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Analysis of the Changes in Metabolic Diversity of Microbial Community in pH-gradient Microcosm

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The Biolog redox technology was carried out for evaluation of acidification effect on microbial communities at each stage of pH gradient microcosm. While the number of heterotrophic bacterial population and activities of extracellular enzyme decreased as the pH decreased, the number of total bacteria in the microcosm was not affected. The average color development of sample at each pH-gradient showed a sigmoidal curve, and at higher pH, more overall color development appeared in Biolog plates. Average color development value in Biolog plates was stabilized at 50 hours as an optimum incubation time. The color production in the Biolog plates was caused by cell density at above pH 5.0, but by cell activity below pH 4.0. Principal component analysis of color responses revealed distinctive patterns among the pH-gradient microcosm samples.

Key words: Biolog redox technology, acidification, microbial communities, microcosm

In order to explain the change of the microbial community structure in a specific environment, we used traditional media selection such as the count of colony forming units; CFUs, directed count by flurochrome dye acridine oranges; AODC (acridine orange direct count) and experiments to identify biochemical charateristics (2). Generally when it is seen that bacteria that is possibly incubated in its natural ecosystem is limited, characterization of the community becomes biased depending on the incubation conditions if the approach methods are based on the isolation of microbes. The development of metabolism-based, biochemical, and molecular techniques directly characterized the structure and function of microbial communities without the need of isolating and culturing individual species. Garland and Mills (7) investigated function based measure for classifying microbial communities on the basis of substrate utilization. This approach was capable of classifying a variety of microbial communities of such as soil, freshwater, and seawater samples (3, 7, 8, 9, 13, 26).

The problem of acid rain is a major concern of today's world. In Korea, damages in agriculture, forestry, and other areas in the ecosystem, influenced by pollutants prevailing the nation are starting to appear (10, 18). Up until now, serious damage by acidification has not get been reported in Korea. This is due to the fact that the pH of most lakes in Korea, which are undergoing or have already undergone eutrophication, easily fluctuate from neutral to alkali due to by the influence of seasonal blooms (19). However as incidences of acid rain increase, accumulating acidic precipitation, studies forecasting various effects of acidification on aquatic ecosystems are becoming very important subjects in Korea.

The typical studies of the influences of aquatic acidification on microbial ecosystem have been compared between acidified areas and non-acidified areas (4, 21, 23), and the changes of ecosystems in specific areas in the process of acidification have been monitored (11, 20, 24). But in Korea, owing to the regional characteristics of freshwater, acidified lakes have not been reported. So, we designed tubing-coupled pH gradostat microcosm. Samples were collected from Wangsong reservoir, which is located in the western region of Korea and in the middle of the industrial area. It can be considered as a representative of those areas affected by acid precipitation carried in by prevailing western wind from China. For the past 3 years (1994~1996), the pH has shown seasonal changes varying from pH 6.0 to 10.2, and no acidification tendency was found. Multiple regression analysis showed a correlation between changes in pH and heterotrophic and total bacteria populations, which signify that the pH greatly affects species compositions and microbial functions in these area (1).

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This research examined the change in metabolic diversity of the bacterial community in the process of acidification in Korean freshwater. For the purpose, the samples of this area were applied to a microcosm designed to change from pH 7.0 to pH 3.0 in stages. An analysis of the change in the function and structure of bacterial communities in the process of acidification was made. In order to achieve this, each sample acquired through pH gradient was inoculated directly with Biolog GN plate. The utilization of 95 sole carbon sources was analyzed through principal component analysis.

Materials and Methods

Microcosm design

A series of five 2-liter borosilicate glass reaction vessels (25 cm high and 15 cm in diameter) with 1-liter sidearm overflow outlets, as described by Hunter *et al.* (17), were arranged in a sequence as steps on the stair in accordance with pH-gradient (pH 7.0, pH 6.0, pH 5.0, pH 4.0, pH 3.0) (Fig. 1). Each vessel was supported by a magnetic stirrer (Hankuk eng., Korea) and contained a 40-mm magnetic stirring bar to achieve complete mixing at 140 rpm. The freshwater (F1) was delivered from a 5-liter (35 cm high and 20 cm in diameter) glass vessel to the top vessel (V1) at a rate of 1.5 ml/min by a multi-channel peristaltic pump (IPC, Ismatec SA, Switzerland) with tygon tubes(SC 0005, Ismatec SA, Switzerland). The acidified water (pH 2.5; F2) was delivered from a 5-liter (35cm high and 20 cm in diameter) glass vessel to the bottom (V5) vessel at a rate of 0.8 ml/min by a multi-channel peristaltic pump (IPC, Ismatec SA, Switzerland) with tygon tubes (SC 0003, Ismatec SA, Switzerland). A pumped upflow of pH 3.0 to pH 4.0 at a rate of 0.8 ml/min and pH 4.0 to pH 7.0 at a rate of 1.5 ml/min and a passive downward overflow of pH 7.0 to pH 3.0 achieved the pH gradient. The pH measurements were taken every 12 hours by a pH meter (Horiba, Japan). Samples were taken from each vessel every 7 days by a sterilized 50 ml syringe for a period of 49 days. Freshwater samples with pH 6.8 were collected in clean polypropylene containers from the Wangsong reservoir (36°18′ N.L., 126°55′ E.L.) in Korea.

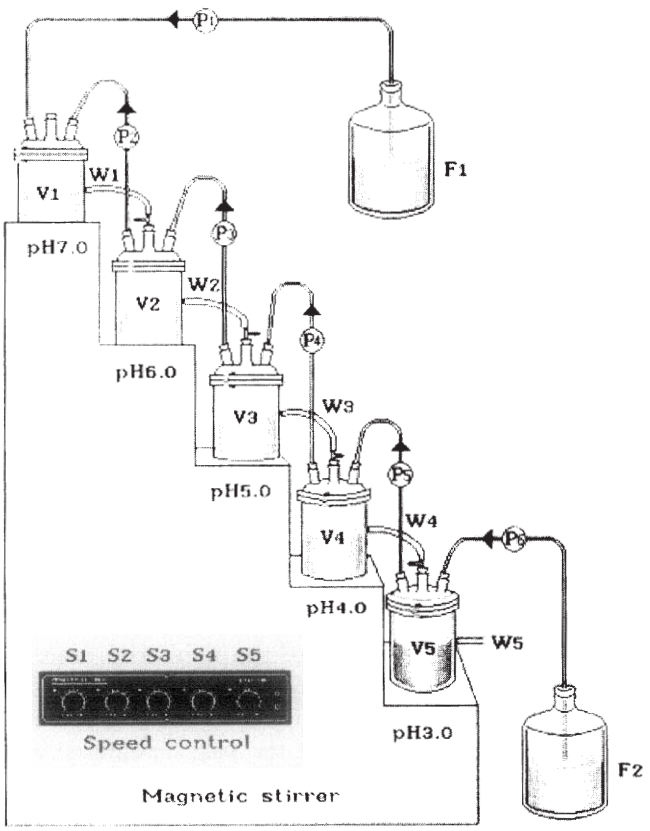

Fig. 1. A diagramatic representation of the tubing-coupled gradostat: V1 to V5 are bioreactor vessels. F1 is freshwater and F2 is adjusting pH 2.5 reservoirs feeding each end of the array. P indicates tubing pumped lines. W and S are overflow weirs and speed control switch.

Microbial analysis

The numbers of total bacteria were counted by an epifluorescence microscope (Axioplane, Ziess, Germany) according to the acridine orange direct count (AODC) as described by Hobbie *et al.* (14). The nutrient agar (10%, Difco) was used for plating viable heterotrophic bacteria. By using nutrient agar, where lipidolytic, amylolytic, cellulolytic, and proteolytic bacteria according to the method of Holding and Collee (15) by Tween 80 (0.1%), starch (0.2%), CMC (0.2%) and gelatin (0.4%) where after incubation at 25°C for 5 days, the colonies were counted. The activity of extracellular enzymes on lipase, phophatase, amylase, cellulase, β-glucosidase, and chitinase was measured using flurochrome 4-methylumbelliferly (MUF) butylate, 4-MUF phosphate, 4-MUF-α-D-glucoside, 4-MUF-β-D-cellobiopyranoside, 4-MUF-β-D-glucoside, and 4-MUF-N-acetyl-β-D-glucosaminide by the spectrofluorometer (RF-510, Shimadzu) at excitation 365 nm and emission 450 nm (16, 25).

Biolog analysis

The metabolic diversity of the pH-gradient microcosm was analyzed with Biolog GN microplates (BIOLOG, Inc.). The 96-well of GN plates were divided by Garland and Mills (7) into 11 categories: polymers (substrates A2 to A6), carbohydrates (substrates A7 to C10), esters (substrates C11 to C12), carboxylic acid (substrates D1 to E12), brominated chemicals (substrates F1), amides (substrates F2 to F4), amino acids (substrates F5 to G12), aromatic chem-

icals (substrates H1 to H4), amines (substrates H5 to H7), alcohols (substrates H8 to H9), and phosphorylated chemicals (substrates H10 to H12). The substrate blank well contained the nutrient medium and dye but no substrate. About 150 μl of each pH gradient vessels samples were inoculated directly into Biolog GN microplate wells by multipipettor. All plates were incubated at 25℃ without agitation. Inoculum density was $0.98\sim1.22\times10^6$ cells per ml. A 590 nm levels in each well were read with a microplate reader (BIOLOG Inc.) equipped with SOFTmax software (version 2.01). Biolog plates from the pH gradient microcosm were read at frequent intervals in order to generate a relationship between average well color development and time. On the basis of this relationship, optimum time was chosen as analysis data of average well color development (AWCD).

Statistical analysis

The average and principal component analyses (PCA) of all data were analyzed using the SAS/STAT (Statistical Analysis System; SAS Institute Inc., Cary, N.C.) version 6.12 under the PRINCOMP procedure. Overall color development in Biolog plates was expressed as average well color development (AWCD). AWCD was derived from the mean difference among optical density values of the 95 response wells and control well as described by Garland and Mills (7). The pattern of sole-carbon-source utilization in each plate was expressed as transformed data. The transformed data was calculated by dividing the response well values for each well by the AWCD of the plate. The relationship among different samples with the basis of the transformed data were determined by the principal component analysis. The principal component analysis calculates the proportion of the variance in each variable explained by given principal components.

Results

Bacterial population and extracellular enzyme activity

The variations in the number of bacterial population and activities of extracellular enzyme are summarized in Table 1. Number of total bacteria and heterotrophic bacteria inclusive of the functional populations of bacteria showed ranges of $0.98\sim1.22\times10^6$ cells $\cdot$ ml^{-1} and $0.47\sim20.36\times10^4$ cfu $\cdot$ ml^{-1}, respectively. There was no significant correlation between total bacteria by acridine orange direct count (AODC) and water pH, but viable counts of heterotrophic bacteria increased as the pH decreased. The activity of extracellular enzyme was between 0.023~1.122 μM $\cdot$ $l^{-1}\cdot hr^{-1}$ and decreased as the pH decreased in the multistage microcosm. Enzymatic activities decreased rapidly below pH 5.0 exclusive of the chitinase, amylase, and cellulase, and then remained between 4~47% at lower pH values.

Sole carbon source utilization

The color production in the control well showed a range between 0.101~0.208 (0.15±0.04) at 50 hour O.D. 590 nm value which was minimal for all pH vessel samples analyzed in this study. Reduction of tetrazolium dye in the control well was more pronounced in pH 7.0 samples than that of pH 3.0 because pH 7.0 samples contained high concentrations of organic material.

Table 1. Changes of microbial populations and extracellular enzymes in pH-gradient microcosm

Factors	pH				
	7	6	5	4	3
TOB (10^6 cells $\cdot$ ml^{-1})	1.22±0.29	1.05±0.24	1.14±0.35	1.01±0.35	0.98±0.35
HEN (10^4 cfu $\cdot$ ml^{-1})	8.53±0.81	7.4±0.7	4.88±0.43	1.15±0.95	0.68±0.37
HEZ (10^4 cfu $\cdot$ ml^{-1})	11.24±0.81	7.93±0.66	6.08±0.11	2.67±0.18	0.83±0.55
PRB (10^4 cfu $\cdot$ ml^{-1})	16.07±0.76	10.69±0.88	8.68±0.18	1.49±0.15	0.71±0.07
LIB (10^4 cfu $\cdot$ ml^{-1})	15±0.98	7.87±0.63	8.85±0.74	2.02±0.12	0.48±0.37
CEB (10^4 cfu $\cdot$ ml^{-1})	20.36±0.17	11.21±0.74	6.88±0.52	3.43±0.29	0.84±0.05
AMB (10^4 cfu $\cdot$ ml^{-1})	2.09±0.26	2.03±0.31	1.12±0.13	0.49±0.064	0.47±0.07
BGLU (μM $\cdot$ $l^{-1}\cdot hr^{-1}$)	0.702±0.3	0.466±0.11	0.334±0.08	0.246±0.01	0.227±0.01
AGLU (μM $\cdot$ $l^{-1}\cdot hr^{-1}$)	0.308±0.05	0.257±0.04	0.238±0.01	0.236±0.01	0.229±0.009
PHOS (μM $\cdot$ $l^{-1}\cdot hr^{-1}$)	0.427±0.31	0.197±0.07	0.13±0.06	0.079±0.03	0.058±0.005
NACET (μM $\cdot$ $l^{-1}\cdot hr^{-1}$)	0.05±0.008	0.069±0.03	0.057±0.02	0.035±0.001	0.031±0.001
BCEL (μM $\cdot$ $l^{-1}\cdot hr^{-1}$)	0.067±0.01	0.078±0.05	0.056±0.03	0.026±0.002	0.023±0.001
BUT (μM $\cdot$ $l^{-1}\cdot hr^{-1}$)	1.122±0.42	0.922±0.44	0.407±0.36	0.135±0.016	0.046±0.01

TOB; Total bacteria, HEN; Heterotrophic bacteria in nutrient agar medium, HEZ; Heterotrophic bacteria in Zobell medium, PRB; Proteolytic bacteria, LIB; Lipolytic bacteria, CEB; Cellulolytic bacteria, AMB; Amylolytic bacteria, BGLU; Glucosidase, AGLU; Amylase, PHOS; Phosphatase, NACET; Chitinase, BCEL; Cellulase, BUT; Lipase

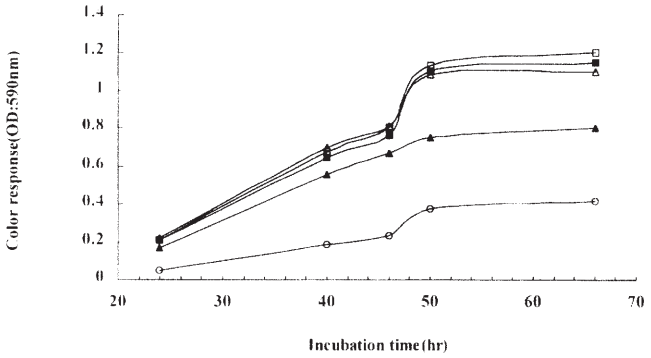

Fig. 2. Color development with incubation. Plots present the color response in five pH gradient microcosm samples. The plot of average well color development (AWCD) represents the mean color response for all 95 response wells. Open cubic , pH 7; closed cubic, pH 6, open triangle, pH 5; closed triangle, pH 4; open circle, pH 3.

The overall rate of color production in a plate with respect to acidification is obtained by dividing the AWCD value for an early incubation during the linear increase in color development by the length of incubation (Fig. 2). The sole carbon source utilization varied greatly among response wells depending on acidification. The response color of the wells developed rapidly and reached a maximum value within 50 hour. On the basis of this relationship, 50 hour was chosen as an optimum time for analysis to compare metabolic diversity depending on acidification. Also, the color production followed a sigmoidal curve with incubation time and also increased, as the pH increased.

Effects of inoculum density and extracellular enzyme activity

The analysis of the correlation of inoculum cell density and extracellular enzyme versus AWCD value was determined for the relative importance of inoculum size versus community activity on the rate and extent of color development (Table 2). AWCD values positively correlated with inoculum density, and the correlation coefficient decreased as the pH decreased. Also, heterotrophic bacteria including functional bacteria like inoculum densities positively correlated with AWCD values above pH 5.0 and negatively correlated with inoculum density below pH 4.0. However, the activities of extracellular enzymes negatively correlated with AWCD values above pH 5. 0 and positively correlated with AWCD values below pH 4.0. This result suggests that color production is caused by inoculum density above pH 5.0 but is caused by cell activity below pH 4.0.

Patterns of sole carbon source utilization

The percentage of AWCD on plates after incubation for 50 hours was compared to sole carbon source utilization according to acidification (Table 3). The utilization of amino acids (substrates F5 to G12) among 11 categories at pH 7.0 ranked the highest at 46.7%, phosphorylated chemicals (substrates H10 to H12) the lowest at 17%. Also at pH 3.0, other amino acids (substrates F5 to G12) ranked the highest at 19.9% and phosphoylated chemicals (substrates H10 to H12) ranked the lowest.

The research of different microbial habitat and relative composition of community affected significantly density and activity. So, statistical significance of the transformed data analyzed it for correlation of the first principal components (PC) with AWCD va-

Table 2. Pearson correlation coefficients (n=30, P<0.05) between average well color development (AWCD) and bacterial populations and extracellular enzymes in pH gradostat microcosm (blank: not significant)

Factors	pH				
	7	6	5	4	3
TOB	0.745	0.641			
HEN	0.385				-0.5
HEZ		0.499		-0.476	-0.477
PRB		0.436			-0.41
LIB		0.651		-0.386	-0.488
CEB		0.514	-0.486		-0.589
BGLU			0.511	0.651	0.391
PHOS	-0.419	-0.416		0.419	
AGLU	0.473				
NACET	-0.521	-0.435	-0.446	0.465	
BUT	-0.383	-0.678		0.773	
BCEL		-0.738		0.672	

TOB; Total bacteria, HEN; Heterotrophic bacteria in nutrient agar medium, HEZ; Heterotrophic bacteria in Zobell medium, PRB; Proteolytic bacteria, LIB; Lipolytic bacteria, CEB; Cellulolytic bacteria, BGLU; Glucosidase, PHOS; Phosphatase, AGLU; Amylase, NACET; Chitinase, BUT; Lipase, BCEL; Cellulase

Table 3. Change of sole-carbon sources utilization(%) in BIOLOG GN microplates according to pH-gradient samples

Carbon sources	pH				
	7	6	5	4	3
Polymers	24.4	24.6	29.4	25.6	12.6
Carbohydrate	35.1	32.9	36.6	28.5	10.9
Esters	27	26	26.5	24.5	14.5
Carboxylic acids	38.9	36.1	35.6	33.2	17.2
Brominated chemical	22	23	19	24	13
Amides	24	21.7	21.6	12.6	10.3
Amino acids	46.7	46.3	46.8	39.8	19.9
Aromatic chemicals	37.3	33.3	33	28.8	16.5
Amines	36.7	40	39.3	27.6	12
Alcohols	22.5	22.5	22	21	11.5
Phosphorylated chemicals	17	12.7	16	13.3	4.3

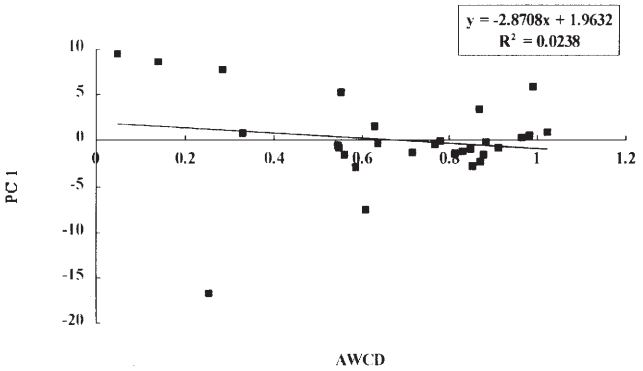

Fig. 3. Comparison of average well color development with scores for the first principal component on the basis of principal component analysis of transformed data for 50-h incubation of pH-adjusted samples. Lines represent the least-square regressions (n=30). R2 values are the squares of correlation coefficients.

lues (Fig. 3). Correlation of samples for the first PC with AWCD values much lower (r^2=0.0238), the eigenvalue values higher (23, 43). Transformation of the data by dividing each raw difference by the AWCD of the plate significantly reduced the influences of rate of color development on the classification of sample (7).

Comparison of substrate utilization data from the pH gradient microcosm

Analysis of the principal components of the transformed data varied in sole-carbon source utilization at different pH. The samples from different pH-gradients had distinctive patterns of sole-carbon-source utilization on the basis of principal component analysis of transformed color response data (Table 4). The result of principal component analysis selected two

Table 4. Pearson's regression coefficient of carbon source variables to principal components (PC) for analysis of pH-gradient samples.

Well No.	Carbon source	PC 1					PC 2				
		pH 7	pH 6	pH 5	pH 4	pH 3	pH 7	pH 6	pH 5	pH 4	pH 3
	Polymer										
A3	Dextrin	0.89	−0.98							0.91	
A4	Glycogen	0.91								0.91	
A5	Tween40		0.88								
A6	Tween80			−0.93		0.97					
	Carbohydrate										
A8	N-Acetyl-D-glucosamine		−0.99								
A10	L-Arabinose	−0.91			−0.82						
A12	Cellobiose		−0.99								
B1	I-Erythntol			0.98	0.93		−0.89				
B2	D-Fructose		−0.97		0.97						−0.72
B3	L-Fucose	0.95			0.98						
B5	Gentiobiose		−0.99		0.91						
B6	α-D-Glucose			−0.97		0.84					
B9	Lactulose			0.92	−0.90						
B10	Maltose		−0.98								
B11	D-Mannitol				−0.85	−0.80					
B12	D-Mannose		−0.92		0.79		−0.91				
C2	β-Methly-D-glucoside		−0.98								
C3	D-Psicose			0.99							
C4	D-Raffinose		−0.99		0.81						
C5	L-Rhamnose			−0.99							
C6	D-Sorbitol		−0.93			0.89					
C7	Sucrose			−0.99		0.93					
C8	D-Trehalose			−0.94	−0.89	−0.67					
C10	Xylitol										0.84
	Esters										
C11	Methyl-pyruvate	0.93									
C12	Mono-methyl-succinate	0.95									
	Carboxylic acid										
D1	Acetic acid			0.96	0.93						
D2	cis-Aconitic acid									−0.88	
D3	Citric acid							0.98		−0.86	
D4	Formic acid		−0.98	0.99	0.99						
D5	D-Galactonic acid lactone	−0.92			−0.79						

(Continued)

Table 4. Continued

Well No.	Carbon source	PC 1					PC 2				
		pH 7	pH 6	pH 5	pH 4	pH 3	pH 7	pH 6	pH 5	pH 4	pH 3
D6	D-Galacturonic acid	−0.91		−0.98	−0.85						
D7	D-Gluconic acid	−0.98			0.83			0.94			
D8	D-Glucosaminic acid	−0.89			−0.96						
D9	D-Glucuronic acid	−0.92									
D10	α-Hydroxybutyric acid		0.97	0.98							
D11	β-Hydroxybutyric acid					0.69					
D12	γ-Hydroxybutyric acid									0.88	
E1	ρ-Hydroxy phenylacetic								0.95		
E3	α-Keto butyric acid			0.99				−0.95			
E4	α-Keto glutaric acid									−0.83	0.66
E5	α-Keto valeric acid		0.95	0.92							
E6	D,L-Lactic acid									−0.81	
E7	Malonic acid							0.92			
E8	Propionic acid		0.95	0.94	0.92						
E9	Quinic acid	−0.95		−0.96							
E10	D-Saccharic acid									−0.87	
	Brominated chemical										
F1	Bromo succinic acid			0.97	0.98						
	Amides										
F3	Glucuronamide		0.93	0.98	0.83						
F4	Alaninamide		0.95	−0.99							
	Amino acids										
F5	D-Alanine		0.95	−0.99							
F6	L-Alanine			−0.97							0.84
F8	L-Asparagine	−0.95		−0.96							
F9	L-Asparti acid	−0.98									
F10	L-Glutamic acid	−0.96		−0.99	−0.85	0.62					0.71
F11	Glycyl-L-aspartic acid	0.92		0.99							
F12	Glycyl-L-glutamic acid		0.94								
G1	L-Histidine							0.93			
G2	Hydroxy L-proline	−0.98									
G3	L-Leucine		0.98	−0.97							
G6	L-Proline			−0.97	−0.88						
G7	L-Pyroglutamic acid	−0.99		−0.97							
G8	D-Serine		0.98								
G9	L-Serine	−0.82		−0.96							
G10	L-Threonine		0.95								
G11	D,L-Camitine		0.96				0.82		0.92		0.89
G12	γ-Amino butyric acid	−0.96				0.71					
	Aromatic chemicals										
H1	Urocanic acid	−0.99									
H3	Uridine						−0.83				
H4	Thymidine	0.89	−0.99	0.96							
	Amines										
H5	Phenyl ethlamine			−0.97						0.85	
H7	2-Amino thanol						−0.81				
	Alcohols										
H8	2,3-Butanediol		0.98	0.99							

(Continued)

Table 4. Continued

Well No.	Carbon source	PC 1					PC 2				
		pH 7	pH 6	pH 5	pH 4	pH 3	pH 7	pH 6	pH 5	pH 4	pH 3
	Phosphorylated chemicals										
H10	D,L-α-Glycerol phosphate			0.99							
H11	Glucose-1-phosphate	0.93		0.95							
H12	Glucose-6-phosphate	0.97	−0.99								

principal components that explained 78.2% of the variance in the data. The first principal component (PC 1) decreased from 75.9% to 47.7% as the pH decreased from 7.0 to 3.0. In contrast, the second principal component (PC 2) increased from 16.5% to 30.5% as the pH decreased from 7.0 to 3.0.

The result of the PC 1(explained 75.9%) indicated that pH 7.0 microbial communities show positive correlation with polymers and phosphorylated chemicals and negative correlation with esters and amino acids among the 95 sole carbon sources compared with other carbon sources. On the basis of PC 2 (explained 16.5%) analysis, microbial communities associated positively with amino acid and negatively with carbohydrate, aromatic chemical, and amine. The PC 1 of pH 6.0 samples, which explained 75.09% of the variance in the data, negatively correlated with amino acid and carbohydrate. The PC 2 explained 18.8% of the variance in the data, positively correlated with esters and amino acid. The PC 1 of pH 5.0 samples, which explained 54.8% of the variance in the data, negatively correlated with carbohydrate and amino acid. PC 2 explained 26.2% of the variance in the data positively, correlated with amino acid and carboxylic acid. The PC 1 of pH 4.0 samples, which explained 58.4% of the variance in the data, correlated with negatively with amino acid. PC 2 explained 28.7% of the variance in the data, correlated positively with polymer, carboxylic acid, and amine. The PC 1 of pH 3.0 samples, which explained 47.7% of the variance in the data, correlated positively with amino acid. The PC 2 explained 30.5% of the variance in the data, correlated positively with amino acid and carboxylic acid. The principal component, which explains the variance in the color response of a sole carbon source, was related to differences in the response in pH gradient microcosm samples with different coordinate values for the principal component.

Discussion

For the past 3 years, we have studied the effects of environmental factors that affect regional and seasonal changes of microbial populations at several reservoirs in industrial areas and big cities by multiple regression analysis. Population of bacteria at small reservoirs with depths 5 meters or less were greatly influenced by the concentrations of organic and inorganic nutrients from external inflows and by seasonal algal blooms caused by eutrophication. On the contrary, the changes in bacterial populations at big lakes were explained by the influence of pH as 40% in the case of total bacteria and 68% in the case of heterotrophic bacteria (1). Therefore, we tried to predict the effects of acidification on an aquatic microbial ecosystem in this area by analyzing the structure of the microbial community in a multistage microcosm with artificial pH gradient from pH 7.0 to pH 3.0.

Microcosms are easily replicated and offer precise control over environmental factors as well as being easy to manipulate and to control spatial heterogeneity (6). However, numerous criticisms have been raised concerning the validity of laboratory microcosm research (5, 22). Peters (22) concludes that model systems are weak analogies to natural systems, lacking the complexity found outside the laboratory, and thus are inadequate to generate usable theories. So Carpenter (5) reported that the microcosm experiments after appropriately scaled field studies become irrelevant and diversionary. In this study, the changes in microbial population and enzyme activity showed various patterns during incubation of 48 days with tubing coupled microcosm. In contrast, no changes in each vessel were found. Therefore, in this study we applied the influence of acidification on the multistage microcosm that was exclusively spatial and temporal heterogeneity

The microbial community and function according to acid precipitation were studied on an acidified reservoir (21, 23, 24). However, these studies were characterizations of microbial community structure and function and required isolation and culturing of individual species. More recently, Hiorns *et al.* (12) investigated the bacterial communities by amplification and sequencing of 16S ribosomal DNA

in the affected acidic precipitation lakes. But molecular approaches still require culture-based information to develop the data base needed for these interpretations. An alternative and relatively simple method that characterizes community function based on net sole-carbon-source utilization patterns is the Biolog analysis. Since then, several authors have applied this method successfully in their field of research (7, 9, 13). An important advantage of the Biolog method is its ease of use and thus feasible for use in large-scale field studies.

Inoculum density and composition has been demonstrated to be an important confounding variable in the interpretation of Biolog substrate utilization patterns (7, 26). The effects of color development on the Biolog plate were confirmed by correlation analysis with inoculum size and community activity according to pH gradient. In our study, the color development on the Biolog plate affected the community activity much more than inoculum size association specific to environmental variables that stressed environment. Even though the number of such facts in the worst environment is small it is true that high specific activities and growth rates are shown by rapid color development (9).

The influence of density is a confounding factor when the analyzed structure and function of microbial communities are from acidified sample. Several methods suggested overcoming this potential influence of inoculum density on color response data (7). For optimal characterization of pH gradient samples, we used multiple readings over a 6-hour interval and transformation of the data accounted for different inoculum density. The transformed data set reflects the intrinsic pattern of different microbial communities and the evenness in distribution of functional abilities within a community. There are a number of statistical approaches for evaluating multivariate data such as Biolog substrate utilization patterns. The particular objectives of a study will determine whether it is more appropriate to use a classification technique, such as cluster analysis, to classify similar samples into discrete groups, or an ordination technique, such as principal component analysis. Also, the direct incubation of acidified samples in Biolog plates produced useful patterns of metabolic response in the characterization of microbial communities.

On the basis of several years field study at several reservoirs in industrial areas, we analyzed the effect of acidification on the metabolic diversity of microbial communities by using an artificial pH gradient microcosm. Until now, serious damage by acidification has not been reported in Korea. But it is expected that this study will be helpful in predicting structural and functional changes of microbial communities in Korean freshwater in the process of acidification.

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