Aerobic Heterotrophic Bacterial Populations of Sewage and Activated Sludge

V. Analysis of Population Structure and Activity

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Two procedures, the confidence interval method and Mountford's index, were tested in analyses of the microbial populations of 11 laboratory activated sludges acclimated to aromatic compounds. The two methods gave somewhat different results but indicated that the populations were quite dissimilar. The activity of seven of the sludges correlated well with the population structure. Some considerations in analysis of microbial population structure are discussed.

In previous investigations (12, 13), we tested methods for enumeration and characterization of the bacterial populations in actual and model waste treatment systems. In this report, somewhat different analyses of a similar problem of population adaptation are described.

Because of the breadth and complexity of adaptation mechanisms in biological waste treatment or, for that matter, in any mixed population of microbes, and because of the laborious methods required, there have been no generally accepted methods of approach to population analyses for bacteria. Methods of population analyses have been more highly developed for the morphologically differentiated forms of biota than for the bacteria because of the relative ease of classification, whereas the laborious efforts of characterizing bacteria are often defeated by the incompleteness of taxonomic systems.

In recent years, several investigators have reported on the microbial populations of activated sludges (1, 9, 17). In these studies, the emphasis in the sphere of population distribution was essentially on the dominance or the variety of identifiable microbial species; however, more precise and sensitive methods of population analysis would be desirable.

In our previous work (13), to avoid the largely fruitless efforts along classic taxonomic lines, we availed ourselves of Lochhead's nutritional classification (8). When, however, we investigated another microbial adaptation system exemplified by an activated sludge (14), a different method seemed to hold some promise.

The experimental activated sludge in question (14) was developed on glucose, nutrient broth solids, and salts ("artificial waste") seeded with settled raw sewage. After a period, aromatic compounds were gradually substituted for the glucose and nutrient broth solids until each of 11 units was subsisting solely on an aromatic compound, ammonium phosphate, and other salts. It appeared that a significant and more sensitive classification of organisms for our requirements would be based on the ability to utilize the aromatic compounds. This report records attempts to describe microbial populations and to evaluate the direction and degree of adaptation by the populations when given the opportunity to utilize 11 aromatic compounds as exogenous sources of carbon. Analyses of the data by a statistical method used in ecological population evaluations provided quantitative and objective evidence that the resulting populations were quite divergent in their microbial composition during the period of investigation. An essential part of the method was to utilize an arbitrary system of classification created for the purpose.

MATERIALS AND METHODS

Activated sludges were developed and acclimated to aromatic compounds as described by McKinney, Tomlinson, and Wilcox (10). The detailed procedure of maintaining the acclimated sludge units and methods for obtaining plate counts on various aromatic media were described in a previous report (14). Individual fill-and-draw sludge units were acclimated to one of the following aromatic substrates: sodium benzoate, sodium mandelate, sodium phenylacetate,
sodium p-hydroxybenzoate, anthranilic acid, benzyl alcohol, o-cresol, m-cresol, p-cresol, catechol and phenol.

**Replica plating.** The method of Lederberg and Lederberg (7) was used to transfer the colonies from a master plate to a series of aromatic plating media to permit ascertaining whether aromatic compounds would support growth. The applicability of this method for the characterization of heterogeneous populations was reported earlier (13).

**Master plates.** Samples of acclimated sludge were homogenized, diluted, and plated on homologous medium (i.e., a medium containing the aromatic compound to which the sludge had been acclimated) containing glucose, 0.5 g; nutrient broth, 0.5 g; potassium phosphate (dibasic), 1.0 g; triammonium phosphate, 0.325 g; sodium chloride, 0.05 g; calcium chloride, 0.05 g; magnesium sulfate, 0.05 g; ferric chloride, 0.01 g; Lonagro no. 2, 8.5 g; distilled water, 1 liter; and the specific aromatic substrate, 0.5 g. However, catechol sludge extract medium was used for plating catechol-acclimated sludge, because the homologous medium containing catechol did not support the growth of the catechol sludge bacteria (14). The plates were incubated at 28 C for 7 days. Plates made at 10^5 dilution with discrete colonies were chosen as the master plates for replica plating, because master plates from this dilution with these sludges contained the requisite number of colonies that could be handled with negligible smearing during replica plating.

**Respirometry.** Oxygen uptake of the acclimated sludges on aromatic substrates was determined by the method previously described (14). From the observed uptake, the percentages of theoretical oxygen uptake were calculated on the basis of equimolar substrate concentrations and theoretically complete oxidation. This method permitted comparisons between sludges and with the results of McKinney et al. (10).

**RESULTS AND DISCUSSION**

**Nature of the population of aromatic-acclimated sludges.** The gross and microscopic differences between the sludges acclimated to aromatic compounds were strongly indicative of profound changes during the adaptation of the laboratory sludge. We characterized the individual microorganisms harbored by the sludges by their abilities to utilize for growth the 11 aromatic compounds. Colonies on master plates from each of the 11 aromatic-acclimated sludges were transferred to all the heterologous aromatic agars (containing salts and the specific aromatic compound only) by replica plating. All the plates were incubated at 28 C for 7 days. The colonies of each master plate then were matched against the colonies developed irrespective of the amount of growth on the corresponding 10 heterologous replica plates. Colonies thus determined to be utilizing a particular set of substrates were categorized by a “type code.” The viable count of each type in a population is termed the “frequency.”

Table 1 gives the type codes assigned to colonies and the frequencies of such types in the sludges.

Evidently, from the distribution of the types (Table 1), the acclimated populations had diverged from the original and from one another. The environmental selection of the types was very severe in certain cases, as with the sludges acclimated to o-cresol and m-cresol, yet the surviving types in these cases were versatile in substrate utilization.

All the types, including those on the catechol master plate, that grew on a particular master plate utilized the specific aromatic substrate contained in that master plate; however, many types that did not appear on a homologous master plate were able to utilize that particular substrate; i.e., they were present in higher numbers in heterologously acclimated sludges than in the homologously acclimated sludge. For example, of the 44 types able to utilize benzoate, only 14 grew in the benzoate-acclimated sludge, whereas the remaining 30 grew in the sludges acclimated to aromatics other than benzoate. Thus, enrichment with the aromatic compounds established only a minority of types capable of utilizing the aromatic at the dilutions of the master plate inocula.

**Population structure of the acclimated sludges and substrate utilization ability.** To evaluate from the population data the abilities of the bacteria of the acclimated sludges to oxidize the aromatic substrates used in this study, we computed a “substrate utilization ability index” (SUAI) for each sludge-substrate combination and compared it with the “oxidation index” (OI) for the combination. A high SUAI of a sludge for a given substrate indicates a high potential for its utilization based on the abilities of the individuals of the population. A high OI value indicated that the sludge actually had the ability to oxidize the corresponding substrate to a relatively high degree. The results of respirometric tests with the sludge-substrate combinations are tabulated as percentages of theoretical oxygen uptakes in Table 2.

**Computation of SUAI.** The SUAI of any given sludge-substrate combination is obtained by SUAI = Y/X where X is total number of organisms in the sludge, and Y is the sum of the frequencies of the types in the sludge able to use the aromatic substrate under consideration (see Table 1).

**Computation of OI.** The OI for any sludge-
substrate combination is given by the following: 
\[ OI = \%T/\%S, \]
where \( \%T \) is per cent theoretical oxygen uptake exerted on the heterologous substrate by the acclimated sludge under consideration, and \( \%S \) is per cent theoretical oxygen uptake exerted by the acclimated sludge under consideration on its homologous substrate, e.g., benzoate acclimated sludge versus benzoate, etc.

**Table 1.** Type codes designated on the basis of substrate utilization and frequencies of microbial types on master plates from sludges acclimated to aromatic compounds

| Type code | Substrates utilized* | Frequencies in aromatic sludges (10^5/ml) |
|-----------|----------------------|------------------------------------------|
|           | Benzene | Mandelate | Phenylacetic acid | o-Hydroxybenzoate | Antranilic acid | Benzylandanol | o-Cresol | m-Cresol | p-Cresol | Catechol | Phenol |
| 1         | 1-11    |           |                |                 |               |              |          |          |         |          |        | 1      |
| 2         | 1-9, 11 | 1         |                |                 |               |              |          |          |         |          |        | 1      |
| 3         | 1-8, 10, 11 | 18 |                |                 |               |              |          |          |         |          |        | 5      |
| 4         | 1-7, 9-11 | 1          |                 |                 |               |              |          |          |         |          |        | 2      |
| 5         | 1-6, 8-11 | 1          |                 |                 |               |              |          |          |         |          |        | 3      |
| 6         | 1-9     |           |                |                 |               |              |          |          |         |          |        | 1      |
| 7         | 1-8, 11 | 1         |                |                 |               |              |          |          |         |          |        | 2      |
| 8         | 1-7, 9, 11 | 1          |                 |                 |               |              |          |          |         |          |        | 2      |
| 9         | 1-7, 10, 11 | 2          |                 |                 |               |              |          |          |         |          |        | 1      |
| 10        | 1-7, 9, 10 | 1          |                 |                 |               |              |          |          |         |          |        | 10     |
| 11        | 1-6, 8, 9, 11 | 3          |                 |                 |               |              |          |          |         |          |        | 1      |
| 12        | 1-6, 8, 10, 11 | 1          |                 |                 |               |              |          |          |         |          |        | 1      |
| 13        | 1-6, 9-11 | 1          |                 |                 |               |              |          |          |         |          |        | 1      |
| 14        | 1-5, 7-9, 11 | 1          |                 |                 |               |              |          |          |         |          |        | 1      |
| 15        | 1-4, 6-9, 11 | 2          |                 |                 |               |              |          |          |         |          |        | 1      |
| 16        | 1, 2, 4-9, 11 | 1          |                 |                 |               |              |          |          |         |          |        | 1      |
| 17        | 1-8     | 1          |                 |                 |               |              |          |          |         |          |        | 1      |
| 18        | 1-7, 11 | 5         | 8                |                 |               |              |          |          |         |          |        | 61     |
| 19        | 1-7, 10 | 1          |                 |                 |               |              |          |          |         |          |        | 1      |
| 20        | 1-6, 8, 9 | 1         |                 |                 |               |              |          |          |         |          |        | 1      |
| 21        | 1-6, 9, 11 | 17        |                 |                 |               |              |          |          |         |          |        | 4      |
| 22        | 1-6, 9, 10 | 1          |                 |                 |               |              |          |          |         |          |        | 45     |
| 23        | 1-6, 10, 11 | 3          |                 |                 |               |              |          |          |         |          |        | 1      |
| 24        | 1-4, 6-8, 11 | 1          |                 |                 |               |              |          |          |         |          |        | 1      |
| 25        | 1-4, 6, 9-11 | 1          |                 |                 |               |              |          |          |         |          |        | 1      |
| 26        | 1-7     | 1         | 7                |                 |               |              |          |          |         |          |        | 1      |
| 27        | 1-6, 11 | 4         | 3                |                 |               |              |          |          |         |          |        | 2      |
| 28        | 1-6, 10 | 1         |                 |                 |               |              |          |          |         |          |        | 16     |
| 29        | 1-6, 9  | 5         | 2                |                 |               |              |          |          |         |          |        | 1      |
| 30        | 1-4, 6-8 | 1         |                 |                 |               |              |          |          |         |          |        | 3      |
| 31        | 1-4, 6, 7 | 1         |                 |                 |               |              |          |          |         |          |        | 1      |
| 32        | 1-4, 6, 9, 11 | 11       |                 |                 |               |              |          |          |         |          |        | 2      |
| 33        | 1-6     | 2         |                 |                 |               |              |          |          |         |          |        | 3      |
| 34        | 1-4, 6, 7 | 1         |                 |                 |               |              |          |          |         |          |        | 2      |
| 35        | 1-4, 6, 11 | 4         |                 |                 |               |              |          |          |         |          |        | 2      |
| 36        | 1-4, 6, 11 | 14        |                 |                 |               |              |          |          |         |          |        | 3      |
| 37        | 1-2, 4-6, 11 | 10       |                 |                 |               |              |          |          |         |          |        | 2      |
| 38        | 1-2, 6, 7, 10, 11 | 1         |                 |                 |               |              |          |          |         |          |        | 1      |
| 39        | 1-2, 6, 7, 11 | 1         |                 |                 |               |              |          |          |         |          |        | 1      |
| 40        | 1-4     | 1         |                 |                 |               |              |          |          |         |          |        | 2      |
| 41        | 1-2     | 2         |                 |                 |               |              |          |          |         |          |        | 2      |
| 42        | 1-2, 6, 7, 11 | 1         |                 |                 |               |              |          |          |         |          |        | 1      |
| 43        | 1-4     | 4         |                 |                 |               |              |          |          |         |          |        | 1      |
| 44        | 1-3     | 1         |                 |                 |               |              |          |          |         |          |        | 1      |
| 45        | 1-2, 4, 6 | 4         |                 |                 |               |              |          |          |         |          |        | 1      |
| 46        | 2-4, 6  | 3         |                 |                 |               |              |          |          |         |          |        | 1      |
| 47        | 1-2     | 2         |                 |                 |               |              |          |          |         |          |        | 1      |
| 48        | 1-2     | 2         |                 |                 |               |              |          |          |         |          |        | 1      |

*Substrate numbers: 1, sodium benzoate; 2, sodium mandelate; 3, sodium phenylacetate; 4, sodium p-hydroxybenzoate; 5, anthranilic acid; 6, benzylandanol; 7, o-cresol; 8, m-cresol; 9, p-cresol; 10, catechol; 11, phenol.
### Table 2. Oxygen uptake of sludges acclimated to aromatic compounds

| Test substrate          | Per cent theoretical oxygen uptake by sludge acclimated to the compound indicated in 6 hr<sup>a</sup> |
|------------------------|------------------------------------------------------------------------------------------------|
|                        | Benzoate | Mandelate | Sodium phenyl-acetate | p-Hydroxybenzoate | Anthranilate | Benzy alcohol | o-Cresol | m-Cresol | p-Cresol | Catechol | Phenol |
| Endogenous<sup>b</sup> | 1.3      | 1.78      | 2.19                | 2.3               | 3.6         | 1.07         | 0.95      | 1.6      | 3        | 0.65     | 1.4     |
| Benzoate               | 10.1     | 8.6       | 12.0                | 1.5               | 10.4        | 10.7         | 10        | 11       | 12.6     | 11.5     | 11.2    |
| Mandelate              | 14       | 16.3      | 10.4                | 6.7               | 6.1         | 1.1          | 3.1       | 12.2     | 11.0     | 5.9      | 12.1    |
| Sodium phenyl-acetate  | 11.3     | 11.5      | 10.7                | 1.7               | 15.6        | 11.8         | 5.1       | 15.7     | 16.4     | 13.2     | 14.1    |
| p-Hydroxybenzoate      | 13.5     | 1.2       | 15.7                | 13.2              | 16.1        | 19.8         | 11.9      | 28.8     | 23.9     | 2.4      | 13.4    |
| Anthranilic acid       | 1        | 17.7      | 13.5                | 11.5              | 16.7        | 15.8         | 16.5      | 14.8     | 10.7     | 16.7     | 16.5    |
| Benzy alcohol          | 11       | 14.4      | 9.4                 | 11.8              | 7.1         | 19.2         | 10.4      | 19.0     | 14.0     | 10.6     | 15.0    |
| o-Cresol               | 4.2      | 4.3       | 1.1                 | 1.2               | 5           | 10.5         | 12.2      | 15.4     | 4.7      | 10.5     | 10.5    |
| m-Cresol               | 0.7      | 2.7       | 2.1                 | 4.6               | 5.5         | 9.0          | 6.9       | 15.5     | 6.8      | 8.5      | 11.2    |
| p-Cresol               | 3        | 4.8       | 0.7                 | 2.43              | 7.4         | 10.4         | 5.1       | 17.2     | 15.8     | 11       | 14.3    |
| Catechol               | 10.5     | 11.8      | 9.7                 | 9.6               | 10.3        | 10.6         | 11.5      | 12.5     | 11       | 11.5     | 13.2    |
| Phenol                 | 4.2      | 10.2      | 6.3                 | 7.7               | 12.7        |              | 16.4      | 14.6     | 8.3      | 18.2     | 12.5    |

<sup>a</sup> All values calculated on basis of equimolar substrate concentrations and theoretically complete oxidation.

<sup>b</sup> [O<sub>2</sub> (mg/liter) endogenous/O<sub>2</sub> (mg/liter) for theoretical oxidation of homologous substrate] × 100.

The SUAI and the corresponding OI values are presented in Table 3. When the values of the computed OI values were higher than 1.00, they were presented as 1.000 because we considered that the sludges under such situations had at least as much ability to oxidize the heterologous substrates as they had the homologous substrates.

From Table 3, the regression of OI on SUAI of the various acclimated sludges was obtained by fitting straight lines by the method of Johnston (5). Unlike the least squares regression method, often used for fitting a straight line between two variables where only one variable is subject to error, this method takes into account the error involved in measuring the two variables of our study, namely SUAI (X) and OI (Y). In computing the slope of the regression line by this method, we assumed the ratio of error variances of the two variables as 1.0. Table 3 shows the equations relating the SUAI and OI values of the various acclimated sludges.

Inasmuch as there was a reasonable conformity to the relationship, \( Y = \beta X + \alpha \) (\( \beta = 1, \alpha = 0 \)), except for the sludges acclimated to anthranilic acid, o-cresol, catechol, and phenol, it appeared in the greater number of cases that there was a rather good correlation between the frequencies of types in the sludges and their abilities to oxidize particular aromatic substrates. For the exceptions, the significantly positive OI intercept values (\( \alpha \)) indicated that something was amiss in that where the type frequency was zero (SUAI = 0), there should have been no oxidation. It was found that some of the sludge bacteria were unable to grow on unsupplemented, homologous master plates (12). Unfortunately, it was impossible at the time to utilize the supplementation. In the case of catechol organisms, which grew in the sludge but not on plates, there was certainly autooxidation, but partial cooxidation without growth cannot be excluded.

Where there were many potentially competent cells in the sludges (high SUAI) but actual oxidation was relatively low (e.g., benzyl alcohol sludge oxidizing mandelate), an explanation may lie in the possibility that we are dealing with slow oxidizers and slow growers which had ample time to develop in the acclimated sludges and on enumeration plates but could not show much activity during the 6-hr respiration tests. As to the degree of oxidation, it must be obvious that even the maximum percentage of oxidation must necessarily appear to be low in relation to the theoretically complete oxidation chosen as a fixed base of reference. The short respiration period was chosen to minimize effects of growth.

When the implicit assumptions which underlie attempts to correlate viable counts with activity are examined, it is perhaps most surprising that there was such a high degree of correlation in the case of the seven sludges. Although activity might be expected to follow the counts broadly, it is clear that the viable count need not be a measure of biomass, nor need the unit of biomass of different types be equally active in the same or in
| Sludge acclimated to | Indexes | Substrates<sup>a</sup> | Regression equation<sup>c</sup> ($y = \beta x + \alpha$) |
|----------------------|---------|------------------------|----------------------------------|
|                      |         | Benzoate | Mandelate | Sodium phenyl-acetate | $\beta$-Hydroxybenzoate | Anthranilic acid | Benzylic alcohol | $\alpha$-Cresol | $m$-Cresol | $\beta$-Cresol | Phenol |
| Benzoate             | SUAI    | 1        | 1         | 0.988              | 0.952              | 0.226              | 1        | 0.297              | 0.024 | 0.024 | 0.452 |         |
|                      | OI      | 1        | 1<sup>b</sup> | 1<sup>b</sup> | 1<sup>b</sup> | 0.099              | 1        | 0.416              | 0.069 | 0.297 | 0.416 |         |
| Mandelate            | SUAI    | 1        | 1         | 1                  | 0.445              | 1        | 0.510              | 0.27        | 0.243 | 0.98  |         |
|                      | OI      | 0.527    | 1         | 0.706              | 0.074              | 1<sup>b</sup> | 0.885              | 0.264        | 0.167 | 0.294 | 0.626 |         |
| Sodium phenyl-acetate| SUAI    | 1        | 1         | 0.989              | 0.399              | 0.978              | 1        | 0.258              | 0.043 | 0.032 | 0.29  |         |
|                      | OI      | 0.174    | 0.114     | 0.51               | 1                  | 0.88               | 0.895     | 0.09                | 0.348 | 0.184 | 0.585 |         |
| $\beta$-Hydroxybenzoate | SUAI  | 0.474    | 1         | 1                  | 1                  | 0.664              | 1        | 0.72                | 0.615 | 0.641 | 0.82  |         |
|                      | OI      | 0.625    | 0.365     | 0.935              | 0.965              | 1                  | 0.425     | 0.299              | 0.329 | 0.443 | 0.76  |         |
| Anthranilic acid     | SUAI    | 1        | 1         | 1                  | 1                  | 1                  | 1        | 0.562              | 0.261 | 0.644 | 0.931 |         |
|                      | OI      | 0.56     | 0.057     | 0.62               | 1<sup>b</sup> | 0.825              | 1        | 0.55                | 0.468 | 0.543 |       |         |
| Benzylic alcohol     | SUAI    | 1        | 1         | 1                  | 1                  | 1                  | 1        | 1                  | 0.565 | 0.418 | 1     |         |
|                      | OI      | 0.82     | 0.254     | 0.418              | 1.975              | 1<sup>b</sup> | 0.855              | 1        | 0.975 |       | 0.498  |         |
| $\alpha$-Cresol      | SUAI    | 1        | 1         | 1                  | 1                  | 1                  | 1        | 1                  | 0.565 | 0.418 | 1     |       |
|                      | OI      | 0.82     | 0.254     | 0.418              | 1.975              | 1<sup>b</sup> | 0.855              | 1        | 0.975 |       | 0.498  |         |
| $m$-Cresol           | SUAI    | 1        | 1         | 1                  | 1                  | 1                  | 1        | 1                  | 0.565 | 0.418 | 1     |       |
|                      | OI      | 0.71     | 0.79      | 1.10               | 1.15               | 1<sup>b</sup> | 0.955              | 1<sup>b</sup> | 0.99 | 1     | 0.81  |       |
| $\beta$-Cresol       | SUAI    | 1        | 1         | 1                  | 1                  | 1                  | 1        | 1                  | 0.565 | 0.418 | 1     |       |
|                      | OI      | 0.799    | 0.696     | 1.1<sup>b</sup> | 1<sup>b</sup> | 0.676              | 0.885     | 0.297              | 0.431 | 0.696 | 0.091 |       |
| Catechol             | SUAI    | 1        | 1         | 1                  | 1                  | 1                  | 1        | 0.178              | 0.056 | 0.2   | 0.78  |       |
|                      | OI      | 1        | 0.512     | 0.209              | 1<sup>b</sup> | 0.925              | 0.915     | 0.74                | 0.96  | 1<sup>b</sup> | 0.447  |       |
| Phenol               | SUAI    | 1        | 1         | 1                  | 1                  | 1                  | 1        | 0.726              | 0.094 | 0.142 | 1     |       |
|                      | OI      | 0.895    | 0.97      | 1<sup>b</sup> | 1<sup>b</sup> | 1<sup>b</sup> | 1<sup>b</sup> | 1<sup>b</sup> | 0.84  | 0.895 | 1<sup>b</sup> | 0.478  |

<sup>a</sup> SUAI and OI for all the sludges with respect to catechol as the substrate were not given because the characterization of the acclimated-sludge bacteria on catechol-agar medium was unsuccessful. For blanks, all values were considered ‘0’. For each sludge-substrate combination a value of ‘0’ was assumed for both SUAI and OI on the premise that when there are no organisms present in a system there will not be any oxygen uptake. We made this assumption to include the datum point ‘0/0’ in the regression analysis of each acclimated sludge.

<sup>b</sup> These values were slightly more than one and were corrected to one.

<sup>c</sup> SUAI = $x$ and OI = $y$ in the regression equation.
different media. Furthermore, the counts of separated colonies may not reflect the biochemical interactions of flocs in suspension. The assumption that the unamended aromatic agars were capable of supporting the growth of all the organisms in the sludges was demonstrably untrue; however, had the members on the master plates been increased by supplementation in the four deviating sludges, their data might have shown a higher degree of correlation with respiration.

Comparative study of the structure of population of the aromatic-acclimated sludges. Objective criteria in making comparisons between the different populations were needed. Except in the case of the few extremes, comparisons for judging similarity or difference may be made in terms of several criteria; however, in this report, two methods based on degree of similarity of distribution of bacterial types are discussed, the confidence limit method and Mountford’s index of similarity.

Analysis of unadjusted population data. In this analysis, the activated sludges are compared to one another in a series of pairs, taking into account the bacterial types and the frequency of their occurrence on the master plates. The term “unadjusted” is used here to denote that the plate counts were used in all the comparisons without scaling to a standard number as mentioned later.

Since there were 11 sludges to be compared in pairs, there were 11! / 9! x 2! = 55 comparisons. In each comparison of the data of Table 1, the differences between the frequencies of each type were recorded as absolute numerical values. When a type was absent from a master plate, it was given a frequency of zero. The total number of differences in each comparison of paired sludges was obtained by adding all the individual differences between the types.

When arranged as a frequency distribution, it appeared that the differences followed approximately a normal distribution.

When there are few differences between the populations of two sludges, it is reasonable to say that the two populations are similar, but, as differences accumulate beyond a critical limit, the populations differ increasingly. It should thus be possible to set confidence limits on the mean of the differences and decide which sludges are extremely similar or dissimilar from the lower and upper confidence limit values.

From the data, confidence limits around the mean (μ) of the total differences were calculated (2): confidence limit = 54.3 < μ < 192.9 at 0.10 level of significance. The level of significance was chosen as 10% because the replica plate technique used in this study has a precision of only 90%.

The conclusions drawn from employing the above confidence limits are as follows. (i) The populations of the o-cresol and m-cresol sludges were highly similar. (ii) The populations of the o-cresol-phenol sludges were highly dissimilar. (iii) Although the o-cresol compared to sodium phenylacetate and o-cresol compared to catechol sludges cannot be classified as very dissimilar on the basis of statistical evaluation, we feel that they could be received as dissimilar because of the very narrow margin by which they fell into the confidence interval.

In consideration of the derived confidence limit, the spectrum of similarity-dissimilarity as indicated by the difference values (total differences in a paired comparison) in the populations supported the visual and respirometric observations (14) that divergencies in the microbial composition of the sludges had taken place during adaptation to the various aromatic compounds.

Analysis of adjusted population data. Another kind of comparison of the populations was attempted by using calculations based on equal numbers of total cells in the populations. The type counts for each sludge were adjusted as follows: C = 106/a x b, where C is weighted frequency of the type, a is total number of colonies on a given sludge master plate, b is number of colonies of a specific type on a given sludge master plate, and 106 is an arbitrary standard number (total colonies on phenol-sludge master plate). In most cases, the adjusted data were treated as previously described.

In contrast to the unadjusted data, the distribution of total differences for the 55 comparisons was severely skewed; hence, the arc sine transformation (2) was used to make the data approximate the normal distribution. From this, the confidence limits were found: 103 < μ < 216 at 0.10 level of significance. In relation to the confidence limits, we may conclude: the sludges benzoate-phenylactic acid and o-cresol-m-cresol were most similar within their pairs, and p-hydroxybenzoate and p-cresol sludges were most dissimilar. All other comparisons were spread over a wide spectrum of dissimilarity and no conclusions can be drawn.

Analysis of population data using Mountford’s index of similarity. Although the results obtained by using the confidence interval approach to the population data gave an indication as to which pairs of sludges were very similar and dissimilar in the extremes, it did not prove to be a sensitive tool for grouping communities on the basis of similarity. Several indexes of similarity have been used in classificatory problems (3, 6, 15, 16). Mountford (11) derived an index of similarity
which was less dependent on sample size. The method makes use not only of the index of similarity between a pair of sludges, but also of an index of similarity between two groups of sludges. We have used this method for classifying the aromatic-acclimated sludges in two ways: (i) by comparison of the microbial types common to any two or more acclimated sludges without considering the frequency of each type and (ii) by a comparison of the counts of colonies common to any two or more acclimated sludges without adjustment of the total counts to a common basis.

Classification of sludges acclimated to aromatic compounds based on indexes of similarity computed considering the types of species only. In a comparison of two or more ecosystems where the qualitative description of the species is available but quantitative data on the frequency of individuals of a species are lacking, Mountford's index of similarity can be used profitably. For example, in comparing the aromatic sludges, we considered initially only the types common to any pair of sludges and did not take into consideration the numbers of each type. Table 4 represents the total of types in each sludge and the types common to any given pair of sludges. Table 5 shows the similarity indexes computed from the data on Table 4. Higher values of similarity indexes represent a high similarity. Figure 1 represents the classification of the sludges and values of indexes of similarity between groups of sludges.

Classification of sludges based on indexes of similarity considering the frequencies of types common to pairs and groups of sludges (unadjusted data). Here we weighted the types according to the frequencies of individuals common to pairs of sludges and computed the similarity indexes for the pairs. In such computation, although some
types are common to a pair of sludges, the frequency of the type, i.e., colony count of a given type, determines the degree of similarity. Thus, sludges having similar types containing more individuals in common will have higher similarity indexes. Table 6 represents the frequency of types common to pairs of sludges. The values of similarity indexes are given in Table 7 for all possible pairs of sludges. Figure 2 represents the grouping of sludges based on the similarity indexes between groups of sludges.

Analysis by Mountford's method provided quantitative comparison over the entire set of sludges and, furthermore, allowed comparison of any desired groupings through the lowest common similarity values. The grouping differed quite sharply depending on whether the types alone were considered or whether the individuals were taken into account. Unless types were the sole immediate concern, the comparison of populations based on the numerical balance between the types seems to be the more desirable. Figure 2 illustrates the latter comparison. Catechol sludge was widely divergent and alone. This result substantiated our previous observations on the peculiar aspects of the catechol-adapted sludge (14). In accord with the confidence limit method, o-cresol-m-cresol and benzoate-phenylacetic acid sludges were very similar as paired; moreover, p-hydroxybenzoate and p-cresol sludges were consistently dissimilar.

Our attempts at population analyses indicated that each acclimated sludge differed greatly in microbial constitution. At present, we do not have evidence based on repeated experiments to say that the various types are characteristic for a specifically acclimated sludge. The occurrence in the sludges of different types able to attack the various substrates suggests that predominance may easily be shifted by conditions not necessarily associated with the presence of a particular aromatic compound. From Table 1 we see, simply from the occurrence of type 1, that the potential

Table 6. Colony counts of sludges acclimated to aromatic compounds and the counts common to pairs of sludges (unadjusted data)

| Sludges acclimated to | Colony counts (10^6/ml) | Numbers of types common to pairs of sludges (10^6/ml) |
|-----------------------|-------------------------|-----------------------------------------------------|
|                       | Mandelate | Phenylacetate | p-Hydroxybenzoate | Anthranilic acid | Benzyl alcohol | o-Cresol | m-Cresol | p-Cresol | Catechol | Phenol |
| Benzoate              | 84        | 30           | 60             | 3               | 12            | 32       | 2        | 1        | 2        | 1      | 16     |
| Mandelate             | 45        | 24           | 4              | 14              | 18            | 2        | 1        | 1        | 1        | 1      | 16     |
| Phenylacetate         | 93        | 5            | 1              | 17              | 18            | 2        | 1        | 1        | 1        | 1      | 16     |
| p-Hydroxybenzoate     | 19        | 11           | 1              | 17              | 18            | 2        | 1        | 1        | 1        | 1      | 15     |
| Anthranilic acid      | 39        | 13           | 14             | 18              | 2             | 0        | 0        | 0        | 0        | 0      | 2      |
| Benzyl alcohol        | 73        | 6            | 0              | 17              | 18            | 2        | 1        | 1        | 1        | 1      | 15     |
| o-Cresol              | 102       | 8            | 0              | 17              | 18            | 2        | 1        | 1        | 1        | 1      | 15     |
| m-Cresol              | 82        | 8            | 0              | 17              | 18            | 2        | 1        | 1        | 1        | 1      | 15     |
| p-Cresol              | 76        | 8            | 0              | 17              | 18            | 2        | 1        | 1        | 1        | 1      | 15     |
| Catechol              | 90        | 8            | 0              | 17              | 18            | 2        | 1        | 1        | 1        | 1      | 15     |

Fig. 1. Diagrammatic classification of sludges acclimated to aromatic compounds and values of indexes of similarity between groups of sludges based on numbers of common types. Heavy horizontal line represents value of similarity index at level of divergence.
TABLE 7. Values of index of similarity for data on colony counts of sludges acclimated to aromatic compounds (unadjusted data)

| Sludges acclimated to | Mandelate | Phenylacetate | p-Hydroxybenzoate | Anthranilic acid | Benzylic alcohol | o-Cresol | m-Cresol | p-Cresol | Catechol | Phenol |
|-----------------------|-----------|---------------|-------------------|------------------|-----------------|---------|---------|---------|---------|--------|
| Benzoate........... | 0.01626   | 0.02398       | 0.00208           | 0.00883          | 0.00023         | 0.00014 | 0.00014 | 0.00032 | 0.00013 | 0.00216 |
| Mandelate.......... | 0.00948   | 0.0055        | 0.01075           | 0.00878          | 0.00259         | 0.00148 | 0.00233 | 0.00051 | 0.00448 |
| Phenylacetate...... |           |               | 0.00336           | 0.00517          | 0.00216         | 0.00021 | 0.00013 | 0.00044 | 0.00012 | 0.00193 |
| p-Hydroxybenzoate. |           |               | 0.004104          | 0.007451         | 0.00029         | 0.00029 | 0.0001058 | 0.000492 |
| Anthranilic acid... |           |               |                   | 0.00897          | 0.00029         | 0.00029 | 0.0001058 | 0.000492 |
| Benzylic alcohol... |           |               |                   | 0.0064           | 0.00072         | 0.00072 | 0.000492 | 0.000492 |
| o-Cresol........... |           |               |                   | 0.000107         | 0.000853        | 0.00471 | 0.00029 | 0.000492 |
| m-Cresol........... |           |               |                   | 0.07968          | 0.01325         | 0.01325 | 0.00098 | 0.000724 |
| p-Cresol........... |           |               |                   | 0.00113          | 0.00113         | 0.00113 | 0.00011 | 0.000058 |
| Catechol........... |           |               |                   | 0.00123          | 0.00123         | 0.00123 | 0.000060 | 0.000024 |

Fig. 2. Diagrammatic classification of sludges acclimated to aromatic compounds and values of indexes of similarity between groups of sludges (unadjusted data) calculated by frequencies of common microbial types. Heavy horizontal line represents value of similarity index at level of divergence.

for utilizing all the substrates was present in all the sludges but two. These two sludges lacked strains able to utilize catechol or catechol and p-cresol. Possibly, at lower plating dilutions, utilizers other than fungi previously reported (14) might have been found.

Our method of adjusting numbers upward to put populations on an even numerical footing cannot take into account the presence of the "minor" organisms missed in the higher plating dilutions. The loss on dilution of organisms present in low numbers is probably not serious in accounting for transformations carried out by the entire biomass, but these "minor" organisms provide the potential for adaptation of the microbial population to changing environmental conditions, especially when they comprise a variety of types. Some means for adequate sampling of these organisms will be necessary in studies of adaptation. At present there are two methods available: nutrient enrichment combined with "most probable numbers," and continuous culture (4). In this connection, our regression equations are based on data describing populations within the limitation of our methods and thus do not contain inherent prediction powers. It would be interesting, however, to try to verify predictions on aromatic oxidation based on combinations of known proportions of the described sludges. Unfortunately, we did not have the resources to attempt this or to retrace many of our steps. Had it been possible to do so, it would have been of high priority to learn more about the organisms present in the sludges that required nutrients not present in the aromatic agars. Some of these, as shown (14), required accessory nutrients besides the aromatics; others may have been unable to utilize the aromatics at all.

It was not anticipated that the populations would be so divergent. Keeping in mind that Mountford's index is based fundamentally on the prevalence of species common to several populations, the values for the sludges, which ranged from 0.0 to 0.2, indicated only very slight similarity. For comparison, Mountford (11) cited indexes for populations of soil fauna in woodlands on different soils as ranging from 0.0669 to 0.211.
The numbers of animals common to pairs of sites was from 12 to 39 in a total of 77 species.

The extreme divergence of our sludges minimized the value of the confidence limits approach. Such a method would be more serviceable when seeking tolerable limits of dissimilarity in several populations when the mean of differences is small.

In retrospect, the need of further tests of the sensitivity of methods of population analysis of bacteria is evident. The need is as great for Lochhead's system as for those we have tried, as well as for further innovation. More subtle changes in one or several environmental variables would be appropriate, as for example: temperature, rate of feeding, pH, salt tolerance, and others.

This report, as well as those preceding it in the series, has been an exposition of attempts to characterize populations of aerobic heterotrophs in such a way that the populations were sufficiently described that their evolution or adaptation could be followed. In spite of the acknowledged deficiencies of such methods, it has been possible to show trends in adaptation qualitatively and quantitatively.

Our use of arbitrary systems of classification is based on nutritional and biochemical methods long in use. The significance of the characteristics chosen was that they were matters of concern in the systems being investigated. The arbitrariness was justified to some degree by the deficiencies of bacteriological taxonomy in general.

The experiments we have described are presented as exploratory attempts to find procedures for sampling microbial populations broadly and describing them at least functionally. The more completely such objectives can be attained, the more completely will it be possible to study population adaptation in such situations as the soil, water, mud, the gut, and other environments in which there is an interplay with a complex microbial flora.

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