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Association of Multiple-Antibiotic-Resistance Profiles with Point and Nonpoint Sources of *Escherichia coli* in Apalachicola Bay†

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A total of 765 *Escherichia coli* isolates from point and nonpoint sources were collected from the Apalachicola National Estuarine Research Reserve, and their multiple-antibiotic-resistance (MAR) profiles were determined with 10 antibiotics. *E. coli* isolates from point sources showed significantly greater resistance ($P < 0.05$) to antibiotics and higher MAR indices than isolates from nonpoint sources. Specifically, 65 different resistance patterns were observed among point source isolates, compared to 32 among nonpoint source isolates. Examples of this contrast in MAR profiles included percentages of isolates with resistance to chlortetracycline-sulfathiazole of 33.7% and to chlortetracycline-penicillin G-sulfathiazole of 14.5% for point source isolates versus 15.4 and 1.7%, respectively, for nonpoint source isolates. MAR profile homology, based on coefficient similarity, showed that isolates from point sources were markedly more diverse than isolates from nonpoint sources. Seven clusters were observed among point source isolates, with a coefficient value of approximately 1.8. In contrast, only four clusters were observed among nonpoint source isolates. Covariance matrices of data displayed six very distinct foci representing nonpoint source *E. coli* isolates. Importantly, *E. coli* isolates obtained directly from human and animal feces also clustered among point and nonpoint sources, respectively. We conclude that *E. coli* MAR profiles were associated with point and nonpoint sources of pollution within Apalachicola Bay and that this method may be useful in facilitating management of other estuaries.

Fecal pollution decreases water quality in many estuaries. This pollution can originate from point sources (PSs), such as industrial and municipal effluents, or from nonpoint sources (NPSs), such as land runoff and septic tank seepage that disperse over wide areas (13).

The Apalachicola National Estuarine Research Reserve (ANERR) is a unique environment to study PS and NPS fecal pollution. The ANERR encompasses more than 193,000 acres of land constituting two barrier islands, the lower 20 miles of the Apalachicola River and its floodplain, adjoining uplands, and the Apalachicola Bay system (12). In the ANERR, extensive protected uplands and wetlands are a major habitat of local wildlife.

The ANERR is also a significant harvest area for shellfish and a variety of finfish. Oysters are the most important commercial invertebrate in the bay system (12). For this reason, effective management of ANERR resources requires tools to identify sources of fecal pollution that decrease water quality and lead to closure of shellfish beds.

The fecal coliform *Escherichia coli* has been used as an indicator of the potential presence of human enteric pathogens for many years (13). However, it is well established that *E. coli* also inhabits the intestines of many warm-blooded animals. Consequently, research is needed to determine potential characteristics of *E. coli* that can be used to identify its points of origin from various sources of fecal pollution. In this manner, remediation efforts can be enhanced.

Antibiotic-resistant *E. coli* have been isolated from rivers and coastal areas (1, 5, 30), domestic sewage (8, 25, 26), surface water and sediments (28, 29), lakes (7), seawater (6), drinking water (7), and hospital environments (24). To a limited degree, multiple-antibiotic resistance (MAR) has been used to differentiate *E. coli* from different sources, including urban and rural waters (14, 23). However, little is known about the application of MAR to differentiate PS and NPS *E. coli* isolates. The present study examined *E. coli* antibiotic resistance as a possible tool to differentiate PS and NPS pollution within the ANERR.

MATERIALS AND METHODS

Collection of samples. Estuarine surface water (500 to 750 ml) was collected on an outgoing tide at monthly intervals over 1 year, in duplicate, in sterile Whirl pack bags (Fisher Scientific, Atlanta, Ga.). Specifically, surface water was collected from two sites in the northeast region of East Bay, which receives extensive drainage from protected marshlands but no known PS pollution (Fig. 1). Samples (250 to 500 ml) of sewage treatment plant effluents were collected from municipal treatment plants on the east and west shores of Apalachicola Bay (Fig. 1). *E. coli* isolates were also collected directly from feces of human volunteers living in Apalachicola, Fla., and from animal feces deposited within the ANERR wildlife reserve. All samples were stored at 4°C and transported via overnight courier.

Isolation and identification of *E. coli*. Sample preparation and bacteriological tests for isolation of *E. coli* were performed by established procedures (2, 3, 22). All samples were processed within 24 h of collection. A predetermined water volume, based on an initial measurement of the *E. coli* most probable number, was filtered through a 0.2-μm-pore-size filter (Gelman Sciences, Ann Arbor, Mich.). Filters were placed on MacConkey agar (Difco Laboratories, Detroit, Mich.) and incubated at 35°C for 18 h, and then all lactose-fermenting *E. coli*-like colonies were screened with 4-methylumbelliferyl-β-D-glucuronide (Sigma Chemical Co., St. Louis, Mo.) (19). Sewage and feces samples were diluted in 10-fold serial increments, and 0.1 ml of each dilution was plated on MacConkey

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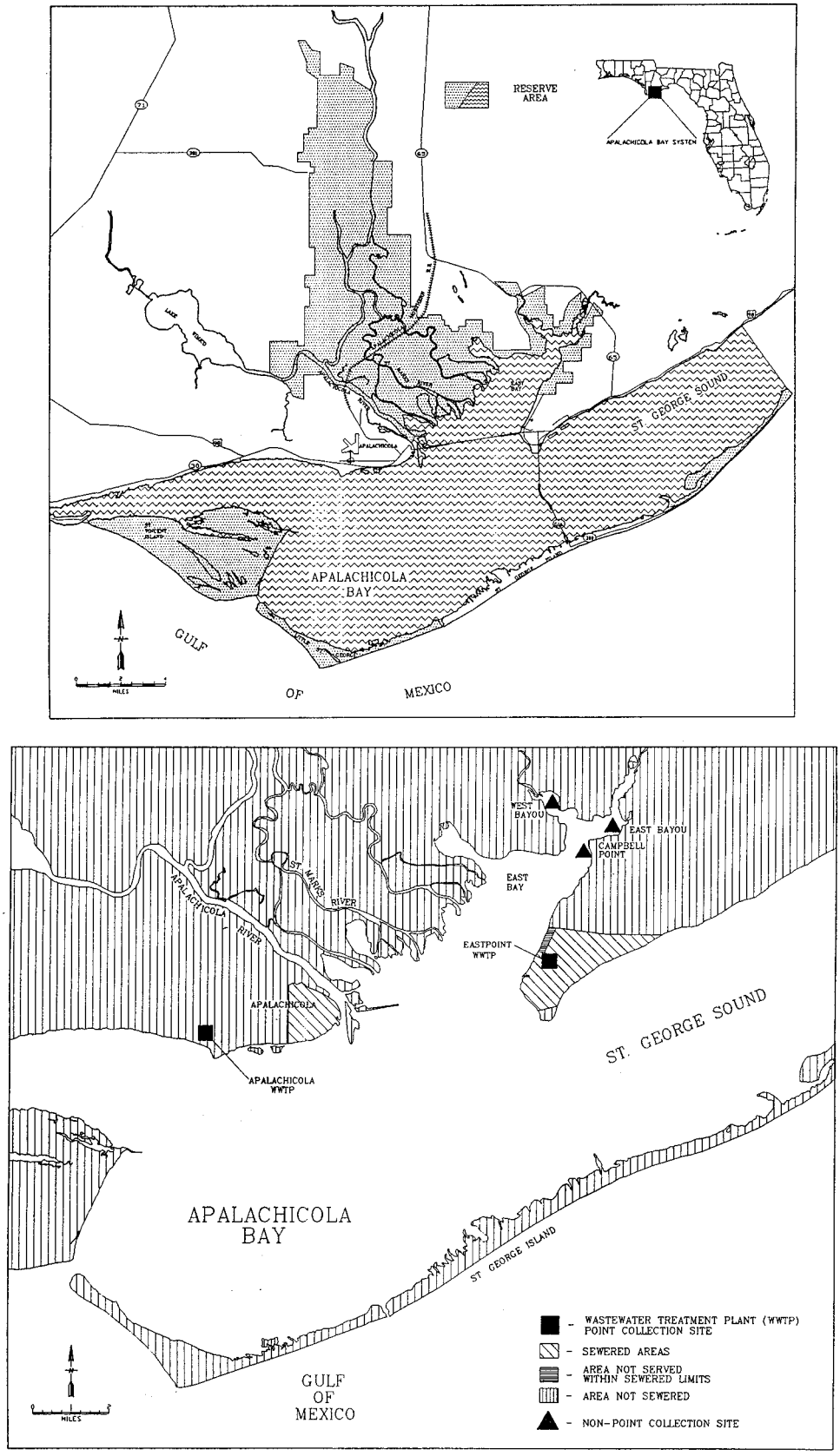

FIG. 1. Major features of the ANERR.

TABLE 1. Percentages of *E. coli* PS and NPS isolates resistant to antibiotics

Antibiotic	% of resistant strains		*P* value[a]
	PS (n = 407)	NPS (n = 358)	
Ampicillin	12.5	4.5	<0.05
Chlortetracycline	88.5	61.2	<0.05
Kanamycin	6.9	0.6	<0.05
Nalidixic acid	2.9	0	<0.05
Neomycin	3.2	0.3	<0.05
Oxytetracycline	10.3	3.4	<0.05
Penicillin G	38.6	37.7	0.86
Streptomycin	10.8	2.8	<0.05
Sulfathiazole	66.3	21.0	<0.05
Tetracycline	10.3	1.7	<0.05
Total	94.6	67.6	<0.05

[a] The *P* value for PS versus NPS isolates was determined by a two-sided binomial test.

agar and incubated at 35°C for 18 h. Typical *E. coli*-like colonies were screened with 4-methylumbelliferyl-β-D-glucuronide. Presumptive *E. coli* isolates were confirmed by standard biochemical tests (2).

MAR. MAR indices were determined by the method of Kaspar et al. (22) with selected antibiotics typically associated with animal feed and/or clinical treatments. Briefly, stock solutions of antibiotics were filter sterilized and stored at 5°C in the dark. The following final concentrations were used: 10 μg/ml for ampicillin, 25 μg/ml for chlortetracycline, 25 μg/ml for nalidixic acid, 50 μg/ml for neomycin, 25 μg/ml for oxytetracycline, 75 U/ml for penicillin G, 12.5 μg/ml for streptomycin, 500 μg/ml for sulfathiazole, and 25 μg/ml for tetracycline. Aliquots of stock solutions were added to tempered (46°C) Mueller-Hinton agar (Difco), mixed, poured into petri dishes, and stored at 5°C for no longer than 2 weeks. *E. coli* isolates were grown in tryptic soy broth (Difco) at 35°C for 4 to 6 h, replica plated onto antibiotic-containing agar plates and control plates without antibiotic, and incubated at 35°C for 18 h. *Pseudomonas aeruginosa* ATCC 27853 and *Staphylococcus aureus* ATCC 25923 were used as positive (resistant to all antibiotics tested except for sulfathiazole) and negative (sensitive to all antibiotics tested) controls, respectively. Isolates were recorded as resistant to an antibiotic if growth, measured with a ruler, was indistinguishable from that on the control plate without antibiotic; more than 10 to 15% reduced growth was recorded as a sensitive reaction to the antibiotic, although growth was normally reduced by more than 90%. The MAR index (number of antibiotics to which the isolate was resistant ÷ total number of antibiotics tested) was determined for each isolate.

Statistical analysis. Significant differences between antibiotic resistance patterns of PS and NPS isolates were determined by a two-sided test of binomial proportion ($P < 0.05$). Data were converted to binary code, and interisolate relationships were measured by the Euclidian metric, average-linkage method (31). Relationships were examined by cluster analysis and demonstrated with plots of principal-component similarity coefficients. Computations were performed with Statistical Analysis System (SAS)/Base and SAS software (15, 33).

RESULTS

Among a total of 765 isolates, approximately 82% were resistant to one or more antibiotics (Tables 1 and 2). Resistance to single antibiotics was significantly higher in PS isolates (94.6%) than in NPS isolates (67.6%; $P < 0.05$) (Table 1).

Examples of single antibiotics which differentiated PS and NPS isolates at a P of <0.05 were ampicillin, kanamycin, nalidixic acid, neomycin, oxytetracycline, streptomycin, sulfathiazole, and tetracycline (Table 1). In contrast, there was no significant difference in levels of resistance to penicillin G of PS and NPS isolates ($P = 0.86$) (Table 1).

The predominant single-antibiotic-resistance and MAR patterns of *E. coli* isolates are shown in Table 2. In general, PS isolates showed higher resistance to single antibiotics and to combinations of antibiotics than NPS isolates. Specifically, 65 resistance patterns were observed for PS isolates compared to only 32 patterns for NPS isolates. In addition, the average MAR index for PS isolates was 0.25 compared to 0.13 for NPS isolates. Among PS isolates, relatively high resistance to chlortetracycline-sulfathiazole (33.7%) and chlortetracycline-penicillin G-sulfathiazole (14.5%) was observed. In contrast, 28.2% of NPS isolates were resistant to chlortetracycline-penicillin G, compared to only 7.4% of PS isolates.

The relationships of antibiotic resistance patterns between PS and NPS isolates, based on coefficients of similarity measured by Euclidian distance, are shown in Fig. 2 and 3. At a Euclidian distance of approximately 1.8, seven clusters were formed among PS isolates, designated P_1 to P_7 (Fig. 2; Table 3). In contrast, four clusters were observed among NPS isolates, designated N_1 to N_4 (Fig. 3; Table 3). A covariance matrix of principal components clearly showed more diversity among PS isolates (Fig. 4); in contrast, NPS isolates formed six focal groups (A to F). Clusters N_2 and N_4 were found only in groups A, B, and D; cluster N_1 was found only in group C; and cluster N_3 was found only in groups E and F.

In a separate experiment, *E. coli* isolates were obtained directly from human and animal feces; results showed 10 and 5 antibiotic resistance patterns, respectively. The predominant antibiotic resistance pattern for human isolates was chlortetracycline-sulfathiazole (40%), similar to that observed for PS

TABLE 2. Predominant antibiotic resistance patterns of *E. coli* isolated from PS, NPS, humans, and animals

Resistance or sensitivity pattern[a]	% of isolates with indicated resistance or sensitivity pattern from source type:			
	PS (n = 407)	Human (n = 30)	NPS (n = 358)	Animal (n = 29)
C-Su	33.7	40.0	15.4	ND[b]
C-P-Su	14.5	ND	1.7	ND
C	7.9	16.7	8.4	ND
C-P	7.4	ND	28.2	6.9
A-C-P	3.2	ND	1.1	ND
A-P	ND	ND	ND	10.3
A-P-Su	ND	ND	ND	6.9
Su	2.2	6.7	1.4	ND
C-K-N-OX-S-Su-T	2.0	ND	ND	ND
P-Su	1.7	ND	ND	ND
A-C-P-Su	1.5	ND	ND	ND
C-OX-T	ND	6.7	ND	ND
A-C-OX-P-Su	1.2	ND	ND	ND
C-OX-P-Su	1.0	ND	ND	ND
C-S	0.7	ND	0.8	ND
C-P-S	0.7	ND	ND	ND
C-OX-S-Su-T	0.7	ND	ND	ND
C-K-T	0.7	ND	ND	ND
A-C	0.7	ND	0.6	ND
C-T	0.7	ND	ND	ND
A-C-P-S	0.7	ND	ND	ND
A-C-P-S-Su	0.7	ND	ND	ND
P	0.5	ND	2.5	ND
C-Na	0.5	ND	ND	ND
C-Na-Su	0.5	ND	ND	ND
C-K-P-Su	0.5	ND	ND	ND
A-C-Su	0.5	ND	0.8	ND
A-C-OX-P-S-Su-T	0.5	ND	ND	ND
C-OX-P	ND	ND	1.1	ND
Other	9.6	16.6	5.6	3.4
Sensitivity to all antibiotics	5.4	13.3	32.4	72.4

[a] A, ampicillin; C, chlortetracycline; K, kanamycin; Na, nalidixic acid; N, neomycin; OX, oxytetracycline; P, penicillin G; S, streptomycin; Su, sulfathiazole; T, tetracycline. The resistance patterns under the heading of "other" consisted of 39 patterns from PS isolates, 20 patterns from NPS isolates, 5 patterns from human isolates, and 1 pattern from animal isolates, and each pattern derived from only one isolate (not shown).

[b] ND, none detected.

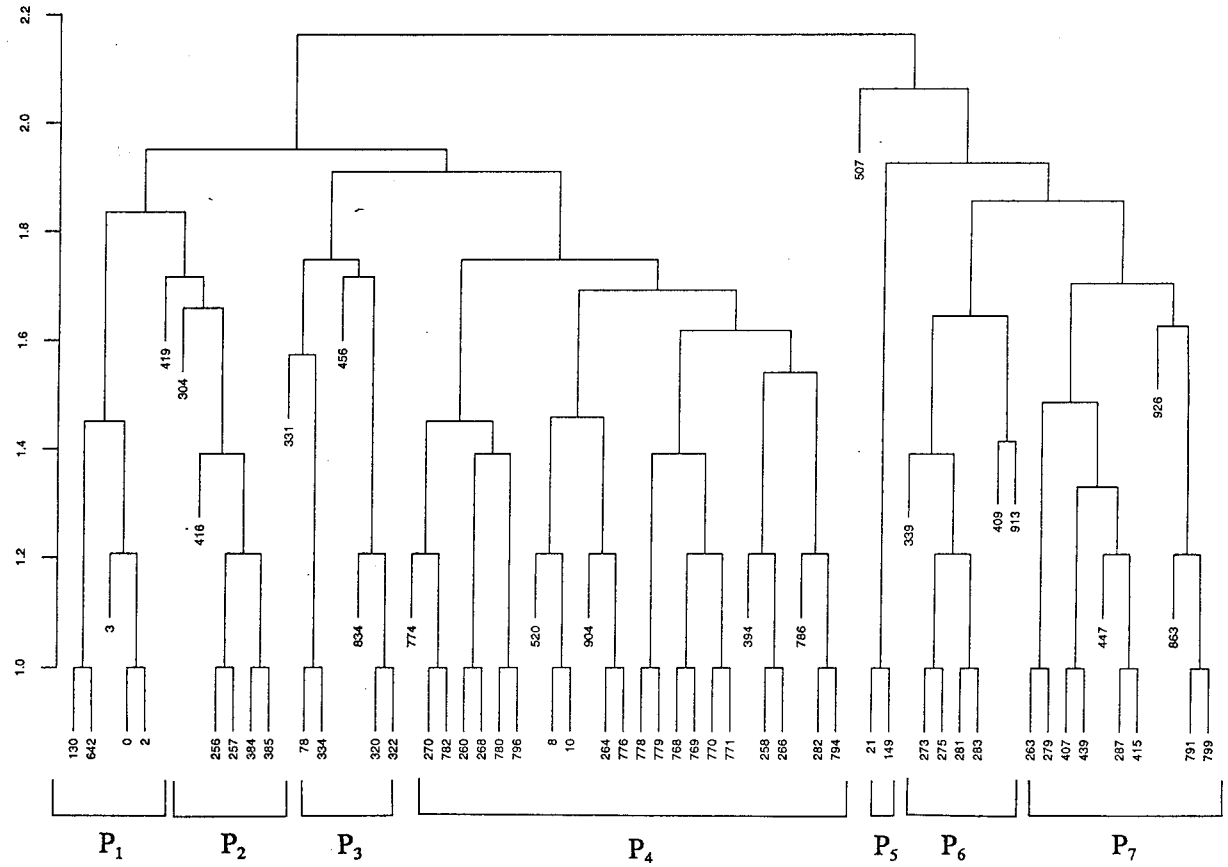

FIG. 2. Dendrogram of antibiotic resistance profiles for PS *E. coli* isolates determined by Euclidean metric, average-linkage analysis. Clusters were defined at a Euclidean distance of 1.8. Numbers on the dendrogram represent antibiotic resistance patterns, not strain numbers.

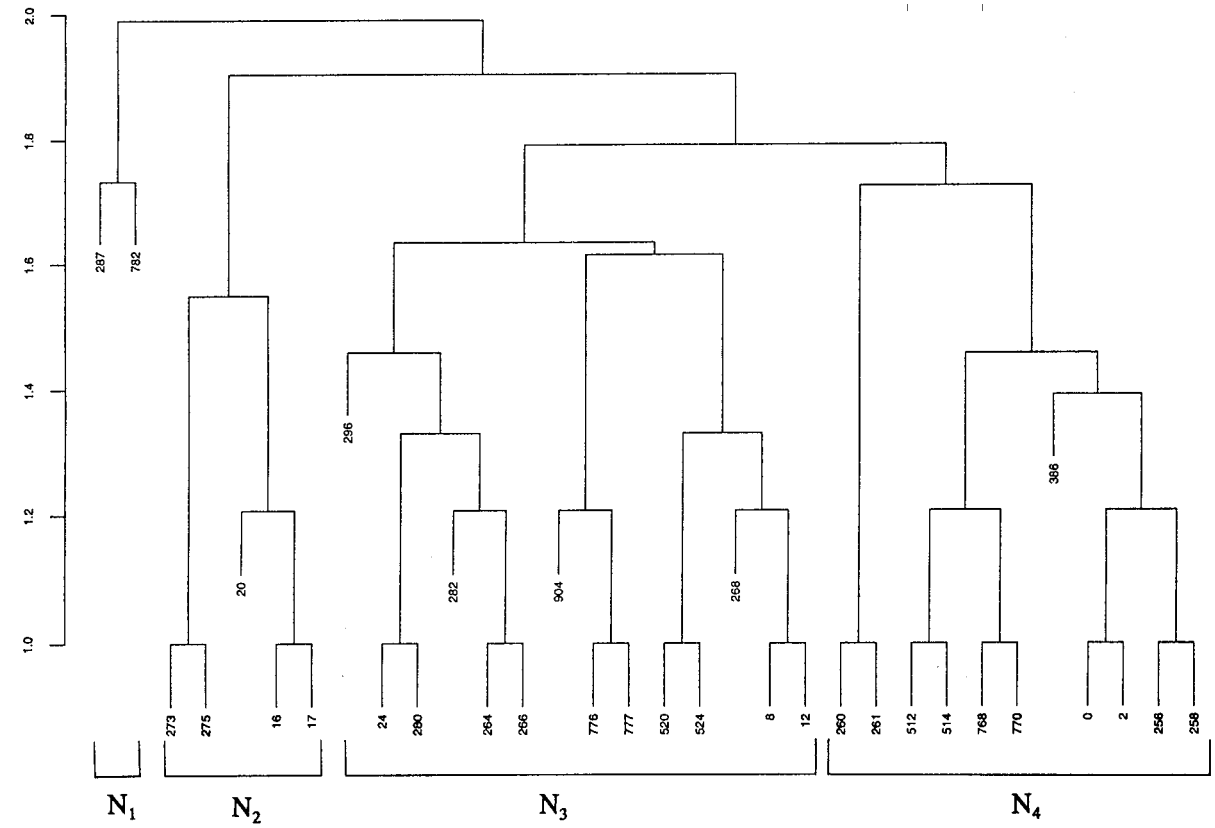

FIG. 3. Dendrogram of antibiotic resistance profiles for NPS *E. coli* isolates determined by Euclidean metric, average-linkage analysis. Clusters were defined at a Euclidean distance of 1.8. Numbers on the dendrogram represent antibiotic resistance patterns, not strain numbers.

TABLE 3. Numbers of PS and NPS strains within individual clusters

Cluster	No. of strains
P_1	34
P_2	42
P_3	9
P_4	291
P_5	2
P_6	7
P_7	21
N_1	2
N_2	5
N_3	133
N_4	218

isolates. In contrast, 72.4% of animal isolates were sensitive to all antibiotics (Table 2).

DISCUSSION

These results indicate that the antibiotic resistance profile of *E. coli* is associated with its source of pollution in the ANERR. The unique foci of NPS isolates observed by covariance matrix analysis (Fig. 4) show that nonhuman sources of *E. coli* may represent clonal forms originating from wildlife in the protected uplands and wetlands of ANERR. This suggestion is supported by independent studies of the MAR profiles of *E. coli* obtained directly from animal and human feces. We suggest that further research is needed to associate specific clusters with specific animal species. In contrast, MAR profiles of PS isolates showed wide diversity likely related to widespread use of antibiotics in human and domestic animal populations.

The level of antibiotic resistance observed in this study among all PS and NPS isolates (82%) is similar to that of a previous report for urban and rural waters by Kaspar et al. (22). They found 90% of all isolates were resistant to one or more antibiotics. In another study, investigators showed that 80% of strains from municipal waste and river and estuarine water displayed antibiotic resistance (32). Much lower resistance, ranging from 31 to 72%, has been reported for *E. coli* isolates from various aquatic environments (9, 10, 11, 16, 20).

The occurrence of antibiotic resistance among PS *E. coli* isolates is probably due to widespread use of chemotherapeutic drugs and may reflect the occurrence of plasmid transfer in the alimentary tracts of humans and in the microbial milieu of sewage systems (13, 16, 17, 34). Several studies have shown

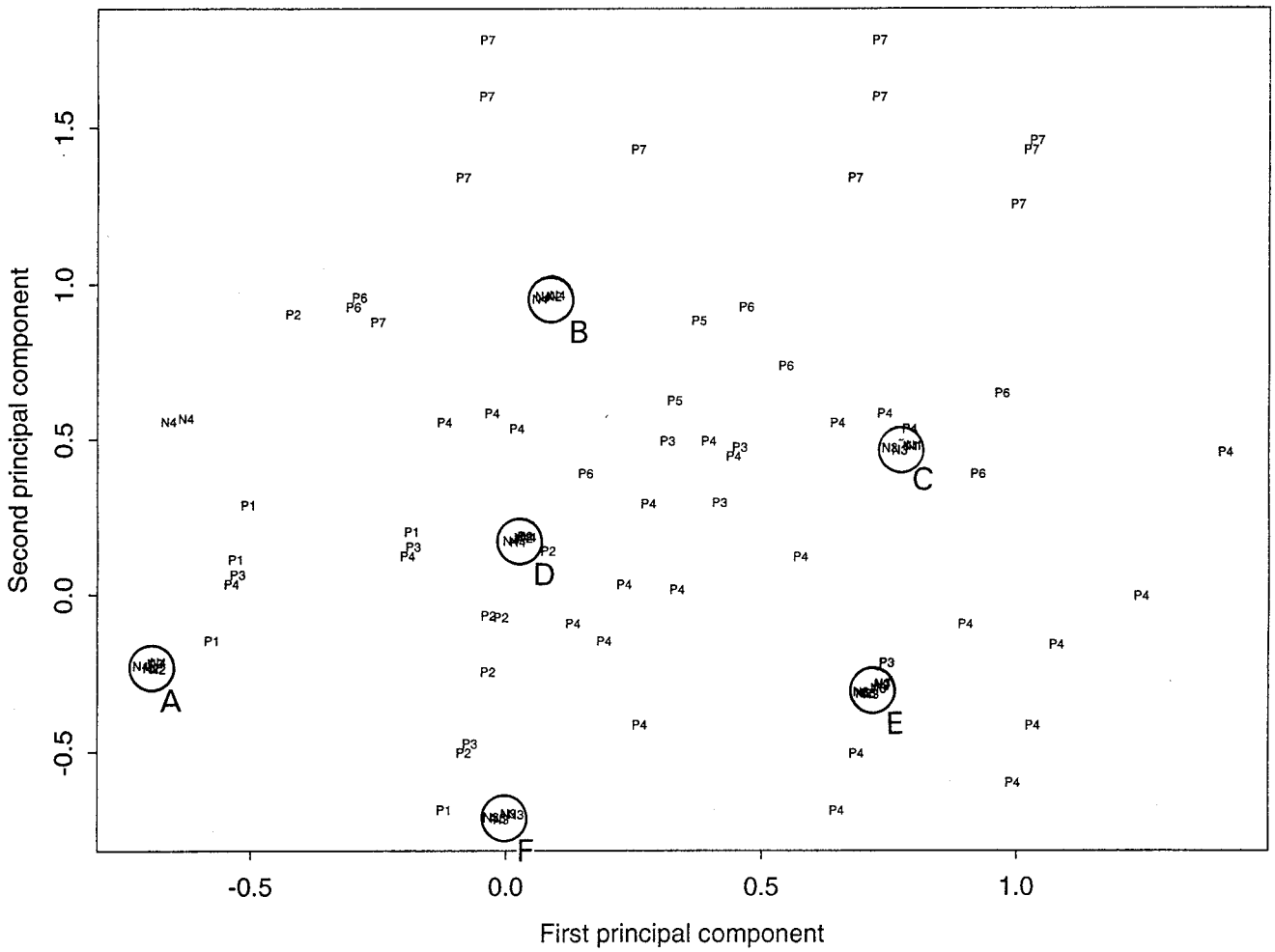

FIG. 4. Two-dimensional plot of antibiotic resistance patterns by principal-component analysis. Circles mark focal groups A to F. The specific clusters and associated numbers of isolates by group are as follows: 117 N_4 and 3 N_2 isolates in group A, 59 N_4 isolates and 1 N_2 isolate in group B, 2 N_1 and 7 N_3 isolates in group C, 36 N_4 isolates and 1 N_2 isolate in group D, 113 N_3 isolates in group E, and 13 N_3 isolates in group F.

that plasmid exchange readily occurs between *E. coli* and other coliform bacteria in stagnant areas of wastewater systems (17, 18).

Reported ampicillin resistance among *E. coli* isolates varies from 5.6% in spring water (11) to 54% in sewage and shellfish (10). In our study, 12.5% of PS and 4.5% of NPS isolates were resistant to ampicillin. One possible explanation for wide variation in ampicillin resistance among reported studies may be due to the composition of bacterial species in different environments and the exchange of R factors. For example, the species composition of a sample has been shown to be influenced by the frequency of fecal input, type and proportion of input, and type of recipient water (10, 21). Furthermore, a high proportion of ampicillin-resistant *Klebsiella* spp. could transfer resistance factors to other members of the *Enterobacteriaceae* (4, 24, 27).

Human isolates showed wide variation in MAR profiles compared to animal isolates, corresponding to differences we observed between PS and NPS isolates. These data provide further evidence that human feces are likely a significant source of PS isolates in the ANERR and that animal feces may contribute to NPS *E. coli* pollution.

Finally, we propose that MAR profiles of *E. coli* may provide a useful tool for measuring and differentiating PS and NPS fecal pollution in the ANERR, thereby facilitating remediation efforts to enhance the quality of this ecosystem. We recognize that a specific *E. coli* MAR profile may not always correlate with PS and NPS pollution in all estuaries. In these instances, extensive databases may need to be formed to develop associations between specific MAR profiles and sources of pollution.

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