

Physiological Characterization of Heterotrophic Bacterial Communities from Selected Aquatic Environments

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Abstract. Three different biotopes, groundwater, surface water, and activated sludge, were examined and the total colony count on nutrient agar determined. The bacteria that could be isolated from the agar plates were identified and their *in vitro* activities investigated. Three principal approaches were used: (1) isolates were identified and the results used in a numerical analysis to determine their similarity; (2) the different physiological properties of isolates originating from a single biotope were compiled and used to characterize the community (collective total activity); and (3) the diversity of the physiological properties of the isolates of all populations was determined; on the basis of main characters a “heterotrophic” diversity index was calculated. The possibility of using a polyphasic ecological study to characterize aquatic bacterial communities is considered.

Introduction

The study of bacterial communities involves the isolation, cultivation, and identification of large numbers of strains. An aim of ecological investigations is to determine the physiological activities of the total community.

Some biotopes are so well defined that only a few physiological tests are needed to determine the important genera and species in them [10, 11, 18, 25], e.g., bacteria associated with particular diseases of humans. Such diagnostic procedures are based on a wealth of available taxonomic information [5, 15, 26].

This does not apply to the ecological examination of aquatic biotopes. Although isolated bacterial groups have been intensively investigated, little attention has been given to their relation to the aquatic environment, and little is known about the numerous aerobic heterotrophic bacteria occurring in unpolluted environments. For their characterization, numerical taxonomic procedures have been applied [4, 14, 16, 22], which are useful only if a large number of features of many isolates are examined. The chief advantage of numerical taxonomy is an objective description of individuals, but the lack of correlation between well-defined species and the phenons of a cluster still remains a problem [3, 17, 22].

From the numerical description of bacterial relationships it is possible to establish synthetic indices, such as the diversity index, that allow analysis of the development of population structures [1, 13, 19, 21, 26]. Thus, with the help of these indices, changes in heterogeneity of bacterial populations in response to man-made environmental stress can be described, although such an application [13] has led to a loss of information and made interpretation more difficult. The purpose of this study was to determine the possibilities and limits of establishing a procedure for investigating aquatic communities, because these communities reflect the conditions occurring in the aquatic environment and, thus, might be used as indicators of water quality. As not all the bacteria of a community can be isolated by any one procedure, it was necessary to see whether a satisfactory assessment of the environment could be made with the fraction of bacteria that could be isolated from the whole community.

Our interest focused on the different methods of describing biological activities in aquatic communities. For this study, we determined the *in vitro* utilization of specific substrates and the range of the total testable physiological activities exhibited by single isolates of a community [13, 20, 23]. Furthermore, we were interested in obtaining results relatively quickly in order to evaluate these measurements as a means of practical assessment of water quality. The procedure needed to be tested using bacterial isolates from several extremely different aquatic biotopes.

Materials and Methods

Water samples were taken from three selected biotopes: groundwater, surface water from an oligotrophic artificial lake, and an activated sludge plant.

The groundwater samples originated from the waterworks Meindorf situated in the Rhine-Sieg lowland near Bonn. The water treatment plant is one of the major suppliers for the municipal water system of the federal capital. The water is delivered from the aquifer (depth 15 m) into horizontal filter wells. The pumping capacity is about 3,000 to 6,500 m³/hour. The groundwater is influenced by bank-filtered water of the river Sieg. The samples were taken from a continually running tap in the waterworks.

The surface water was derived from the Wahnbach reservoir located 12 km northeast of Bonn. The reservoir is the second source of supply for the municipal water system. It has a surface area of about 2.15 km², an average depth of 19.3 m, and an estimated storage capacity of 41.4 × 10⁶ m³. The reservoir was created in 1956 by impounding the main river Wahnbach, which drains approximately 69.3 km² of rural area. Since 1978 the total inflowing water of the Wahnbach river has been treated by a phosphate-elimination plant to avoid eutrophication. The water samples were taken from the top layer of the lake near the intake tower (depth 0.5 m). Because of the artificial aeration of the hypolimnion the lake was destratified.

The activated sludge samples originated from the compact sewage treatment plant at Weilerswist, near Bonn. The plant was built for 16,500 population equivalents and contains a primary clarification tank, a centrifugal aerator tank, and a peripheral feed settling tank. The main domestic sewage has an average BOD₅ of 67 mg/liter for the rain drainage inlet and 334 mg/liter for the dry-weather inlet, respectively. The samples were taken from the middle of the aerator tank.

Sterile 1,000-ml screw-capped bottles were used for collecting the samples. They were transported in a cold-storage bag and used immediately in the laboratory. Serial dilutions of the samples (10⁻¹ to 10⁻⁸ using 0.5% NaCl-solution) were inoculated (0.1 ml spread plates) on the surface of nutrient agar (MERCK 5450) by means of sterile, L-shaped glass rods. Additionally, groundwater samples were drawn through membrane filters (0.2 μm pore size), which were subsequently incubated on

nutrient agar. After 5 days of incubation at 20°C, all separate colonies on an appropriate agar plate or membrane filter were isolated and cultivated on the same medium.

For each of the three biotopes, 90 pure cultures of bacteria were chosen randomly. Five-day old colonies were characterized morphologically, including 15 features with 67 variables. Colony characteristics included growth, diameter, margin, shape (elevation and edge), surface, transparency, translucency, consistency, diffusible pigment, pigmentation of colonies, odor, aerial mycelium, and substrate mycelium. The cell morphology was described following observations under a phase contrast microscope with 1,000-fold magnification (10 features with 37 variables) including length of the vegetative cell, diameter, relation between length and breadth, configuration, end of the cell, prostheca, granula, motility, grouping of the cells, and endospores. For the physiological characterization of the isolates, 70 liquid and 25 solid media were used in a microtitration system as described previously [6]. The results of these tests were evaluated after 3 and 6 days of incubation. Growth media and physiological tests included the following:

Carbohydrates (1%): glucose, galactose, fructose, mannose, arabinose, rhamnose, xylose, sorbose, methyl-glycopyranose, lactose, maltose, sucrose, cellobiose, trehalose, melibiose, raffinose, melezitose, starch, dextrin, glycogen, inulin, cellulose (0.05%), chitin (0.2%), xylan (0.5%), pectin (0.5%), and alginate.

Glycosides (1%): aesculin (0.1%), arbutin, amygdalin, and salicin.

Alcohols (1%): dulcitol, mannitol, sorbitol, inositol, and adonitol.

Organic salts: citrate (2%), acetate (2%), oxalate (0.1%), Na-tartrate (1%), gluconate (4%), malonate (3%), and hippurate (1%).

Amino acids (1%): glycine, serine, histidine, and glutamic acid.

Enzyme reactions (1%): lysine decarboxylase (0.2%), ornithine decarboxylase (0.2%), arginine dehydrolase, tryptophan deaminase, phenylalanine deaminase (0.2%), oxidase, catalase, urease, galactosidase, aminopeptidase, phosphatase (0.01%), and DNAase (0.2%).

Proteolytic activity (1%): gelatin, casein, and lecithin.

Lipolytic activity (1%): Tween 20, Tween 40, Tween 60, Tween 80.

Metabolism of nitrogen compounds (0.1%): NO₃-NO₂, NO₃-N₂, NO₂-N₂, NO₃-NH₃, and NO₂-NH₃.

Growth: 5% NaCl, 10% NaCl, 4°C, 20°C, 30°C, 37°C, 45°C, 54°C, pH 4, pH 7.

Selective agents: penicillin (0.2%), cyanide cetrinide, SS agar, and McConkey agar.

Metabolic products: H₂S, indole, acetoin, pyocyanine, and fluorescein.

All features were marked separately for each of the isolates using data check sheets. The data were stored on magnetic tape with the help of a reader and were further evaluated using a computer.

Empirical and numerical methods were used for the taxonomic evaluation. The overall similarity of the bacteria was determined by a mixed coefficient, e.g., simple matching coefficient applied for morphological data and an unnamed coefficient for physiological data [7]. The latter calculates the similarity level only on the basis of positive agreements.

The clusters were formed by the "single linkage" method on the basis of 30 morphological and 95 physiological features.

A detailed description of the practical use of the "single linkage" method and of the applied cluster algorithm is given in a previous paper [7]. For the calculation of the diversity index, the simple matching and the unnamed coefficient were used.

The empirical taxonomic evaluation and nomenclature were performed according to Bergey's manual [2], using additional identification keys [10, 11, 18, 25].

To observe whether there was a relationship between deterministic and numerical taxonomy, the bacterial names were added to the left side of the dendrograms obtained with numerical taxonomy.

The different physiological activities of populations were described by so-called activity indices [20]. In our case, "in vitro activity" expressed the ability of the bacteria to utilize a given number of substrates, using standard bacteriological test methods under laboratory conditions. The total in vitro activity of a community (collective total activity) was calculated as the quotient of the sum of all positive physiological features divided by the number of all the tested features (sum of all isolates and all features).

Table 1. Characters used to obtain the three physiological indices

Polymeric substrates	Nonpolymeric substrates	Nitrogen compounds
Cellulose	Glucose (oxidation and fermentation)	Arginin dihydrolase
Chitin		Lysine decarboxylase
Starch	Fructose	Ornithin decarboxylase
Xylan	Xylose	Phenylalanine deaminase
Pectin	Lactose (oxidation and fermentation)	Tryptophan deaminase
Glycogen		Urease
Alginate	Sucrose	Histidine
Lecithin	Cellobiose	Glutamate
Tween	Raffinose	Serine
Gelatin	Melizitose	Glycine
Casein	Dulcitol	Nitrite production
Dextrin	Inositol	Nitrogen gas production
Inulin	Citrate	Ammonification
DNA	Malonate	
	Oxalate	

Moreover, *in vitro* activity indices were calculated from data on the particular groups of substances: polymeric substrates, nonpolymeric substrates, and nitrogen compounds (Table 1). These three groups were treated separately from the total number of features.

The single *in vitro* activity of a community (collective single activity) was calculated as the quotient of the number of bacteria with a positive particular feature divided by the number of all isolates.

For the total activity of a single isolate all 95 physiological tests were taken into account. This average activity of an isolate was calculated by comparing the sum of all positive features with the total number of all 95 features tested. Subsequently, all bacteria isolated from a biotope were arranged according to their average activity [8].

The diversity index [Shannon-Weaver; 24] was used to express the overall homogeneity of all bacteria originating from a particular biotope. Therefore it was necessary to divide the community into certain groups, i.e., C_1, \dots, C_z .

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, it follows that the unit of average information, H , is defined by:

$$H = \ln N - \frac{1}{N} \sum_{i=1}^z N_i \ln N_i$$

where Z is the total number of clusters.

If the community is divided into N clusters ($Z = N$), each with only one isolate ($N_i = 1$), it is extremely inhomogeneous, and H takes its maximal value ($H = \ln N$).

If the community contains only one cluster ($Z = 1$) with N isolates ($N_i = N$), it is extremely homogeneous, and H takes its minimal value ($H = 0$).

The community was split into clusters C_1, \dots, C_z by dendrograms calculated on the basis of nine features (gelatin, starch, glucose, fructose, trehalose, cellobiose, sucrose, and lactose utilization, and oxidase production). All isolates with 80% similarity were summarized in a group, C_i , according to Mills and Wassel [21]. Samples of 15, 30, 45, 60, 70, 80, and of all 90 bacteria of a community were arranged randomly to determine whether the diversity index could be obtained with a smaller number of isolates. To measure the uncertainty in the valuation of H , a bootstrap estimate of the standard error [9] was used. For each sample size, five subsamples were drawn and the standard deviation was used as an estimate.

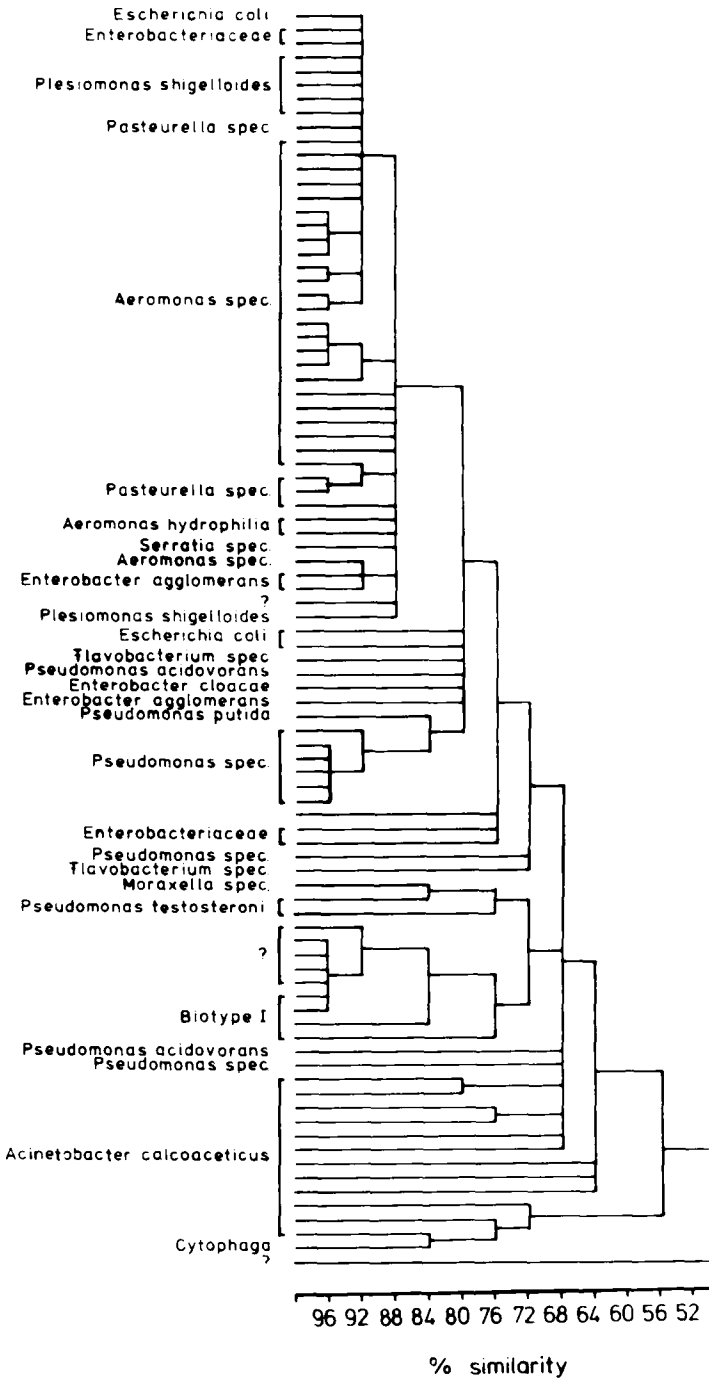


Fig. 1. Relationship of dendrogram to the deterministic results for bacteria isolated from activated sludge.

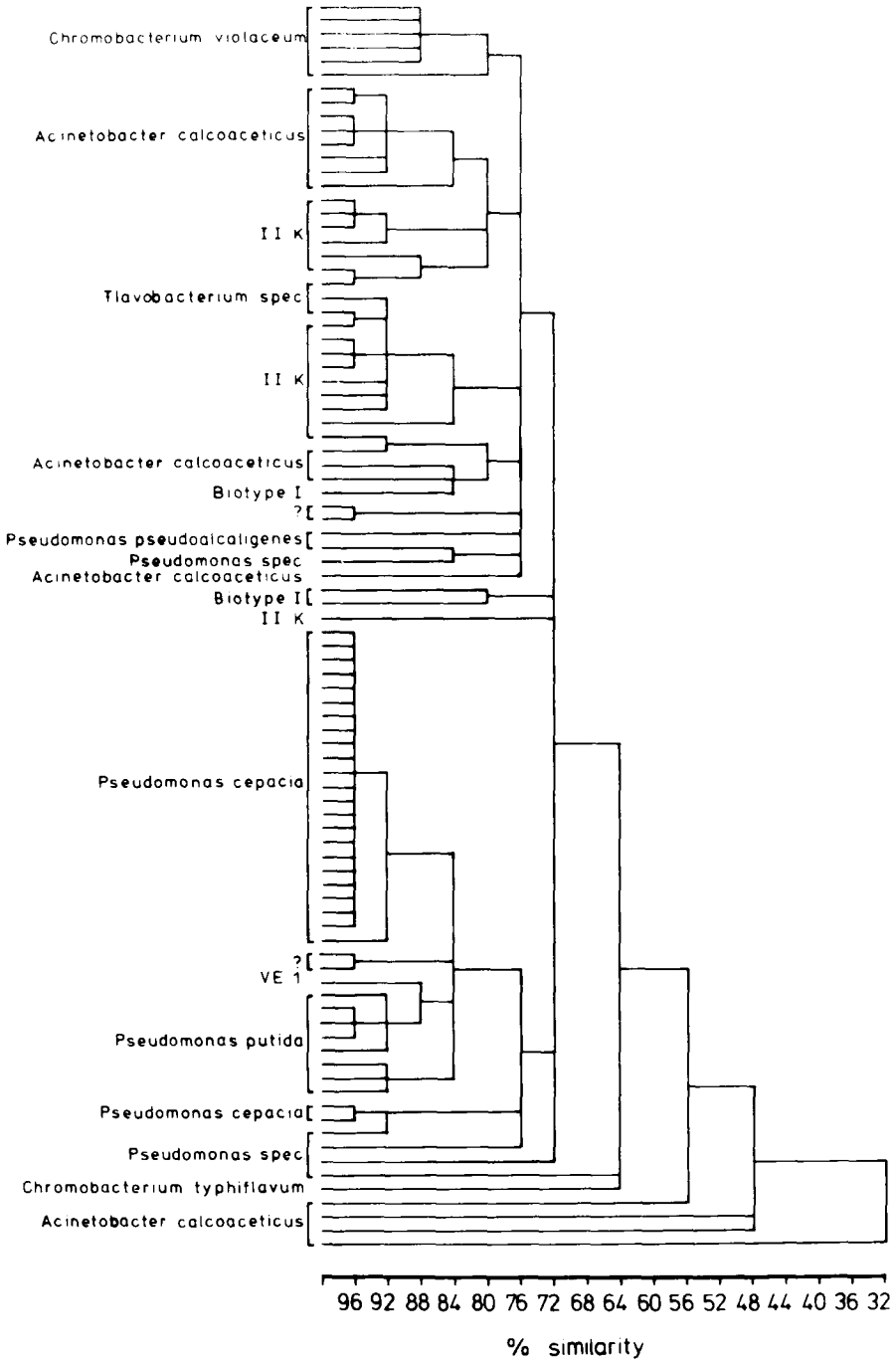


Fig. 2. Relationship of dendrogram to the deterministic results for the bacteria isolated from surface water.

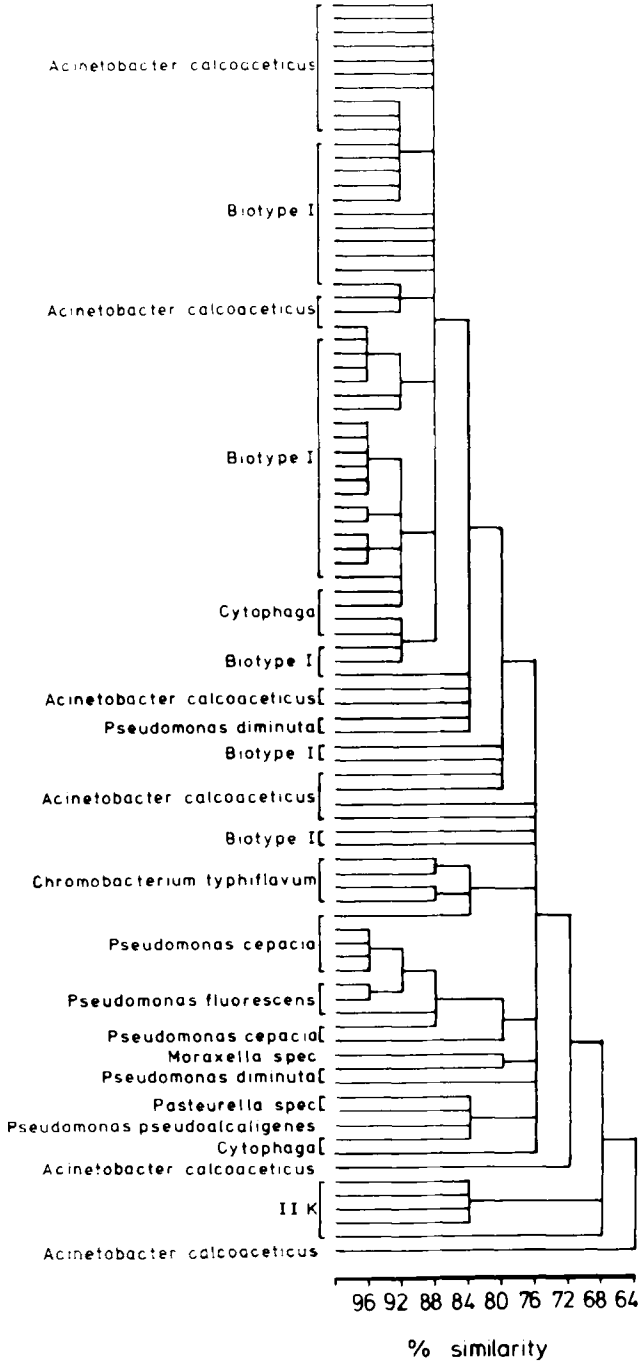


Fig. 3. Relationship of dendrogram to the deterministic results for the bacteria isolated from groundwater.

Results

Deterministic and Numerical Taxonomy

The isolates of the three examined biotopes were arranged according to their similarity in dendrograms by cluster analyses. Figures 1, 2, and 3 show the dendrograms of the isolates from activated sludge, surface water, and groundwater, respectively.

Most isolates of the groundwater were gram-negative, oxidase-negative rods, which showed in the applied physiological test systems no, or only a few, positive responses. They were grouped together and designated as Biotype I. Additional isolates belonged to the genera *Acinetobacter*, *Pseudomonas*, *Chromobacterium*, *Cytophaga*, *Pasteurella*, and *Moraxella*.

Fifty percent of the bacteria isolated from the surface water belonged to the genus *Pseudomonas*. The remainder consisted of members of the genera *Chromobacterium*, *Acinetobacter*, *Flavobacterium*, and the so-called Biotype I. Enterobacteriaceae were found neither in the groundwater nor surface water.

In contrast, the activated sludge isolates consisted mainly of members of the Enterobacteriaceae (*Escherichia*, *Serratia*, *Enterobacter*) and *Pseudomonas* species, as well as fermentative bacteria, which had similar reactions to the Enterobacteriaceae (*Plesiomonas*, *Aeromonas*, *Flavobacterium*). In some cases the genera *Moraxella*, *Pasteurella*, *Acinetobacter*, and *Cytophaga* were identified.

In Figs. 1 through 3, it can be seen that the dendrograms bear no consistent relationship to the deterministic identification. For example, the dendrogram of isolates from activated sludge (Fig. 1) showed that the genera *Escherichia*, *Plesiomonas*, *Pasteurella*, and *Aeromonas* belong to a single phenon on the level of 92% similarity, whereas in Fig. 2, nearly all species of the genus *Pseudomonas* isolated from surface water had a similarity of only 76%.

Characterization of Bacterial Communities by Different Activity Indices

The main in vitro activities of the communities (collective single activity) are given in Table 2. As expected, each substrate could only be utilized by a small fraction of isolates from the groundwater. In contrast, the proportion of isolates from the surface water and activated sludge able to utilize a particular substrate was higher, although the spectrum of substrates used by each biotope community was different. For example, the number of isolates with enzymatic activities such as dehydrolase and decarboxylase was relatively high for surface water, whereas the utilization of some substrates like fructose, chitin, casein, and Tween occurred most commonly with the isolates originating from activated sludge.

The activity indices (collective total activity) for the utilization of polymeric and nonpolymeric substrates, for enzymatic reactions of nitrogen metabolism, as well as the total activity of each community are given in Table 3 and are illustrated in Fig. 4 (all data in percentage). The highest collective total activity was found within the activated sludge isolates and the lowest within the bacteria

Table 2. Comparison of the proportion (%) of isolates in the communities showing particular activities (collective single activities)

Test or substrate	Active isolates (%)		
	Acti- vated sludge	Surface water	Ground- water
Arginin dihydrolase	16.7	55.6	12.2
Lysine decarboxylase	20.0	48.9	12.2
Ornithin decarboxylase	6.7	44.4	14.4
Citrate	30.0	45.6	10.0
Acetate	27.8	22.2	21.1
Oxalate	0.0	1.1	1.1
Fructose	71.1	51.1	16.7
Xylose	11.1	56.7	11.1
Sucrose	70.0	72.2	21.1
Starch	70.0	25.6	8.9
Chitin	31.1	5.6	3.3
Casein	65.6	41.1	23.3
Lecithin	45.6	34.4	24.4
Tween	26.6	3.3	9.4
Phosphatase	18.9	20.0	6.7
Nitrite production	73.3	16.7	27.8

Table 3. Comparison of the utilization indices and the collective total activity of the different communities (data in percentage)

Indices	Acti- vated sludge	Sur- face water	Ground- water
Polymeric substrates	32.0	12.8	9.0
Nonpolymeric substrates	32.3	26.7	5.8
Nitrogen compounds	17.1	15.9	6.9
Collective total activity	32.8	24.0	12.8

from groundwater (Fig. 4). Comparing the three biotopes, there were no significant differences in the relationship of total activities whether 95 or 49 characters were used. The main difference between the total activity of activated sludge bacteria and those derived from the surface water was due to the utilization of polymeric substrates, whereas the difference between surface water bacteria and groundwater bacteria was caused by the utilization of nonpolymeric substrates and nitrogen compounds.

Physiological Activity of the Isolates Related to the Total Community

The total activity of a community can also be expressed in terms of the single characters or character groups of the single isolates, and the bacteria can be

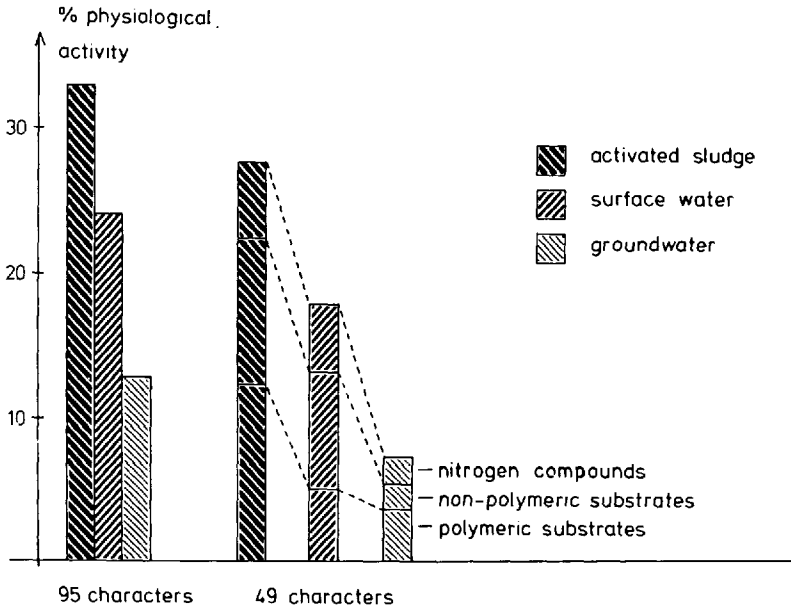


Fig. 4. Physiological activity of the bacteria isolated from activated sludge, surface water, and groundwater (in percent). Comparison of the results based on 95 characters (collective total activity) with those of 49 characters including the three different utilization indices (utilization of polymeric and nonpolymeric substrates, tests for nitrogen compound metabolism).

arranged according to increasing metabolic activities. Such an arrangement is given in Fig. 5. The distribution of the isolates results in different patterns for the three different biotopes. The groundwater contained numerous bacteria with low activity (below 10%), whereas the activities of many activated sludge bacteria ranged between 40% and 50%. The pattern obtained for surface water consists of two parts, indicating the occurrence of two different bacterial communities.

Diversity Indices of the Communities Relative to Selected Physiological Characters

A further possible way of describing the heterogeneity and homogeneity of microbial communities and their changes is by means of a diversity index. The diversity indices were calculated on the basis of 80% similarity level. The results (Fig. 6) show the clear difference between the diversity indices of the examined communities if the unnamed coefficient was used. In this case the diversity indices of random samples with more than 45 isolates varied insignificantly for all the three biotopes examined. The application of the simple matching coefficient led to a significant decrease of the diversity indices for the microbial communities of surface water and groundwater. On the strength of negative agreements which were taken into consideration, especially the physiological inactive members of the groundwater showed a very low diversity index.

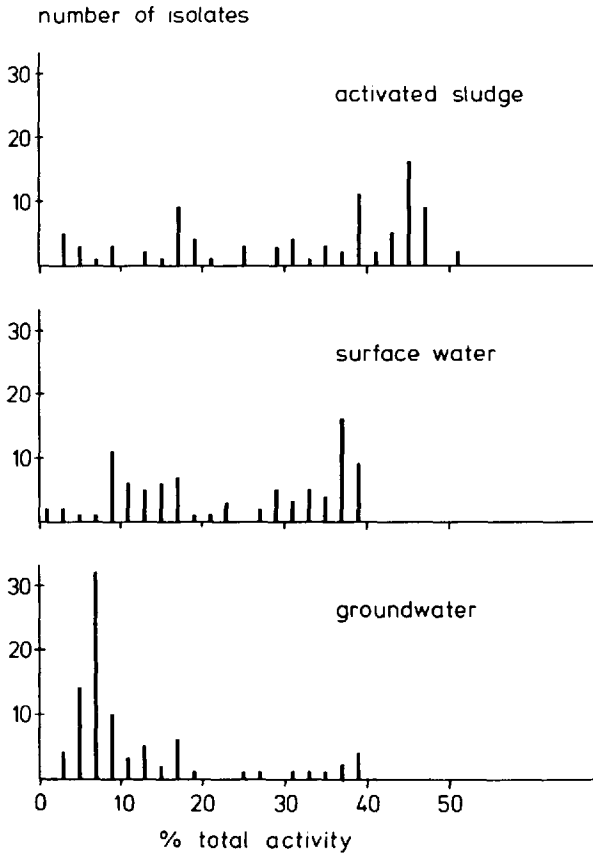


Fig. 5. Distribution of the isolates according to their total physiological activity.

Quantitative Aspects of Ecological Investigations of Water

All results mentioned above are based on qualitative results of the tests for *in vitro* utilization and enzymatic activities which might be theoretically available *in situ*. On the other hand, the true utilization and its rate *in situ* is influenced mainly by the number of bacteria in the biotope. These numbers were evaluated as viable counts on nutrient agar (5 days at 20°C), which showed that the three biotopes differed greatly in bacterial numbers (Table 4).

Discussion

The determination of a large number of features for each isolate increases the problems of comparing and handling the data. Therefore, numerical principles need to be applied, but these are disadvantageous in that all features are equally weighted and regarded as totally independent [1], and this may not reflect the true situation. Intensive research in the field of numerical taxonomy has worked out the limits of comparison between numerical phenons and taxonomic units

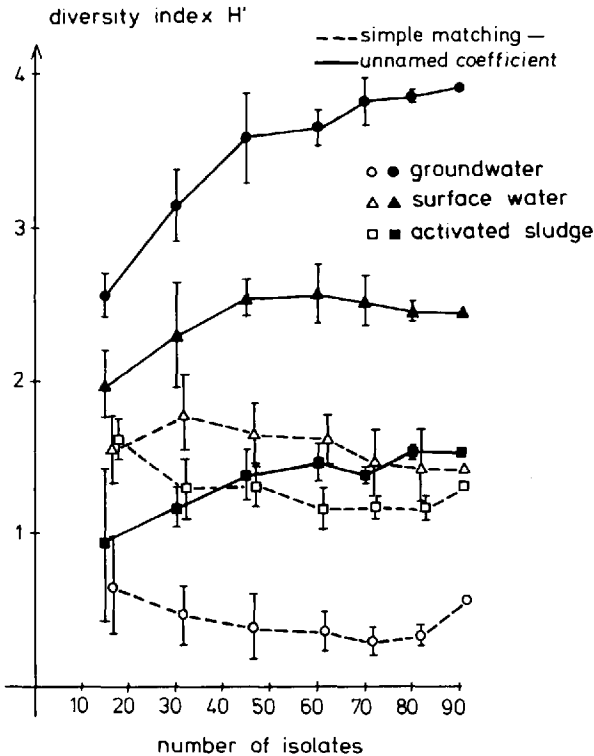


Fig. 6. Comparison of the average diversity index of the community depending on the number of isolates and the different coefficients (unnamed and simple matching), respectively. The standard deviation for each diversity index is given in the figure.

Table 4. Results of the viable count on nutrient agar

	Activated sludge	Surface water	Ground water
Viable cells/ml	4.95×10^7	1.01×10^3	4.7

of deterministic identification systems [3, 4, 14, 17, 22]. As a replacement for deterministic procedures, numerical taxonomy is only applicable in a limited way. In only a few cases, phenons can coincide with the accepted names at the generic and species level, i.e., similarity groups of numerical procedures [4, 14]. Nevertheless, for ecological investigations of aquatic biotopes, numerical taxonomy can be used as a first survey of the similarity structure of a community. It shows, for example, remarkably large phenons or gives a first hint on the heterogeneity of the microbial population.

If a large number of physiological features of the isolates can be determined (as is necessary for numerical taxonomy), it is possible to calculate the activity indices as described by Krieg and Lockhart [14]. In this study, three indices (collective total activity), which gave an indication of the utilization of polymeric and nonpolymeric substrates and metabolism of nitrogen compounds, were used and could distinguish the different bacterial communities investi-

gated (Fig. 4). As expected, the sample of activated sludge had the highest levels of activity, and the groundwater isolates had the lowest, with respect to all three indices.

In general the same conclusions can be drawn regarding the frequency of single characters (collective single activity), but some differences did occur, e.g., the proportion of isolates with decarboxylases was highest in the population from surface water. These results correlate with the taxonomic ones, because these activities are characteristic of *Pseudomonas* spp. (Fig. 2).

Another way of utilizing the data is to order the isolated bacteria according to their total activity. This is, however, only useful if about 100 isolates of a biotope are tested (Fig. 5). The results are similar to those of the activity indices and show clearly that the bacterial activity in a community could vary distinctly among different sub-populations. In addition, the community could be divided into two groups with respect to the total activity. The results indicate that all biotopes contained active, less active, and inactive isolates, but the number of organisms in each group varies according to the nutrient status of the biotope. The "heterotrophic" diversity index [24], similar to that applied to plant and animal ecology, indicated the extent of heterogeneity of the isolates relative to selected physiological characters.

For the interpretation of the dendrograms it is necessary to know which kind of cluster metric was applied. Theoretically, a low diversity index is an indicator of some form of environmental stress, which selects a specialized homogenous community [13]. This stress can be produced by extreme pollution (e.g., activated sludge) or in water with extremely low nutrient content (e.g., groundwater). However, diversity index values can also be affected by the conditions of the test in several ways. First, in our calculation, the use of only seven features may create the appearance of low diversity as an artifact of the tests chosen [13]. Second, it became evident that the diversity index was strongly dependent on the applied cluster metric. Third, the diversity index can be influenced by selective isolation methods. As with Bianchi and Bianchi [1], it was observed that 45 isolates were sufficient to calculate the diversity index. A further enlargement of the number of isolates did not cause significant changes of the indices. Moreover, in our opinion the diversity index seems to be a parameter that cannot be easily interpreted. Even the literature contains an extensive discussion of the controversy of the interpretation of data obtained in these types of studies [12, 21].

For statements about the true turnover rate of organic material in a biotope, it is necessary to consider not only the qualitative evaluation methods (related to 90 isolates) but also the number of viable bacteria in the biotope. The extreme differences that were found in the biotopes examined make it impossible to add these data as factors for the activity indices. Therefore it seems to be convenient to separate the quantitative and the qualitative results and to regard the number of bacteria only as an additional parameter of ecological investigation.

The main problem with all ecological examinations of water is the limitation caused by the method of isolation. Especially groundwater microorganisms, which are adapted to extreme environments with low nutrient concentrations. On conventional nutrient agar, only 1% to 10% of the original population can

be isolated [23]. Thus, only a small nonrepresentative sample is available for physiological characterization [23]. On the other hand, although a standard isolation and testing procedure allows the assessment of only a limited number of genera, these may occur in all biotopes. If a specific test procedure had been developed for each biotope to maximize counts, a comparison of the results, particularly with regards to the physiological properties of the bacteria, would not have been possible. So the practical and economical aspects necessitate standardized test systems for investigations, which are always compromises between scientific demands and practicability.

Despite the limits of the methods presented, it was possible to obtain information about microbial communities and processes taking place in aquatic environments, which is particularly necessary for the study of water treatment processes [23]. In microbial ecology one should bear in mind that the use of terms describing a bacterial community and its activity are of limited value in water technology unless they are easily understood by all water technologists and not just by microbial workers in the field of water technology.

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