two of those studies investigated streams (Lemly 1998, in press), and one examined wetlands (this paper). As yet, there have been no false positives (i.e., locations in which nutrient enrichment and bacterial growth occur, but there are no discernable impacts on macroinvertebrate populations). Thus, the reliability of the method seems sufficient to justify further application and investigation, particularly with regard to wetlands.

Importantly, detection of the diagnostic endpoint (insects with ≥25% body coverage) is easily accomplished under low magnification (10–20×) with a hand lens or dissection microscope (Figure 5). Detailed quantitative measurements and taxonomic identifications are not necessary; qualitative samples and order-level classification are adequate. Moreover, insects can be scanned on-site, literally in-hand, allowing a screening-level field assessment to be conducted within minutes.

Preservation of insects in ethanol or formalin, or manipulation of insects with collection equipment such as brushes and forceps apparently does not dislodge

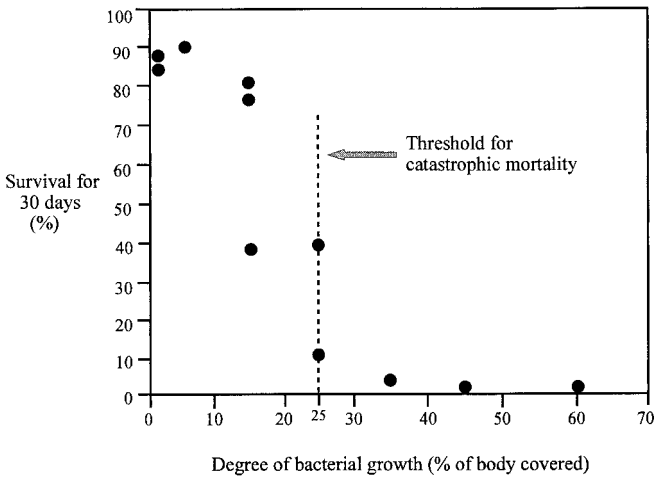

Figure 6. Relationship between degree of bacterial infestation and survival of mayflies (*Ephemerella* sp. and *Drunella* sp.) for 30-d in the laboratory. Each dot represents the percent survival from one infestation-level group for one 30-day study (25–35 individuals in the test group, plotted as the mid-range of the infestation levels for the test group; i.e., 20–30% group is plotted as 25%). A threshold for catastrophic mortality exists at about the 25% level of body coverage. Beyond this level of infestation, very few individuals survive.

the bacteria. Consequently, severity of infestation can be confirmed in the laboratory without loss of accuracy. Archived samples collected as part of a long-term monitoring program or other research purposes can also be evaluated. Immersing individual insects into water or preservative suspends bacterial filaments attached to the lateral edges of the body for easy recognition, particularly on the caudal filaments of heavily infested Ephemeroptera (Figure 5). Individuals whose bodies are ≥25% covered by bacteria (i.e., the indicator level for impact assessment) can be rapidly detected in the field or laboratory.

Application to Wetland Bioassessment

In streams, it is possible to use aquatic insects for rapid assessment of biotic conditions. The EPA Rapid Bioassessment Protocol for Macroinvertebrates (RBP, Plafkin et al. 1989) was developed specifically for that purpose. However, there is no comparable assessment method for wetlands (Danielson 1998). The environmental tolerance ratings that form the foundation for RBP in streams do not convey the same ecological significance when applied to wetlands. For example, a predominance of species that tolerate warm, turbid water and silty substrate indicates poor biotic conditions in upland streams; yet, tolerant species adapted to a wide range of conditions may be characteristic of healthy wetlands. The insect-bacteria bioindicator presented here could be useful in the development of a Wetland Bioassessment Protocol (WBP) or a multimetric index such as IBI (Index of Biological Integrity; Karr and Chu 1997) for application to ecosystems whose macroinvertebrate fauna does not lend itself to evaluation by the classic stream RBP. Positive diagnosis of bacterial growth immediately reveals a probable cause for impaired wetland macroinvertebrate communities, and it can help to focus subsequent investigations because nutrient enrichment is indicated as a major contributing factor. These strengths, combined with the simplicity and speed of the method, suggest that it would be a key element of a WBP or IBI.

In promoting the use of the bioindicator, we do not imply that *Ephemerella* or *Drunella* are the best or only taxa to use in a field assessment or that they are assumed to be ubiquitous in wetlands. Our study wetlands had a noticeable flow, particularly during periods of high water, which may account for the dominance of these typical stream genera in the samples. Importantly, our results show depression of all mayfly genera concurrent with bacterial infestation, including *Caenis* and *Callibaetis,* which are more typical of the swamp taxa found in the Southeast. We selected *Ephemerella* and *Drunella* because they were the only mayflies numerous enough to supply the individuals needed for the laboratory experiments. However, we believe that the bioindicator is applicable to a wide variety of wetlands and that mayflies, as a group, are the best taxon to use in detecting detrimental levels of bacterial growth.

CONCLUSIONS

This study provides evidence that the insect-bacteria bioindicator is valid for application to nutrient assessment in wetlands. Bacterial growth on insects is a practical tool for identifying the existence of detrimental non-point-source nutrient inputs, as well as evaluating the severity of biological impacts from known sources. Rapid field or laboratory screening of macroinvertebrate samples is possible. A discovery of mayflies whose bodies are ≥25% covered by filamentous bacteria is all that is necessary to reliably diagnose harmful impacts of nutrients on wetland macroinvertebrate communities. The information provided by this bioindicator will be useful for detecting nutrient problems as well as monitoring the success of management actions to improve water quality.

Additional investigations are needed to determine if the method performs consistently for different types of wetlands (forested vs. pothole, etc.), nutrients (phosphorus vs. nitrogen), and nutrient sources (chemical fertilizers, industrial and municipal wastewater, animal

wastes), and if the 25% infestation level is the appropriate indicator threshold in those cases. There is also a need to investigate why some insect taxa are consistently more heavily colonized by bacteria than others, i.e., factors such as differences in physiological parameters (chemical composition of body surfaces) or ecological variables (degree of movement, microhabitat usage, etc.). Finally, because mayflies are not common in all wetlands, other taxa (e.g., odonates) should be investigated as a possible surrogate for diagnosing nutrient impacts.

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