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Numerical Analysis of Heterotrophic Bacterial Community in the Sudong Stream

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수동천에서의 종속영양세균 군집에 대한 수리학적 분석

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ABSTRACT: Taxonomic composition and diversity were examined by statistical analysis for bacterial communities in surface waters of the Sudong Stream, a tributary of North Han River. For total 282 isolates, *Flavobacterium*, *Aeromonas* and Enterobacteriaceae was identified by the deterministic schemes as a major group above 50% of total isolates in all sampling sites. Morphological, biochemical and physiological characteristics were numerically analyzed for bacterial isolates from each site and clustered into 15-28 groups. Not all statistically clustered groups were identical to the groups derived from deterministic identification. Especially, consistent relationship was not found in dendrograms for the groups with each a single strain which has peculiar sugar-degrading activity. At a level of 80% similarity, bacterial diversity (H) was ranged as 2.37-3.14, and it was suggested that the research area was oligotrophic-mesotrophic status. Regional distribution of bacterial community was most heterogeneous at the site where large input of allochthonous materials or bacteria were occurred. And that was the significant factor for the compositions of bacterial communities in the Sudong Stream.

KEY WORDS □ Sudong Stream, Heterotrophic bacteria, Numerical taxonomy, Diversity index

Microorganisms are effective competitors for reduced carbon sources in all aquatic ecosystems. It is now recognized not only that microorganisms, especially aerobic heterotrophs are major users of energy and materials but also that they form microbial food webs. In this respect, a number of approaches have taken to demonstrate the diversity of decomposer species, of their activities, and of their patterns of distribution. But few studies have been reported on a bacterial community in natural ecosystems. The problems in measuring species diversity of bacterial communities inherent in the identification of bacterial species have been discussed by several authors (Mandel, 1969; Kaneko *et al.*, 1977; Hauxhurst *et al.*, 1981). Because of the difficulty of identifying bacterial species directly on the basis of their morphological or colonial attributes, few studies on bacterial diversity can rely on morphological observations as the primary criteria for distinguishing taxa. Studies on the diversity of bacterial communities, therefore, have most often employed the techniques of numerical taxonomy as defined by Sneath and Sokal (1973). The taxon as defined clustering in numerical taxonomy is functionally equivalent to a species and can be used as such in the calculation of the diversity index. Thus, with the help of these methods, changes in heterogeneity of bacterial populations in response to physicochemical water quality or man-made en-

vironmental stress have been described in particular biotopes (Lighthart, 1975; Bell *et al.*, 1982; Gehlen *et al.*, 1985).

In this study, we investigated the seasonal fluctuation of bacterial communities in a stream, where allochthonous organic materials input containing animal-originated sewage, farmland outfall and domestic sewage were occurred. And the relationships between the results from deterministic and numerical taxonomy were discussed. By the application of taxonomic diversity index derived from the numerical approach to bacterial populations, the effects of environmental factors such as hydrographic locations and seasonal changes on the bacterial communities were also observed.

MATERIALS AND METHODS

Sample collection and Isolation of Bacteria

Five samples were taken from the surface water of the Sudong Stream, a tributary of North Han River (Fig. 1). The principal source rises from the Mt. Sori and several subsidiary streams flows into the main stream. These subsidiary streams increase stream discharge and organic input which contains domestic, farmland, animal originated sewage. Especially, fecal discharges from farm animals (3,500 pigs) were present between the principal source and the Site 2. Thus great abundances of aquatic vegetations could be seen at the Site 2. The stream bed was mostly gravel or sand except the principal source which shows some accumulation of fallen leaves. And in the main river, it was mainly composed of claylike basin. The sampling dates were chosen as Oct. 23, Dec. 11, 1986 and Apr. 16, Jun. 17, 1987 to reflect seasonal effects on bacterial community. Samples were collected in sterile 2L screwcapped polypropylene bottles (Nalgene, USA) between 9:00 a.m. and 1:00 p.m. on sampling date. Using a cold-storage box (4°C), they were transported and analyzed immediately in the laboratory.

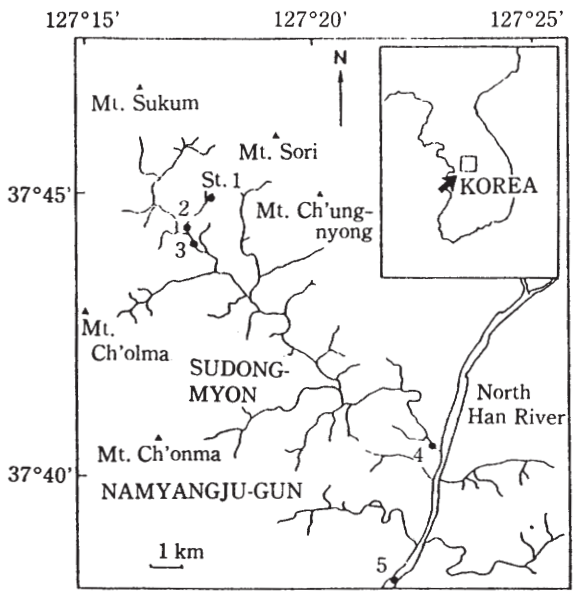


Fig. 1. *Map of the research area and sampling sites.*

The pour plate method was used to estimate viable heterotrophs on a ZoBell's 2216e medium with distilled water. Plates were incubated at 25°C in dark room, and colony-forming units (CFUs) were counted after 2 weeks. The plates were selected that contains 30-300 developed colonies for each sample. Colonies for taxonomic analysis, grown on the above agar plates, were picked from the plates at random. Simultaneously, colony characteristics which included form, elevation, opacity, diameter, pigmentation and texture were observed. And purified as a single colony through re-streaking to fresh medium.

Numerical taxonomy

A range of 53 tests, which were morphological, biochemical and physiological characters, was selected for numerical taxonomy. The tests included ability to grow on the following compounds as a sole source of carbon (1% w/v, except 0.5% w/v salicin); adonitol, arabinose, dulcitol, fructose, galactose, inositol, lactose, mannitol, raffinose, rhamnose, sorbitol, sucrose and salicin. Ability to use inorganic nitrogen (200 μg of N/ml for $N\text{-}NO_3$), citrate, malonate and ability to grow at medium pH 4.0, 10.0 and at temperature 5°C, 37°C and 44°C were included. Tolerance to 1.6%, 3.5%, 5.0% (w/v) NaCl, 0.0075% (w/v) KCN and hydrolysis of casein, gelatin, Tween 80, esculin and starch were tested. Production of catalase, oxidase, urease, indole, arginine dihydrolase, H_2S on triple sugar iron agar and gas from glucose-

peptone water were observed. Methyl red and Voges-Proskauer reactions, O/F reactions, Gram reactions and motility were included. And several observed colony morphological characters were also included.

Similarity coefficients were calculated according to Goodfellow (1977) and described in the previous paper (Hong *et al.*, 1985). Eighty percent similarity level clusters were formed by the unweighted-pair group method with arithmatic average linkage cluster analysis. Identification of the strains to the genus level was performed by the schemes described from Shewan *et al.* (1960) and reference to Bergey's manual of systematic bacteriology (Krieg and Holt, 1984), Manual for the identification of medical bacteria (Cowan, 1974), and Biochemical tests for identification of medical bacteria (MacFaddin, 1980).

The diversity index (Shannon and Weaver, 1963) was used to express the overall heterogeneity of bacterial community from a particular location and season. The community was split into clusters C_i, ..., C_z by dendrograms calculated on the basis of total 53 tests. All isolates with 80% similarity were summarized in a group, C_i. If N_i is the number of bacteria of the cluster C_i and $N = \sum_{i=1}^{Z} N_i$, where N is the total number of isolates of a community, the diversity index (H) is calculated by:

$$H = \ln N - 1/N \sum_{i=1}^{Z} N_i \ln N_i$$

where z is the total number of clusters.

RESULTS

The total 282 isolates were categorized to 13 groups by deterministic taxonomy. *Flavobacterium, Aeromonas* and Enterobacteriaceae were the major groups, consisting above 50% of total isolated strains in each sampling sites (Table 1). Only small portions of isolates were gram-positive or cocci, which include the genera *Aerococcus, Staphylococcus, Micrococcus* and *Bacillus*. The remainder consisted of members of the genera *Acinetobacter, Pseudomonas, Vibrio, Zymomonas, Lucibacterium* and *Photobacterium*. And it could be seen that there was no evident shift of generic composition according to the stream flow. The occurrence of Enterobacteriaceae group and *Aeromonas* spp., however, shows relatively larger

Table 1. *Variations of the bacterial population in the Sudong Stream between sampling sites. Flavobacterium, Aeromonas and Enterobacteriaceae were the major group in all sampling sites.*

Group	No. (%)[a] St. 1	St. 2	St. 3	St. 4	St. 5
Flavobacterium	12 (21.4)	15 (25.0)	15 (22.7)	14 (28.0)	14 (28.0)
Aeromonas	18 (32.1)	14 (23.3)	14 (21.2)	14 (28.0)	13 (26.0)
Enterobacteriaceae	12 (21.4)	10 (16.7)	14 (21.2)	8 (16.0)	14 (28.0)
Acinetobacter	2 (3.6)	5 (8.3)	5 (7.6)	1 (2.0)	2 (4.0)
Pseudomonas	—	1 (1.7)	4 (6.1)	3 (6.0)	1 (2.0)
Vibrio	2 (3.6)	4 (6.7)	3 (4.5)	1 (2.0)	—
Aerococcus	—	3 (5.0)	5 (7.6)	1 (2.0)	2 (4.0)
Zymomonas	2 (3.6)	2 (3.3)	—	1 (2.0)	2 (4.0)
Staphylococcus	2 (3.6)	2 (3.3)	2 (3.0)	3 (6.0)	1 (2.0)
Micrococcus	—	—	2 (3.0)	1 (2.0)	1 (2.0)
Lucibacterium	5 (8.9)	2 (3.3)	—	2 (4.0)	—
Photobacterium	—	2 (3.3)	2 (3.0)	—	—
Bacillus	1 (1.8)	—	—	1 (2.0)	—
No. of strains	56	60	66	50	50

a; Total of individuals for each bacterial group and percentage of each group isolated on any one sampling site.

fluctuation (16.0-28.0%, 21.2-32.1%, respectively) than that of the genus *Flavobacterium* which was 21.4-28.0%. Despite small number of isolates (50 isolates), more diverse genera were found at Site 4.

Table 2 which represents seasonal fluctuations of bacterial flora, shows a more specific pattern than that of regional fluctuations. The proportion of *Flavobacterium, Aeromonas* and Enterobacteriaceae group were found to be similar in April and June, 1987. In contrast, the isolates consisting of the members of Enterobacteriaceae were about 10% fewer in Dec., 1986 than in the other months. And the number of identified genera was maximum (13 genera) in April, 1987. The isolates of the genus *Bacillus* were found only in this month. In winter, the members of *Zymomonas, Micrococcus* and *Bacillus* spp. were not occurred.

The analysis of isolated bacteria strains for each sampling sites yielded in 15-28 clusters. The clustering was done at the 80.0% similarity level on the average. In Figure 2, it can be seen that the dendrograms bear no consistent relationship to the deterministic identification. For example, the dendrogram of isolates from Site 3 (Fig. 2c) showed that the genera *Flavobacterium, Acinetobacter, Vibrio* and group Enterobacteriaceae belong to a single phenon on the level of 81.3% similarity, whereas in Fig. 2a, nearly all species of the genus *Aeromonas* isolated in June, 1987 had a similarity of only 74.6%.

Generally, the fermentative bacteria which had reactions similar to the case of Enterobacteriaceae (*Aeromonas, Flavobacterium*) were clustered together to a single phenon.

Some tendencies were found with regard to the seasonal relationships. In many cases, the isolates from each season were clustered closely together to a single phenon. At Site 2 (Fig. 2b), the eighteenth group includes the genera *Vibrio, Aeromonas, Photobacterium* and *Lucibacterium* to a single phenon on the level of 80.7% similarity. All of them were isolated in April, 1987 and clustered together.

Frequently, phenetic groups which contain only a single strain were found. It shows no relatedness to any other strain at 80.0% similarity level. Upon analysis of these strains, they were found to have some peculiar physiological characteristics. Most of them have the ability to utilize some sugar as a sole carbon source and then produce acids. Therefore, it is necessary to regard these clusters

Table 2. *Variations of the bacterial population in the Sudong Stream between sampling dates. Members of Enterobacteriaceae were fewer in Dec. 1986 than the other seasons about 10 %.*

Group	No. (%)			
	Oct. 1986	Dec. 1986	Apr. 1987	Jun. 1987
Flavobacterium	18 (27.3)	19 (36.5)	19 (20.7)	14 (19.4)
Aeromonas	14 (21.2)	15 (28.8)	24 (26.1)	20 (27.8)
Enterobacteriaceae	13 (19.7)	7 (13.5)	22 (22.9)	16 (22.2)
Acinetobacter	4 (6.1)	1 (1.9)	6 (6.5)	4 (5.6)
Pseudomonas	2 (3.0)	1 (1.9)	4 (4.3)	2 (2.8)
Vibrio	5 (7.6)	2 (3.8)	2 (2.2)	1 (1.4)
Aerococcus	4 (6.1)	3 (5.8)	3 (3.3)	1 (1.4)
Zymomonas	3 (4.5)	—	3 (3.3)	1 (1.4)
Staphylococcus	1 (1.5)	2 (3.8)	2 (2.2)	5 (6.9)
Micrococcus	1 (1.5)	—	3 (3.3)	—
Lucibacterium	—	1 (1.9)	1 (1.1)	7 (9.7)
Photobacterium	1 (1.5)	1 (1.9)	1 (1.1)	1 (1.4)
Bacillus	—	—	2 (2.2)	—
No. of strains	66	52	92	72

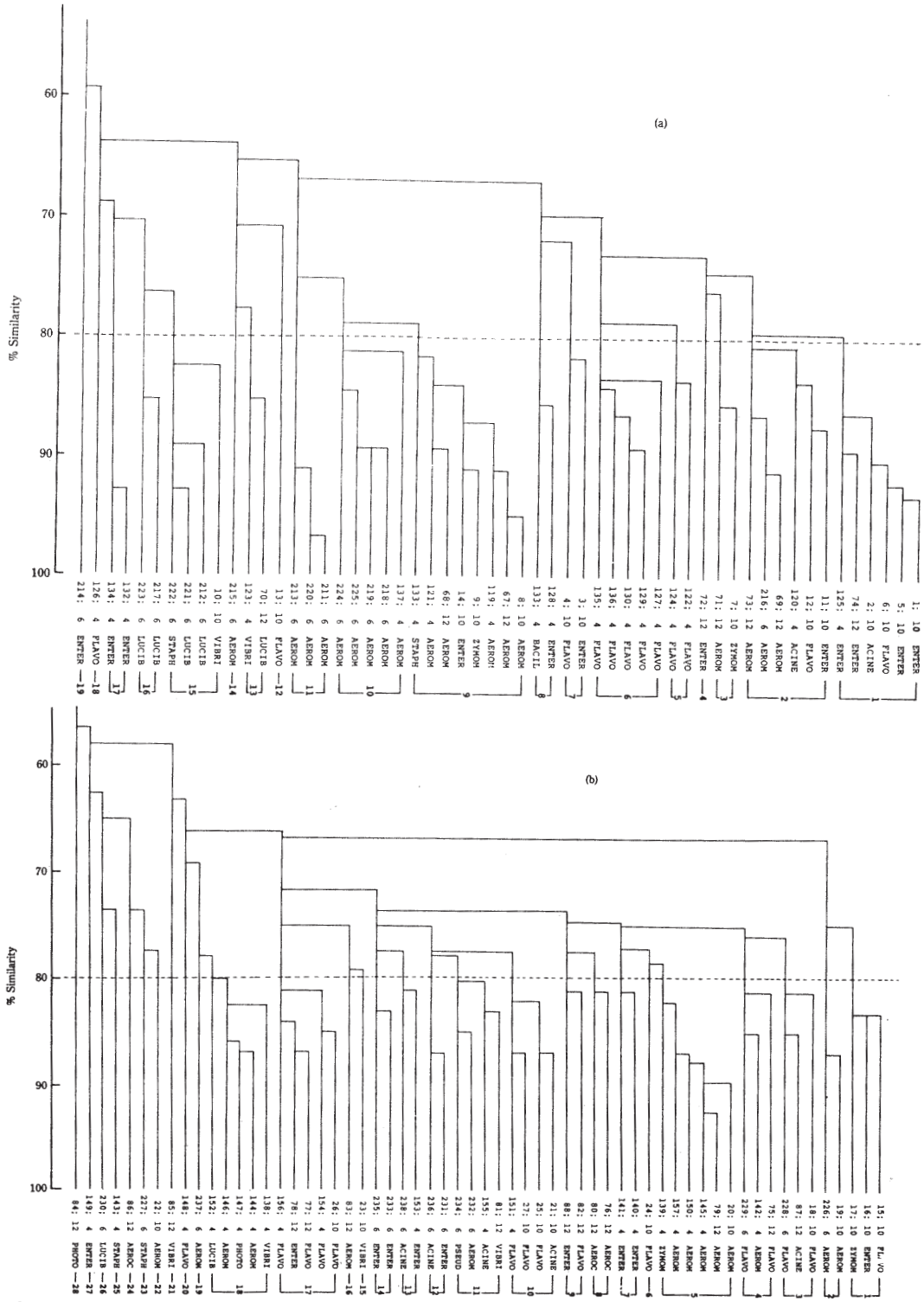
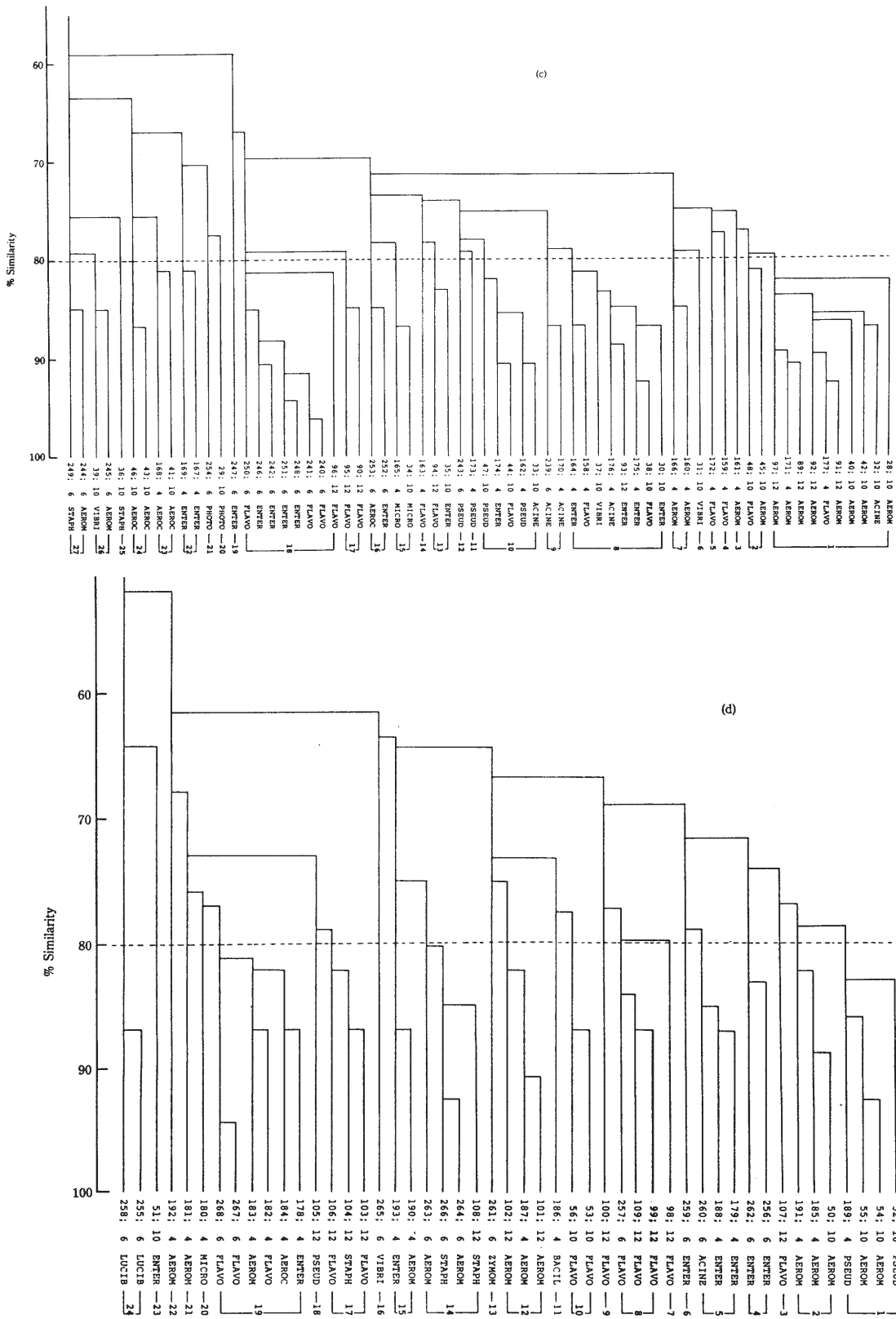
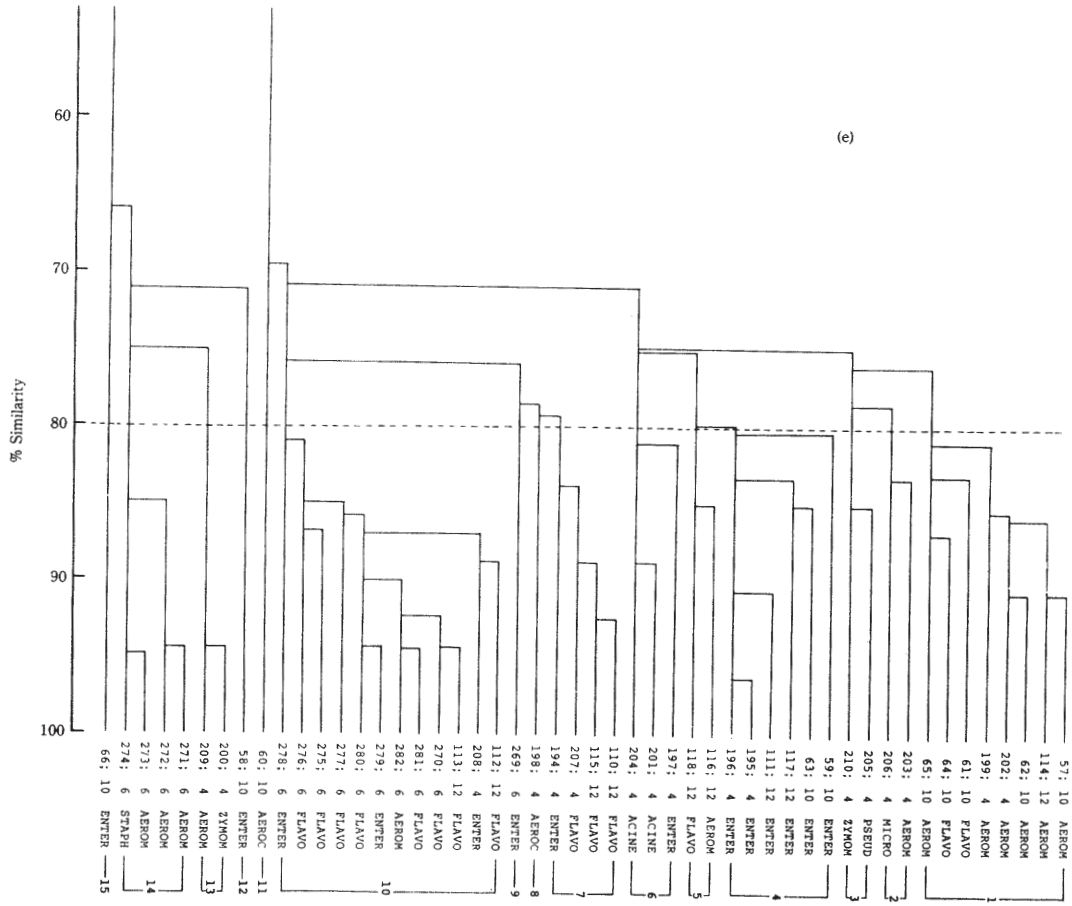


Fig. 2. *Dendrograms of bacterial strains isolated from the Sudong Stream at a level of 80.0% similarity.*
Relationship of phenetic group to the deterministic results were represented. Each Figures (a) through (e), shows bacterial community at Site 1, 2, 3, 4 and 5, respectively. Months of isolation, names of identified group and phenetic group numbers were listed following to each strain number.


% Similarity
100
90
80
70
60
(c)
(d)



each consisting of only one isolate as physiologically diversified groups.

By means of a diversity index, it is possible to describe the heterogeneity and homogeneity of microbial communities and their changes. The diversity indices were calculated on the basis of 80% similarity level. The results show some differences between the diversity indices of the examined communities in each sampling sites and/or dates (Fig. 3). Notable aspects of bacterial distribution due to hydrographycal differences were as follows. At the flow of Site 2, which contains more concentrated animal-originated fecal discharge, the value of the diversity index was greater than that of any other region. In contrast, it can be shown that the principal source and the main stream flows consisted of a relatively more specialized and homogeneous community.

The pattern was similar between the variation

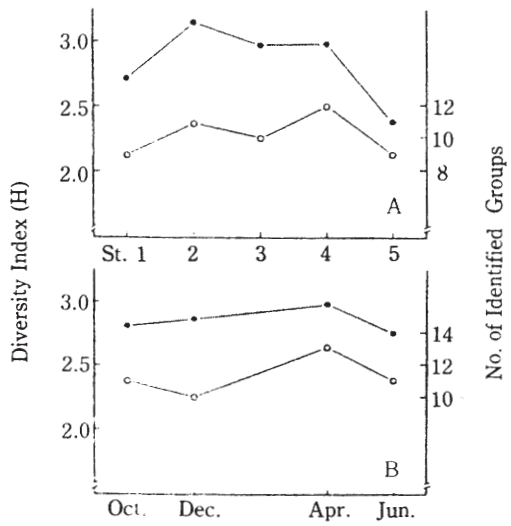


Fig. 3. *Fluctuations of the bacterial diversity index (● – ●) and the number of identified groups (○ – ○) depending on the sample location (A) and season (B), respectively.*

of the diversity index and the numbers of identified genera with only one exception. Though the values of the diversity index fluctuated less widely in seasonal aspects, specific variations of heterogeneity of bacterial community were observed. The highest bacterial diversity index was found in April, 1987 and the lowest in June, 1987. A similar pattern was also observed in the fluctuations of the number of identified genera.

DISCUSSION

Although numerical techniques have been applied mainly in the redefinition of existing taxa, they have also been successful in distinguishing between groups of bacteria occurring in large mixed populations in aquatic habitats. Furthermore, the measurement of microbial diversity provides insight into the ecological functioning of the community (Atlas, 1984).

In this study, we conducted clustering analysis with the advantages of a computer, and considered the clustered phenon as a single species at 80% similarity level. As shown in the results, evident features of the variations of bacterial diversity index were identified according to hydrographycal characteristics. At the site with fecal contaminations, the bacterial community shows the most heterogeneous composition. However, significant changes of generic composition were not found. And the clusters composed with only a single strains have particular activities on the metabolizing or degradation of some carbon sources. Thus it could be assumed that the bacterial classification with numerical techniques has the merit of being an indicator of physiological activities of a community which is overlooked in deterministic taxonomy. In this respect, numerical taxonomy with the isolates relative to selected physiological characters can provide not only some knowledge of structural heterogeneity of bacterial community but also the insight to the role of heterotrophic bacteria.

The effects of environmental stress on community structure have been examined by several authors with the effects of offshore dumping of phamarceutical wastes (Peele *et al.*, 1981), water temperature (Odum, 1971) and phenolic compounds (Milner, 1986). Theoretically, a low diversity index ($H<3.0$) is an indicator of some form of environmental stress, which selects a specialized homogeneous community. This stress could generally be produced by extreme pollution or in water with extremely low nutrient content (Hauxhurst *et al.*, 1981; Gehlen *et al.*, 1985). Thus we may propose the possible trophic status of the sampling sites ($H=2.37$-3.14), as an oligotrophic-mesotrophic status. And a possible reason for the increase of diversity index at Site 2, could be the effects of inputed allochthonous microorganisms in the animal-originated sewage which were non-toxic synthetic chemical but naturally occurring material. Therefore, it could be thought that the input of the fecal contaminants acted as a stimulator (nutrients) and not an inhibitor for bacterial growth.

Kaneko *et al.*, (1977) proposed the threshold level of bacterial population size and up to that level the bacterial diversity was inversely related to population size. Temperature is one of the most critical factors for bacterial survival and growth. Thus bacterial diversity decreased during summer, but increased during winter. These observations were also true in our research. Presumably the seasonal variations of bacterial diversity indices were characterized as a temperature-dependent factor, having summer lower, winter higher pattern.

In conclusion, the input of allochthonous materials or bacterial populations in a stream acted as an most important factor for the changes of bacterial community structure. They enhanced a diversification and probably metabolic activities of bacterial communities. The increased diversity of bacterial community was prolonged in downstream of the Sudong Stream, despite of continuous dilution effect with water mass.

적 요

북한강의 지류인 수동천 수층에서 세균 군집의 구성 및 다양성을 통계학적으로 조사하였다. 4계절에 걸쳐 분리된 282개의 균주를 계통분류한 결과, 전 정점에서 *Flavobacterium*, *Aeromonas*와 Enterobacteriaceae가 50% 이상으로 주종을 이루었다.

각 정점별로 분리된 세균들은 수리학적 분석을 행하여 각기 다른 형태학적, 생화학적 및 생리학적 특징을 나타내는 15-28개의 집단으로 분류되었다. 각각의 수리학적 세균 집단은 계통분류에 의한 세균 집단(속명)들과 반드시 일치하지는 않았으며, 특히 독특한 당 분해능을 지닌 균주들은 수리학적 분석에 의하면 각각의 균주가 독립된 형질집단을 이룸으로써 일관적인 관련성을 보이지 않았다.

80%의 유사도 수준에서 나뉘어진 각각의 집단을 하나의 종으로 하여 세균 다양성 지표(diversity index ; H)를 구한 결과, 2.37-3.14의 범위로 계산되어 조사 대상 지역은 빈영양-중영양 상태의 다양성을 갖는 세균 군집이 존재함이 밝혀졌다. 유기물질이나 세균 집단의 대량 유입 영향을 직접 받는 정점에서 세균 군집의 다양성 지표가 가장 높았으며 이러한 유입 현상이 전 수동천 생태계에서의 세균 군집의 분포에 가장 중요한 요인으로 작용함이 관찰되었다.

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