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Impact of Coal-Coking Effluent on Sediment Microbial Communities: a Multivariate Approach

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The functional response to and recovery from coal-coking waste effluent was evaluated for sediment microbial communities. Twenty estimates of microbial population density, biomass, and activity were measured five times during a 15-month period. Significant effects on microbial communities were observed in response to both wastewater contamination and diversion of the wastewater. Multivariate analysis of variance and discriminant analysis indicated that accurate differentiation between uncontaminated and contaminated sediments required a minimum of nine estimates of community response. Total viable population density, ATP, alkaline phosphatase, naphthalene, and phenanthrene mineralization rates were found to be highly weighted variables in site discrimination. Lipid and glucose mineralization, nitrogen fixation, and sediment protein also contributed significantly to explaining variation among sites. Estimates of anaerobic population densities and rates of methane production contributed little to discrimination among sites in the environment examined. In general, total viable population density, ATP, and alkaline phosphatase activity were significantly depressed in contaminated sediments. However, after removal of this contamination, the previously affected sites demonstrated greater temporal variability but a closer approximation of the mean response at the control site. Naphthalene and phenanthrene mineralization did not follow the general trend and were elevated at the contaminated sites throughout the investigation. Results of the investigation supported the hypothesis that multiple functional measures of microbial community response are required to evaluate the effect of and recovery from environmental contamination. In addition, when long-term effects are evaluated, select physiological traits, i.e., polyaromatic hydrocarbon mineralization, may not reflect population and biomass estimates of community response.

Developments in energy technology in the areas of coal conversion and gasification may have major impacts on aquatic ecosystems, due to the release of potentially toxic, mutagenic, or carcinogenic contaminants that pose a threat to ecosystem function as well as human health. Coal-coking wastewater has a composition similar to coal-conversion wastewater and may serve as a model for future coal-conversion-derived wastewater (22). A recent report has demonstrated that chronic contamination of a stream receiving coal-coking wastewater results in elevated polyaromatic hydrocarbon (PAH) biotransformation rates (8). It was also noted that elimination of the source of contamination did not result in the immediate reduction in PAH biotransformation rates, suggesting that the microbial community responds slowly to variations in PAH concentration. However, our preliminary studies, performed concurrently on the same stream, indicate rapid changes in response to the presence or absence of the coal-coking wastewater (24).

The goal of this investigation was to describe the response of a natural microbial community to coal-coking wastewater contamination to predict microbial community responses to coal-conversion contaminant-induced ecosystem perturbations. A specific objective of this investigation was to determine whether coal-coking wastewater caused a permanent alteration in the microbial community's ability to respond to temporal changes in the environment and to evaluate that response with respect to PAH biotransformation rates. An equally important objective was to determine those microbio-

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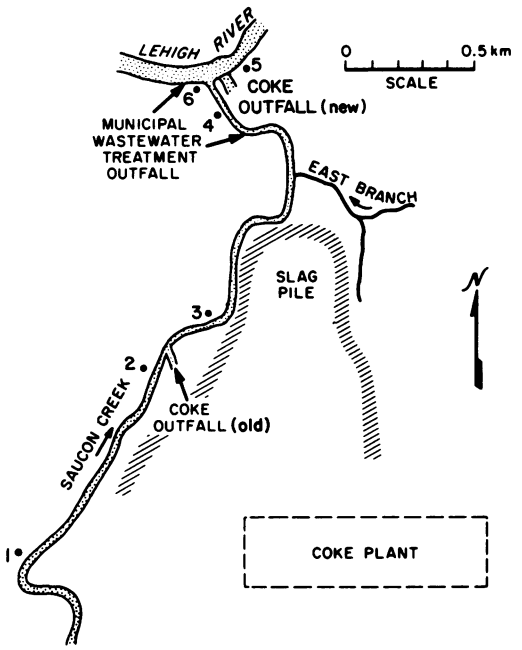

FIG. 1. Field site locations on Saucon Creek and the Lehigh River, Bethlehem, Pa.

logical variables that provide the greatest information in detecting changes or responses within the microbial community of a contaminated environment.

A testable hypothesis of this investigation is that, without prior knowledge of the specific effect of a perturbation, no one or a limited number of microbiological variables can be used as predictors of community change or response. A second testable hypothesis is that after chronic contamination the removal of that contamination represents a perturbation to a previously acclimated microbial community.

MATERIALS AND METHODS

Field sampling. Sediment samples were collected from the Lehigh River and Saucon Creek at Bethlehem, Pa., during 18 to 22 July and 13 December 1978 and 20 March, 19 June, and 27 September 1979 (Fig. 1). Random triplicate subsamples were collected along a transect at each of four locations on Saucon Creek, whereas duplicate subsamples were collected from each of two sites on the Lehigh River on each sampling date. Before the July 1978 sampling, a Bethlehem Steel Co. coking plant waste effluent discharge, which had previously contaminated Saucon Creek in the proximity of site 3, was temporarily diverted upstream to a point designated site 2. After completion of diversion construction (August 1978), the coking effluent discharge was relocated to a point immediately below the confluence of the Lehigh River and Saucon Creek (see Fig. 1), and stream bank reclamation efforts destroyed site 2. In December 1978, site 6 (50 m above the confluence) was added to the sampling schedule to serve as a control site for the newly contaminated site 5, which was immediately downstream from the relocated discharge.

Saucon Creek sediments were composed of large rock, coarse gravel, and sand. Consequently, sediments samples were collected by scraping the bottom substrate with polyethylene beakers or polycarbonate cylinders. All samples were double bagged in sterile Whirl Pak bags (Fisher Scientific Co., Norcross, Ga.). Lehigh River samples were collected with the aid of a petite Ponar grab (Wildlife Supply Co., Saginaw, Mich.) and were treated in the same manner as Saucon Creek samples. Samples collected during July 1978 were processed on site; all other samples were collected and flown immediately to the University of Tennessee, Knoxville, for sample processing. In all cases the sample processing time, from the point of sample collection to completion, ranged from 15 to 18 h.

Physical-chemical characteristics of sediment samples. Physical-chemical parameters assessed during sampling included water and air temperature, dissolved oxygen, conductivity, pH, and Eh. Methods for these determinations have been previously described (18, 23). Lehigh River water flow data for the week preceding sampling were provided by the U.S. Geological Survey. Sediment dry weight and organic matter content determinations have been previously described (18). Dissolved organic carbon of the sediment pore water was determined by direct combustion and infrared CO_2 analyses in a Beckman carbon analyzer.

Sediment nitrate levels were determined by extracting the sediments in 1.0% acetic acid and assessing nitrate levels by a nitrate-specific ion probe and selective ion meter (Orion 407A). Phosphate levels were determined by the ascorbic acid-ammonium molybdate-potassium tartrate method (28) after extraction in dilute acetic acid.

Assessment of microbial biomass and population densities. Sediment ATP-biomass determinations, using the cold sulfuric acid extraction technique (12), have been previously described (24). Sediment protein was determined by ultrasonic treatment of a 1.0-ml sample of sediment in phosphate-buffered saline on ice for 1 min. The sonicated sample was mixed with 5.0 ml of *para*-Coumassie protein-binding dye (BioRad Laboratories, Richmond, Calif.) and incubated for 30 min at 37°C. The dye-sample mixture was centrifuged for 10 min at 5,000 × *g* in a bench top centrifuge. The absorbance of the supernatant was measured at 595 nm on a Bausch & Lomb Spectronic 70 spectrophotometer. Absorbance measurements were converted to micrograms of protein per milliliter of reaction mixture by comparison to a standard curve prepared with bovine serum albumin (Sigma Chemical Co., St. Louis, Mo.). Recovery of bovine serum albumin from sterile acid-washed sand was 93.2 + 3.5%.

Total viable heterotrophic bacteria (TVC) and phenanthrene-resistant bacteria were determined by spread plate inoculation of yeast extract-peptone-glucose agar and phenanthrene agar, respectively (23). Protein, lipid, and starch hydrolytic microorganisms were determined by either spread plate inoculation or replica plating of colonies from TVC enumeration onto protein, lipid, or starch agars, respectively. Protein and starch agars were prepared by incorporating 5 or 1% (vol/vol) skim milk or soluble starch (Fisher Scientific

Co., Pittsburgh, Pa.) in yeast extract-peptone-glucose agar. Lipid agar was formulated with 1% Tween 20 (Fisher) in a basal medium containing the following: peptone (Difco Laboratories, Detroit, Mich.), 10 g; NaCl 5 g; $CaCl_2 \cdot 2H_2O$, 0.1 g; per liter of distilled water. The Tween and basal media were autoclaved separately and mixed; the final pH was adjusted to 7.4.

Nitrifying bacterial populations were enumerated by the most-probable-number dilution extinction technique, using a 5 dilution–10 replication most-probable-number scheme as previously described (25). Total anaerobic bacterial populations were assessed by inoculating prereduced yeast extract-peptone-glucose medium in either anaerobic roll tubes, gassed with a um which was incubated in anaerobic Gas Pak jars (BBL Microbiology Systems) (18). Methanogenic anaerobes were assessed by using a gas sampling tube modification of the most-probable-number technique (18).

Assessment of microbial activity. Alkaline phosphatase activity was determined by the sediment sonification–*p*-nitrophenyl phosphate hydrolysis technique (24). Glucose, lipid, starch, protein, phenanthrene, and naphthalene mineralization assays were performed in an identical experimental format. A 5-ml, 20% (wt/vol) sediment slurry mixture was placed in reaction vessels containing a center well and filter paper CO_2 absorber. Each reaction vessel was supplemented with one of the desired radioactive substrates at the appropriate concentration: [9-^{14}C]phenanthrene, 1 μg ml^{-1}, 11.3 mCi mmol^{-1} (Amersham Corp., Arlington Heights, Ill.), and [1,4,5,8-^{14}C]naphthalene, 5 mCi mmol^{-1} (Amersham), 1.18 to 0.5 μg ml^{-1}; [*U*-^{14}C]glucose, 304 mCi mmol^{-1} or 4.86 mCi mmol^{-1}; denatured algal protein, 56 mCi matom of carbon^{-1} (Amersham); and [*U*-^{14}C]oleic acid, 0.0036 μg ml^{-1}, 789 mCi mmol^{-1}; and *U*-^{14}C-labeled starch, 0.01 μg ml^{-1}, 39 μCi mg^{-1} (Amersham). After addition of the substrate, the reaction mixtures were incubated at room temperature for variable time intervals. The reactions were stopped by the addition of 0.5 ml of 2 N H_2SO_4, final pH 2.5, which also liberated dissolved inorganic carbon. KOH (0.2 N), 0.4 ml, was injected via syringe into the center wells to absorb $^{14}CO_2$. The reaction vessels were gently shaken for a minimum of 2 h to allow for complete absorption of the $^{14}CO_2$. The center wells were then removed and placed in 0.8% Omnifluor (New England Nuclear, Boston, Mass.) in dioxane liquid scintillation cocktail. Counting efficiencies ranged from 85 to 95%, as determined by channels ratios. Control samples were established by acidifying samples before the addition of the labeled substrate. All assays were performed in triplicate.

Rates of methanogenesis in sediments were determined by Miller and Wolin's (14) serum bottle modification of the Hungate technique (10) as previously described (18). Nitrogen fixation rates were determined by the acetylene reduction technique (26). A 5- to 15-g sediment subsample was placed in 60-ml serum bottles. The headspace gas was replaced with a mixture of argon (79.36%)-O_2 (20.6%)-CO_2 (0.04%) at atmospheric pressure, sealed with butyl rubber serum stoppers, supplemented with known quantities of acetylene, and incubated; ethylene detection and quantitation were determined by gas chromatography.

Dehydrogenase activity was assessed by the triphenyl tetrazolium chloride reduction assay (13). Sediment slurries, 0.5 to 1.0 ml in phosphate-buffered saline, were sonicated for 30 s, flushed with pure N_2 gas, and incubated in the presence of triphenyl tetrazolium chloride (30 mg ml^{-1}; Sigma) at 37°C. The reaction was terminated, and the reduction product, triphenyl formazon was extracted by the addition of 5 ml of 95% ethanol. Triphenyl formazan was quantitated by determination of the absorbance of the extracted sample at 595 nm and comparison with a standard curve.

For the purpose of this report the following notations and units are used for measurements of microbial population densities, biomass, and activity: (i) TVC—total viable counts (aerobic heterotrophs), $\log_{10}$ gram^{-1}, dry weight; (ii) PHE—phenanthrene-degradative/resistant bacteria, $\log_{10}$ gram^{-1}, dry weight; (iii) METH—methanogen most-probable-number estimates, $\log_{10}$ gram^{-1}, dry weight; (iv) TANAE—total anaerobes, $\log_{10}$ gram^{-1}, dry weight; (v) NITMPN—nitrifier most probable number gram^{-1}, dry weight; (vi) PROTHY—protein hydrolytic microorganisms, $\log_{10}$ gram^{-1}, dry weight; (vii) STARCH—starch hydrolytic microorganisms, $\log_{10}$ gram^{-1}, dry weight; (viii) LIPHYD—lipid hydrolytic microorganisms, $\log_{10}$ gram^{-1}, dry weight; (ix) ATP, ATP biomass—$\log_{10}$ femtograms gram^{-1}, dry weight; (x) PHOS—phosphatase activity, nanomoles of P_i released gram^{-1}, dry weight, hour^{-1}; (xi) DEHYD—dehydrogenase activity, nanomoles of triphenyl formazan produced gram^{-1}, dry weight, hour^{-1}; (xii) PROTE—total protein, micrograms gram^{-1}, dry weight; (xiii) GLUMIN—glucose mineralization, micrograms respired gram^{-1}, dry weight, hour^{-1} micromoles added^{-1}; (xiv) STAR—starch mineralization, milligrams respired gram^{-1}, dry weight, hour^{-1}; (xv) PROTMN—protein mineralization, picograms respired gram^{-1}, dry weight, hour^{-1}; (xvi) LIPMIN—lipid mineralization, picograms respired gram^{-1}, dry weight, hour^{-1}; (xvii) PHEMIN—phenanthrene mineralization, picograms respired gram^{-1}, dry weight, hour^{-1}; (xviii) NAPMIN—naphthalene mineraliza-

TABLE 1. Microbiological variables contributing to discrimination between control and contaminated Saucon Creek sampling sites

Discriminating variable[a]	Discriminant function coefficient (*Z*)[b]	
	Function 1	Function 2
1. LIPHYD	0.25	−0.55
2. LIPMIN	0.48	0.64
3. NAPMIN	0.09	−1.00
4. GLUMIN	−0.42	−0.13
5. TVC	0.45	0.61
6. PHOS	−0.42	0.27
7. PHEMIN	−0.65	0.15
8. PHE	−0.42	−0.21
9. METH	−0.28	−0.16

[a] Order of entry into stepwise discriminant analysis. Variables not in the analysis did not contribute to discrimination. See text for definitions.

[b] Standardized coefficient. Those variables approaching limits of ±1.0 contribute most to discrimination on an individual discriminant function.

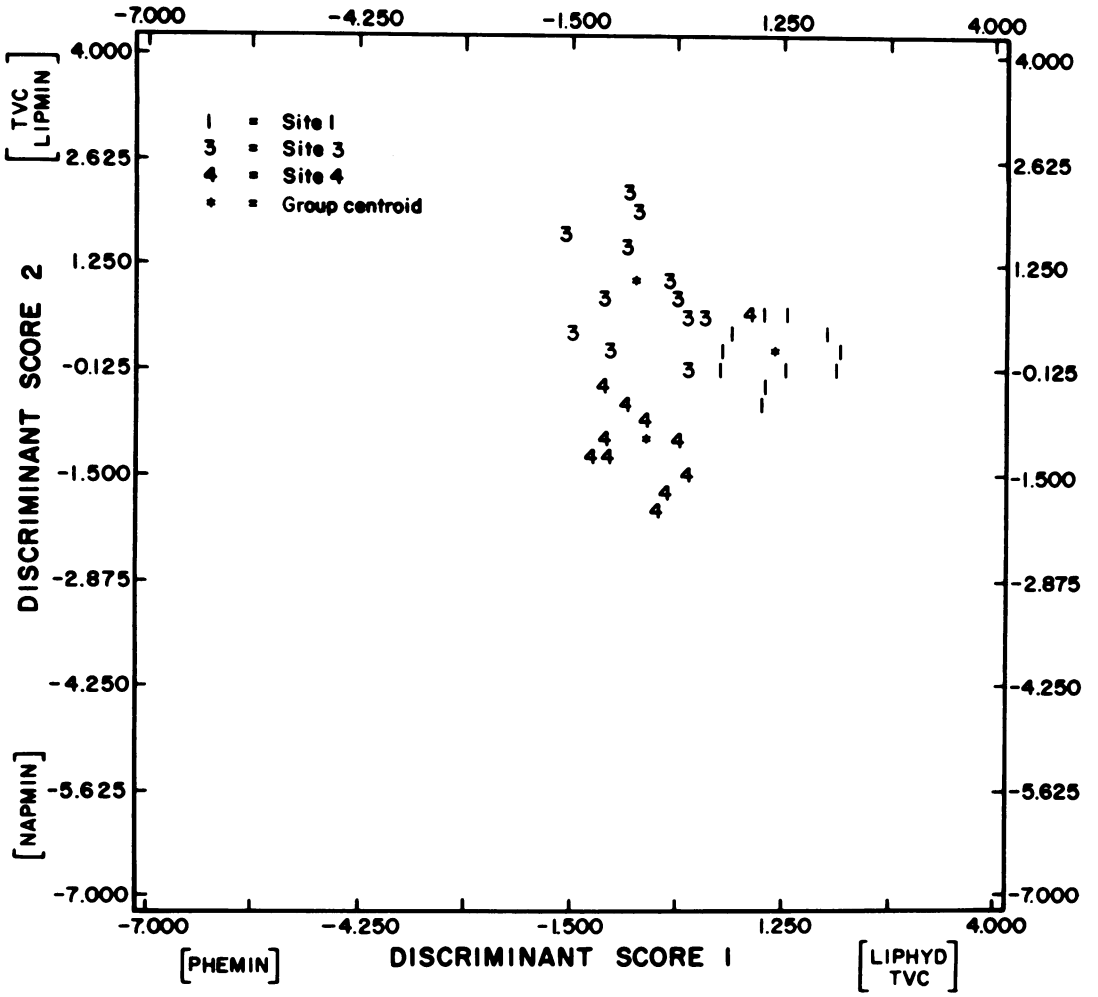

FIG. 2. Discrimination between control (site 1) and contaminated (sites 3 and 4) Saucon Creek sample sites. (See Table 1 for discriminating variables; brackets indicate those microbiological variables providing the greatest information in defining an individual discriminant axis [definitions in text]; Site centroid [*] orientation on a given discriminant axis indicates those discriminating variables contributing to site separation.)

tion, picograms respired gram^{-1}, dry weight, hour^{-1}; (xix) N_2—nitrogen fixation rate (acetylene reduction), nanomoles gram^{-1}, dry weight, hour^{-1}; (xx) METHRT—methanogenesis rate, nanomoles gram^{-1}, dry weight, day^{-1}.

Experimental design and data analysis. Due to the nature of the objectives to be tested, a multivariate experimental design and statistical analysis were used to evaluate the changing relationships among microbiological variables as a function of time, space, and environmental perturbation (3, 6). The relationships among the variables examined describe population function of the original sample and can be used to classify the community and detect differences or changes in communities.

Direct effects of the coking effluent were evaluated by examining changes in relationships of microbiological variables in spatially segregated, contaminated and uncontaminated sites. Such a design was needed due to the lack of preimpact data (6). However, samples were collected before wastewater diversion; consequently, diversion effects were detected by examining changes in both temporal and spatial relationships within the microbial communities examined.

The potential effects of the coking effluent and effluent diversion on the microbial community were unknown. Consequently, variables estimating population densities, biomass, and activity were chosen as potential discriminating variables. The relationships among these variables were examined by using discriminant analysis as the primary statistical tool to distinguish between sites before and after diversion of the coking effluent. The discriminant analysis procedure used in this study consists of four basic components: (i) a two-way, fixed-model, multiple analysis of variance to determine whether there were significant differences between the sites; (ii) a stepwise selection of discriminating variables contributing to maximum

separation between sites; (iii) an analysis to determine whether the created discriminant functions significantly explained variation between the sites; and (iv) a classification of the sites, using the discriminating variables to predict the probability of discrimination between sites. These four components lead to discriminant space analysis of the relationships among microbial communities during ecosystem perturbation.

Logarithmic raw data transformations were made before statistical analysis. The data were analyzed by correlation analysis, factor analysis, and discriminant analysis, using computer programs available in the Statistical Package for the Social Sciences (15). Multivariate analysis of variance (MANOVA) and Duncan's multiple range analysis were performed with the Statistical Analysis System (2) general linear model procedures. A multiple correlation matrix, using pairwise deletion of missing variables, was used as input data for factor analysis. MANOVA and discriminant procedures used listwise deletion of missing variables, using the standardized transformed raw data. Data analysis was performed with the University of Tennessee central computer facilities and used DEC-10/IBM 370 computers. Computational methods and tests of significance are described by Pimentel (19), Tatsuoka (27), and Green (6). All statistical tests were conducted at $P \geq 0.95$.

RESULTS

The raw data are stored in a DEC-10 systems file and are available on request in a punched card 80 column format or correlation matrix. Physical-chemical variables examined for Saucon Creek and Lehigh River samples are characteristic of many lotic environments. On a seasonal basis the sites are relatively similar for most physical-chemical variables examined. Sites 4 and 5 demonstrated a generally more reducing environment (lower Eh) which is most likely the result of deposition of a higher proportion of smaller sediment substrate, ≤ 420 μm. Nitrate and phosphate levels were marginally above the analytical limits of detection and may suggest possible nutrient limitation.

Independently, microbiological variables describing the microbial communities of the Lehigh River and Saucon Creek sediment and Aufwuch samples demonstrated no uniform trends, indicating either effluent or diversion effects. Mineralization rates of organic substrates were generally higher below the coking outfall, yet no uniform trend among all variables was evident seasonally or after diversion of the outfall.

Results of a preliminary MANOVA indicated significant variability between sampling dates and sampling sites and a significant date-site interaction (Wilks criterion, λ; approximation of the F statistic: $F_{24, 32}$, 32.01; $F_{16, 22}$, 8.90; and $F_{48, 58}$, 4.86, respectively). Since there was significant interaction, these results indicate changing relationships among variables for each site during the course of the field investigation. Consequently, statements concerning difference between sites or sampling date alone cannot be made, and discriminant analysis was used to elucidate the interaction effect.

Stepwise discriminant analysis was used to determine which variables could be used to discriminate between sites (a detailed treatment of the method and interpretation is given by Pimentel [19]). Nine of the 20 variables examined were found to significantly contribute to site discrimination (Table 1). The order of entry of the variables into the discriminant analysis is controlled by minimization of Wilks lambda, a test criterion which bases entry level for each variable in the analysis on maximizing the multivariate F statistic. Consequently, those variables entered first explain a greater proportion of the overall variation and provide the greatest discrimination power. Entry level into the analysis is also associated with the absolute value of the standardized discriminant function coefficient, Z (which ranges from +1.0 to −1.0), or the relative weighting of each independent variable on the discriminant functions that describe the linear relationship of the variables contributing to discrimination between sites. Those variables with the highest absolute Z provide the greatest information in defining an individual discriminant function. The two discriminant functions generated by this analysis accounted for 62% of the total between site variation. A graphic representation of site discrimination was obtained by plotting the individual site centroids (vectors derived in part from the means of each independent variable examined for the site) in relation to the two discriminant functions (Fig. 2). Site 1 was differentiated from sites 3 and 4 by its relative position on discriminant function 1 (horizontal axis) which is defined by LIPHYD and TVC ($Z = 0.48$ and 0.45) and PHEMIN ($Z = -0.65$). Figure 2 also indicates discrimination of sites 3 and 4 by their relative positions on discriminant function 2 (vertical axis), defined by TVC and LIPMIN ($Z = 0.64$ and 0.61) and NAPMIN ($Z = -1.00$) and indicating higher NAPMIN at site 4. By using all discriminating variables, the individual samples could be reclassified to the site from which they were obtained with 94.4% accuracy. The results of this analysis are interpreted as a direct effluent toxicity resulting in depressed TVC and LIPHYD population densities at the contaminated sites. The contaminated sites are also characterized by evaluated rates of phenanthrene mineralization as a result of enzyme induction or selection for biodegradative populations by specific components of the waste effluent. The contaminated sites (3 and 4) can be distinguished one from another relative to their orientation to

TABLE 2. Microbiological variables contributing to discrimination between control and contaminated Saucon Creek sampling sites, before and after diversion of a coal-coking wastewater

Discriminating variable[a]	Discriminant function coefficient (Z)[b]		
	Function 1	Function 2	Function 3
1. PROTE	0.72	−0.25	−0.82
2. ATP	−0.25	0.53	−0.67
3. NAPMIN	−0.28	−0.49	−0.56
4. LIPHYD	−0.09	−0.27	0.13
5. GLUMIN	0.03	−0.02	0.23
6. LIPMIN	0.05	−0.17	0.13
7. METHRT	−0.05	−0.23	−0.03
8. PHEMIN	0.01	−0.10	0.03
9. TVC	0.13	−0.10	−0.17
10. STARCH	0.23	0.06	−0.20
11. DEHYD	0.19	−0.03	0.05
12. PROTHY	−0.15	0.02	0.01
13. PHE	0.00	0.15	0.12

[a] Order of entry into stepwise discriminant analysis. Variables not in the analysis did not contribute to discrimination. See text for definitions.

[b] Standardized coefficient. Those variables approaching limits of ±1.0 contribute most to discrimination on an individual discriminant function.

discriminant function 2, which indicates that during the course of the investigation site 4 maintained higher rates of naphthalene mineralization and may indicate accumulation of PAH at the confluence of Saucon Creek and Lehigh River.

To evaluate the effects of diverting the coking effluent, a second stepwise discriminant analysis was performed to detect differences between sites on a temporal and spatial basis (after diversion). For this analysis each individual site within a given sampling period was treated as a separate dependent variable. Thirteen of the 20 microbiological variables examined were found to be significant in site discrimination (Table 2). The discriminating variables resulted in ≥92% accurate reclassification of individual samples. The first three discriminant functions derived accounted for 78.4% (34.5, 27.3, and 16.6%, respectively) of the total variation between the three sites among the four sampling periods and provide adequate discrimination ability to detect changing relationships among the sites before and after diversion of the effluent (Fig. 3). Before diversion, control site 1 could be distinguished from impacted sites 3 and 4 relative to discriminant function 1 defined by sediment protein levels (Z = 0.72) and NAPMIN and ATP (Z = −0.28, and −0.25). After diversion (sampling periods 2, 3, and 4), primary discrimination between sites was based on relative relationships to discriminant function 2, defined by the relative weights of ATP and NAPMIN (Z = 0.53 and −0.49, respectively). However, during the third sampling period, site 4 was clearly differentiated from sites 1 and 3 by its position relative to discriminant functions 1 and 2. All sites within a given sampling period demonstrated a similar relative position on discriminant function 3, defined by GLUMIN (Z = 0.23) and PROTE, ATP, and NAPMIN (Z = −0.82, −0.66, and −0.55, respectively). The differences among site centroids on the various sampling periods, relative to discriminant function 3, are an indication of seasonal variation among sites, not attributed to effluent impact. The results of this analysis indicate that the primary effects of the coking effluent and its diversion are manifested in sediment microbial biomass levels and mineralization of organic substrates. PROTE and ATP, both biomass variables, are significantly depressed below the point source discharge of the coking effluent, and these effects have been previously reported in greater detail (24). Naphthalene mineralization is a highly weighted discriminating variable on all three discriminant axes and, as previously described (Fig. 2), is associated with evaluated naphthalene mineralization at contaminated sites 3 and 4 before and after diversion of the effluent.

The relative spread of individual cases around a site centroid is an indication of the variability of that site over the course of sampling and analysis. Variability within an individual site was less for site 1 than for sites 3 and 4 (Fig. 2). Site 3, which was located nearest the original treatment outfall, demonstrates greater variability than site 4. A similar pattern is observed in Fig. 3, which describes the relationships among sites during individual sampling periods. The relationships represented demonstrate two points. (i) In general, the relative variability between sites (distance between centroids) during one sampling period is maximum before diversion and decreases after diversion of the effluent. (ii) The variability of the control site (distance between site 1 group centroids) is less than either downstream site. In addition, during March and June (sampling periods 3 and 4) discrimination among the two contaminated sites and the control site, relative to discriminant axis 2, is due to significant differences in rates of naphthalene mineralization. This is the result of residual evaluated PAH mineralization activity in the contaminated sediments 1 year after diversion of the coking effluent.

Impact of effluent diversion on microbial activity of Lehigh River sediments. Due to difficulties in obtaining samples from site 6 on the Lehigh River, the impact of the diverted effluent on site 5 was evaluated by discriminant analysis among site 5 samples obtained before and after diversion of the effluent and their relationship to the

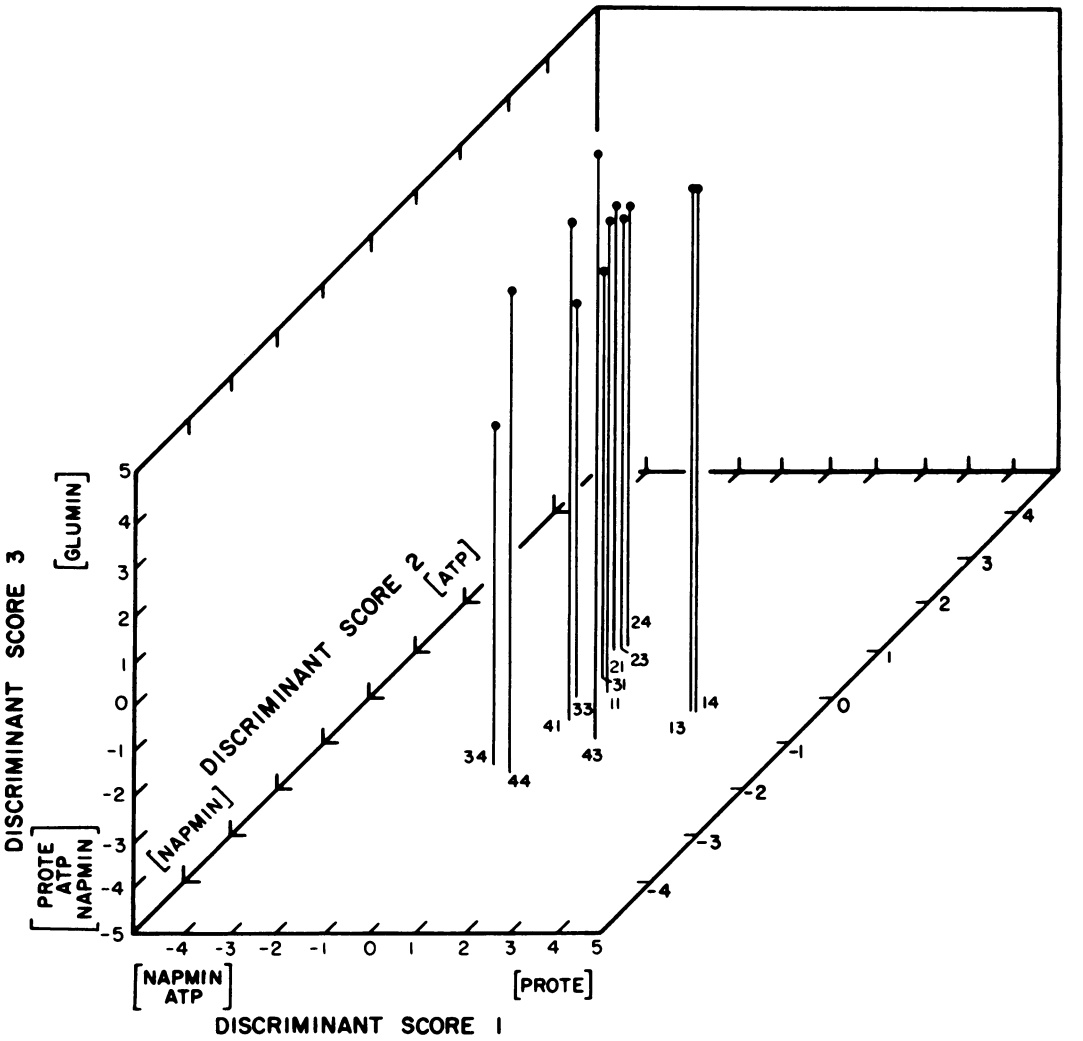

FIG. 3. Effect of coal-coking wastewater diversion on discrimination between control (site 1) and contaminated (sites 3 and 4) Saucon Creek sample sites. (See Table 2 for discriminating variables; ● = site centroid.) Sample code: first digit, sampling date; second digit, sampling site; sampling date 1, before diversion; dates 2, 3, and 4, after diversion (definitions in text).

microbial communities of Saucon Creek samples. Discrimination between Saucon Creek and Lehigh River samples required all microbiological variables except METH, TANAE, NITMPN, and STARH (Fig. 4). The analysis indicated significant difference between the Lehigh River (site 5) and all Saucon Creek samples in July 1978 (before diversion). During the first three sampling periods after diversion, the Lehigh River community response approximated that observed for the Saucon Creek communities. However, during the fifth sampling period, September 1979, microbial community responses at site 5 were significantly different from any of the previous site 5 or Saucon Creek samples examined. Before diversion, discrimination was accomplished relative to discriminant function 1 defined by PHOS and PROTHY (Z = 0.16 and 0.11) and N_2 and PROTE (Z = −0.49 and −0.47). In September 1979, discrimination was accomplished relative to discriminant function 2 defined by PROTE (Z = 0.16) and PHOS, NAPMIN, and N_2 (Z = −0.83, −0.33, and −0.29, respectively). The results of this analysis are interpreted as major differences in the aquatic environment and microbial community function in sediments from the Lehigh River compared with Saucon Creek. These differences, before diversion, are attributable to both the hydrological conditions of a shallow fast-flowing stream and a deeper slower-flowing river. This interpretation is made relative to a comparison of the

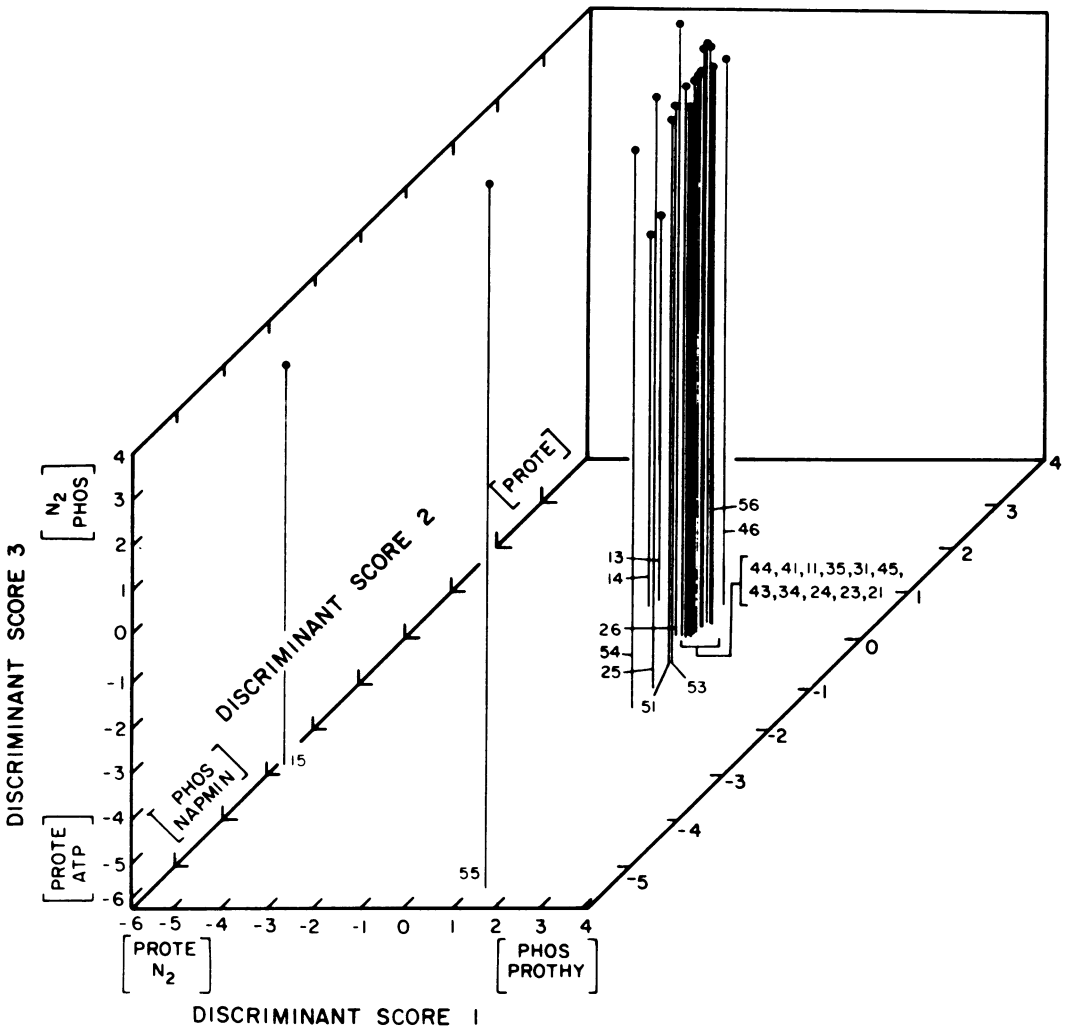

FIG. 4. Comparative discrimination between Lehigh River and Saucon Creek sample sites before and after diversion of coal-coking wastewater (sample code: first digit, sampling date; second digit, sampling site; ●, site centroid). Saucon Creek samples collected during sampling period 5 represent periphyton. Definitions are given in the text.

Saucon Creek control site and the Lehigh River site in July 1978 (site codes, 11 and 15, respectively; Fig. 4). After diversion, in December 1978, March 1979, and June 1979, the original functional community structure at site 5 was altered due to the direct impacts of the coking effluent and demonstrated community characteristics, similar to the previously contaminated Saucon Creek sites. In September 1979, Lehigh River site 5 appeared to reach a new equilibrium characterized by elevated rates of naphthalene mineralization.

Site 5 could also be distinguished from site 6 and the Saucon Creek sites relative to discriminant function 3, defined by N_2 and PHOS (Z = 1.1 and 0.36) and PROTE and ATP (Z = −0.9 and −0.23). The three discriminant functions and defining variables indicate that site 5 was characteristically higher in N_2, PHOS, and microbial biomass before diversion of the effluent, transiently similar to the Saucon Creek sites after diversion, and elevated in NAPMIN 1 year after relocation of the effluent above the site.

Relationships among discriminating variables. The lists of discriminating variables for spatial or temporal discrimination or both (Tables 1 and 2) between Saucon Creek communities, as well as between Lehigh River communities, demonstrate considerable overlap. Culturable methanogens were utilized only in site discrimination, whereas sediment ATP and protein, dehydrogenase activity, and starch and protein hydrolytic

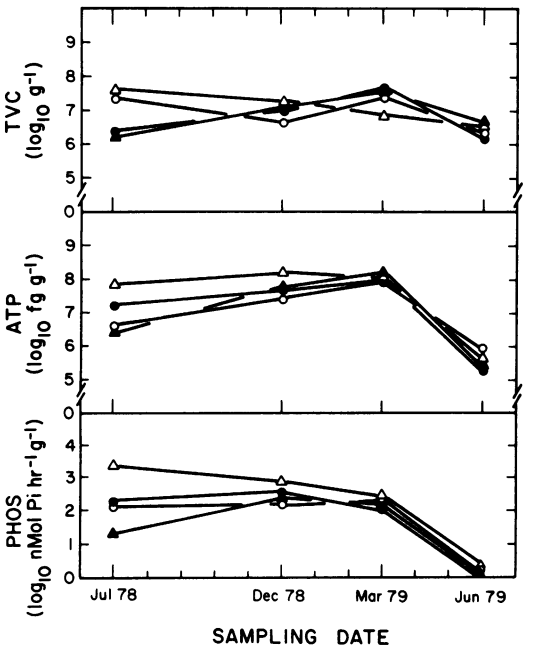

FIG. 5. Comparative relationships among TVC, ATP, and alkaline phosphatase (PHOS) in Saucon Creek and Lehigh River samples before and after coal-coking wastewater diversion. Diversion into the Lehigh River above site 5 (△) occurred in August 1978; ○, ▲, and ● indicate Saucon Creek sites 1, 3, and 4, respectively.

populations were variables used only in discrimination among sites during perturbation.

Discriminant analysis indicated that TVC, ATP, PHOS, PHEMIN, and NAPMIN were highly weighted in defining discriminant functions, resulting in optimal site discrimination. TVC significantly correlated with both ATP and PHOS (correlation coefficient [r] = 0.57 and 0.30, respectively) and ATP and PHOS were significantly correlated (r = 0.41).

These variables describe similar community responses among the samples examined. TVC density, ATP concentrations, and alkaline phosphatase activity significantly increased at impacted sites 3 and 4 after diversion of the effluent and reached a maximum in March 1979 (Fig. 5). Control site 1 demonstrated a similar trend for ATP; however, PHOS remained static and TVC density declined between July and December 1979. All three variables demonstrated a significant decline (one to two orders of magnitude) in all Saucon Creek samples in June 1979. Comparatively, during the initial sampling, site 5 (Lehigh River) demonstrated significantly higher levels of TVC, ATP, and PHOS than did Saucon Creek. Whereas ATP followed the same general trend, both TVC and PHOS at site 5 significantly decreased throughout the study period. The discriminating variables PHEMIN and NAPMIN also reflect this decrease in community response from the March to June 1979 sampling periods (Fig. 6). The observed decrease in mineralization rate constants (k) was less than an order of magnitude. Contaminated sites 3, 4, and 5 demonstrated significantly greater rate constants than did site 1 (except for PHEMIN, June 1979, site 3). In addition, site 4 demonstrated significantly greater NAPMIN and PHEMIN than did site 3 (except for PHEMIN, March 1979).

PHEMIN and NAPMIN were found to be significantly correlated (r = 0.60) and NAPMIN significantly correlated with TVC, ATP, and PHOS (r = 0.44, 0.49, and 0.46, respectively). However, PHEMIN was not correlated with TVC, ATP, or PHOS (r = 0.0, −0.08, and −0.02). In addition, PHE or the TVC/PHE ratio did not correlate with either NAPMIN or PHEMIN.

DISCUSSION

Wastewater perturbation of microbial communities can be evaluated by systematic analysis of the structural (taxonomic) or functional (population biomass and activity) relationships or both of temporally and spatially segregated samples. Specific information is seldom available on the biochemical effects of a waste effluent on individual populations within the community. Consequently, the success of both structural and functional analyses rests on the selection of criteria describing the response of the microbial community. Multivariate methods have been suggested to select criterion variables and analyze their relationships as environmental response measures of the microbial community (3, 9, 21). Relatively few attempts have been made to apply multivariate analyses to perturbation assessment; these have been limited to specific toxic agents or coal leachates and acid mine waters on limited community components (4, 7, 11, 17).

This investigation was an attempt to develop a systematic approach for identifying the effects of environmental contamination on the functional processes of natural microbial communities. A diversity of autotrophic and heterotrophic biochemical processes contributes to the functional equilibrium within any ecosystem. Consequently, the investigation was designed to provide a framework to detect those variables which respond to the presence or absence of the contamination. These variables would provide the greatest information on the fundamental effects of the contamination on the thermodynamic and biogeochemical roles of the microbial community within the ecosystem examined.

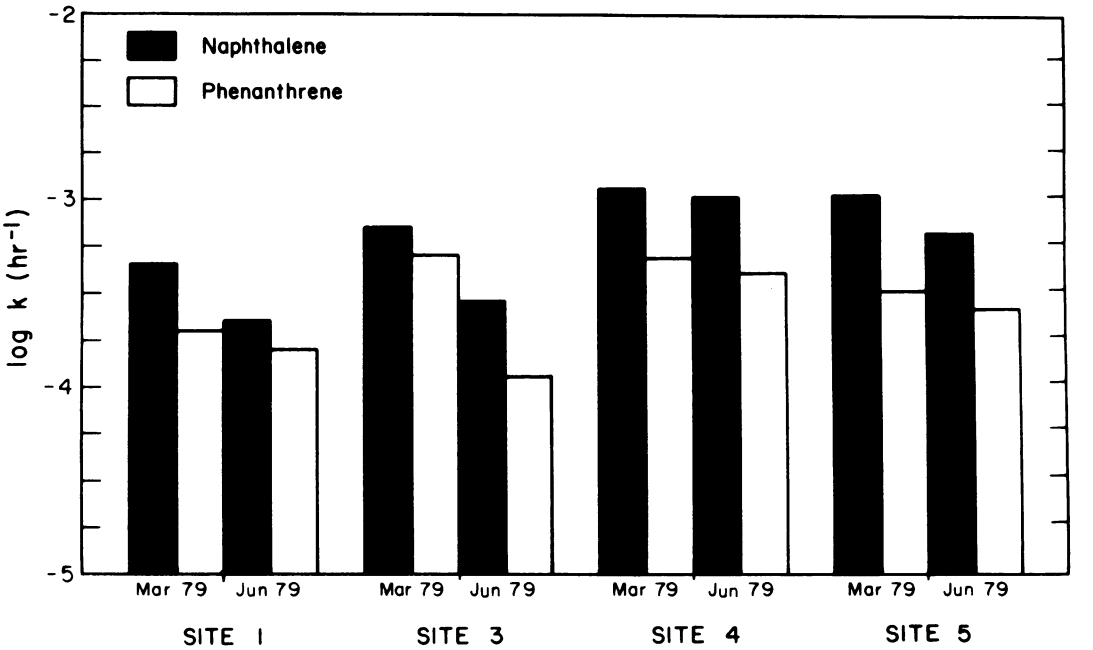

FIG. 6. Comparative naphthalene and phenanthrene mineralization rate constants (k) in uncontaminated and coal-coking wastewater-contaminated Saucon Creek and Lehigh River samples.

Two important results of this investigation are the demonstration of specific variables describing differences between microbial communities and the contribution of these variables in describing the microbial community response to environmental variations both natural (seasonal) and anthropogenic (perturbances). The results of this investigation support the hypothesis that multiple estimates of microbial community function are required to evaluate the effects of coal-coking wastewater perturbation of natural sediment communities. Those variables selected by discriminant analysis as being most informative in explanation of variation between sites (Tables 1 and 2) included TVC and ATP, both of which are commonly evaluated in many environmental and ecological investigations. However, these two variables along with either alkaline phosphatase, dehydrogenase and phenanthrene-degradative populations, or phenanthrene and naphthalene mineralization result in an approximately 50% predicative ability in reclassifying individual samples to the sites from which they were obtained. Comparatively, a ≥90% reclassification success is obtained when the entire list of discriminating variables is used. TVC and ATP estimates can be suggested as good discriminating variables for most communities examined. However, the choice of additional variables used in the multivariate approach is dependent on the environment to be examined. An example of this point is a comparison of those variables discriminating among Saucon Creek sites (Fig. 3) and those discriminating between Saucon Creek and Lehigh River sites (Fig. 4). Whereas naphthalene mineralization was found to represent a good index of comparison for Saucon Creek sites (Fig. 3), this variable was of moderate importance in comparing the sediment communities of the Lehigh River and Saucon Creek (Fig. 4). The results of this analysis were interpreted as general similarity in rates of PAH mineralization for the microbial communities of the two different contaminated aquatic environments (see Fig. 6). Phosphatase activity represented a better index in contrasting the community response of the two environments examined (Fig. 4). This was not an unexpected result in that preliminary data indicated significant depression of alkaline phosphatase activity by coking effluent contamination (24), and alkaline phosphatase activity in Lehigh River sediments was significantly inhibited by diversion of the effluent (Fig. 5). Results as described above demonstrate direct toxicity for a component functional activity of the microbial community with potential long-term effect on biogeochemical phosphorous cycling. However, PAH mineralization rates in Saucon Creek or Lehigh River sediments were little affected by effluent diversion, most likely as a result of long-term PAH enrichment in sediments of all contaminated

sites. Herbes previously demonstrated that microbial PAH biotransformation rates remain elevated in Saucon Creek 1 year after diversion of the coking effluent (8). Although a general decrease in naphthalene and phenanthrene mineralization was observed for all sites, this study confirms the trend that PAH biotransformation remains significantly elevated in the previously contaminated sites. These combined results suggest that in a dynamic environment the microbial community rapidly adjusts to new environmental regimes, yet specific physiological traits may be conserved within the community, i.e., PAH biotransformation potential. This may be a result of threshold levels of PAH in the sediment or genetic adaptation to PAH stress. These results can be important in predicting the ability of microbial communities to respond to and dampen similar ecosystem perturbations.

It is anticipated that discriminant analysis will be used as a tool to select variables that optimize evaluating microbial community response in a given environment. Correlation between variables estimating community response (ATP, TVC, and PHOS) were lower than previously reported (24) or reported by other investigators (5). In addition, correlation between PHE (or their proportion relative to TVC) and PHEMIN or NAPMIN was not observed (such correlations have been suggested as biological indicators of contamination [20]). This result is due in part to changing relationships among functional variables after effluent diversion and pooling data over time.

Ideally, factor analysis can be used to examine these relationships among discriminating variables at each sampling location and time to predict specific effects of the perturbation on the communities. This analysis could not be performed due to insufficient numbers of observations for each variable. Preliminary factor analysis did demonstrate a direct relationship between NAPMIN and TVC and ATP. However, PHEMIN appeared independent with respect to population density or biomass estimates.

Discriminant analysis of temporal variation demonstrated that there was significant seasonal variation among all sites; however, site 1 (control) varied less than any of the contaminated sites. Both sites (3 and 4) demonstrate a closer approximation of microbial community response at site 1 after diversion of the effluent. These results are interpreted as significant resiliency (ability to recover from environmental stress) within the microbial communities of the sites. However, since they demonstrate greater seasonal variability (Fig. 2 and 3) than at site 1, it can be interpreted that these communities are less stable (response to natural environmental variation). Such interpretations conform to the perturbation theory which suggests that community response variation, beyond a specific range observed for a natural community (in this instance the control site), is indicative of either a subsidy (stimulatory) or a stress (inhibitory) perturbation (16).

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