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# Project Summary

# Enumeration and Identification of Heterotrophic Bacteria from Drinking Water

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**Various spread-plating enumeration media and procedures were tested to determine the method of choice for enumerating the highest numbers of heterotrophic bacteria from chlorinated drinking waters. Dilute media, including a caseinate peptone starch medium, a dilute peptone medium, and R2A medium provided greater recoveries than the standard plate count medium currently used. In addition, reduced temperatures of 20°C and prolonged incubation periods of 14 to 28 days resulted in the highest recoveries of heterotrophic bacteria from the waters of the two chlorinated distribution systems examined.**

**In the Seattle, Washington, water treatment and distribution system, unchlorinated source water had higher diversities of heterotrophic bacteria than did chlorinated drinking water samples. The predominant types recovered from chlorinated drinking waters of that system were Gram-negative pigmented bacteria. Most of these could not be readily identified to known genera. Some of these could be placed in the poorly described genus *Flavobacterium*, but the great variety of types of pigmented Gram-negative bacteria included many that could not be readily classified in this group without further studies including DNA hybridization.**

**A number of representatives of known genera were encountered among the isolates from the chlorinated drinking waters. Included were members of the genera *Caulobacter*, *Hyphomonas*, *Aquaspirillum*, *Vibrio*, *Gluconobacter*, *Azomonas*, and *Aeromonas*.**

***This Project Summary was developed by EPA's Water Engineering Research Laboratory, Cincinnati, OH, to announce key findings of the research project that is fully documented in a separate report of the same title (see Project Report ordering information at back).***

## Introduction

Water is universally consumed in large quantities by the public. Thus from a public health perspective, it would be desirable to know the types and numbers of bacteria that are ingested by drinking chlorinated water. Yet numerous investigations have shown that the standard method for enumerating heterotrophic bacteria from drinking waters (i.e., the standard plate count or SPC procedure) frequently provides lower counts of bacteria from drinking waters than other procedures. Furthermore, no adequate procedure currently exists for characterizing and identifying heterotrophic bacteria from drinking waters.

The objectives of this investigation were twofold: a) to evaluate alternative procedures for enumerating heterotrophic bacteria from chlorinated water supplies and b) to develop a procedure that could be used to identify the bacteria that commonly occur in drinking waters.

The City of Seattle water distribution system was used as a test system for the study of enumeration procedures. Various media and methods were compared with the SPC procedure to provide an optimum count of the viable heterotrophic bacteria.

About 100 bacterial strains (50 isolates each from the Cincinnati, Ohio, and Seattle, Washington, water systems) were studied to develop a schema for use in characterizing and identifying the predominant bacteria from treated distribution water. Morphologic and biochemical tests were used to characterize the isolates.

## Materials and Methods

Drinking water samples from the Seattle water system were collected in sterile sample bags or bottles containing sodium thiosulfate to inactivate residual chlorine, as recommended by Standard Methods for the Examination of Water and Wastewater (15th Ed., American Public Health Association, 1980). Samples were iced until processed, and all samples were analyzed within 8 hr of collection.

Enumeration of heterotrophic bacteria was done by the spread plate procedure using several media, including Standard Methods agar (SMA), dilute peptone agar (DP), caseinate peptone starch agar (CPS), and R2A medium. The pour plate procedure was also used for some SMA plates.

Strain diversity in water samples was determined based on colony type. The Shannon index was calculated as an estimate of strain diversity from the formula:

$$H' = -\sum_{i=1}^{s} {}^{n}i/N \; 3.3 \log_{10} {}^{n}i/N$$

where

H' = diversity index value
N = total number of colonies counted
$^{n}i$ = number of colonies of one type (i.e., ith strain)
s = number of strains

Direct counts were made microscopically using acridine orange (AO) staining and an epifluorescence microscope..

Bacterial strains used for the characterization and identification studies were isolated from winter and summer water samples from both the Seattle and Cincinnati distribution systems. Seattle isolates were obtained from DP plates, and Cincinnati isolates were selected from R2A plates. Characterization tests included Gram-stain, colonial and cellular morphology, anaerobic growth, oxidase test, growth on mineral media with carbon sources, oxidation/fermentation reaction, polyhydroxybutarate incorporation, spore formation, growth at pH 4.5, acid production from ethanol, nitrification, cellulose degradation, pigmentation or fluorescence, growth temperature, and other special media tests as required. Media were incubated at 20°C unless otherwise specified. Known baterial strains were obtained for comparison from other investigators and from the American Type Culture Collection.

## Experimental Results

### *Enumeration of Viable Heterotrophic Bacteria from Chlorinated Drinking Water*

Water samples were collected and examined from 22 sites in the Seattle water distribution system, representing both pre- and post-chlorination locations.

All four media were used to examine samples from three surveys. The results showed that the higest counts were obtained after 14 to 28 days of incubation, depending on the sample. However, counts from SMA plates incubated at 35°C for 48 hr were never as high as those obtained by other means, and 20°C SMA counts were almost always inferior to those obtained with other media used in the surveys. Overall, the best medium was DP, followed by CPS and R2A. DP and CPS consistently provided the highest counts for chlorinated water samples, but DP was less satisfactory than CPS for enumerating bacteria in water samples collected before chlorine treatment. Because of the superior performance of DP and CPS, these media were used for subsequent viable plate count analyses.

The length and temperature of incubation strongly affected viable plate counts. After 14 days of incubation, most chlorinated samples showed viable counts at 20°C that were 50% to 100% of the counts obtained after 28 to 30 days at 20°C. Incubation beyond 28 to 30 days at 20°C infrequently showed significant increases in plate counts. Bacterial counts on DP and CPS media were compared after plates were incubated at 20°, 30°, and 35°, and at a dual temperature—20° for 48 hr, then at 30°C for up to 30 days (20° → 30°C). Ranked from highest to lowest counts, the results were 20° > (20° → 30°) > 30°C.

Total direct counts by the AO method were much higher than viable counts, as expected. For unchlorinated water samples, only about 1% of the total direct count bacteria were cultivable; and for chlorinated samples, the percentage of viable organism recovery was even lower. In some reservoirs, the ratio of viable to AO counts exceeded 10% (range 11.8% to 42.4%). Such high ratios indicate that the water was eutrophic rather than oligotrophic.

Diversity indices showed that chlorination of the water caused a dramatic change in bacterial diversity (Table 1). Whereas the unchlorinated source water contained a variety of colony types, including many nonpigmented ones, the chlorinated water contained relatively few colony types and most were pigmented. Since these results were supported by those from an earlier study in which diversity indices were calculated for both chlorinated and unchlorinated waters, it was concluded that the disinfection and distribution processes in the Seattle system commonly (and perhaps always) result in selection of relatively few bacterial species compared with the source water.

### *Characterization and Identification of Bacteria from Chlorinated Drinking Waters*

The major purpose of this portion of the study was to identify the predominant heterotrophic bacteria from two different drinking water systems. The process of identification assumes that the organisms being investigated are known organisms that have been previously characterized, described, and named. We anticipated that most bacteria found in chlorinated drinking waters would be strains of known organisms and that characterization of the isolates and their comparison with known organisms would be rather straightforward. Such was not the case, however. Because of the slow growth of the isolates and their general nonreactivity in biochemical characterization media, they posed special problems for characterization. Tests were developed to permit phenotypic characterization and description of these bacteria, and a dichotomous flow chart for characterization was prepared (Figure 1). Many of the bacterial isolates were not identifiable to known species, but use of the dichotomous key permitted grouping of similar types of organisms.

Most of the bacterial isolates examined were Gram-negative rods, some of which were morphologically distinctive,

**Table 1.** *Diversity Indices of Source Water (CPRI) and Chlorinated Samples Collected September 2, 1981, and Counted after 28 and 40 days*

| | After 28 Days | | After 40 Days | |
|---|---|---|---|---|
| Sample | No. Bacteria/ml | Diversity Index* | No. Bacteria/ml† | Diversity Index‡ |
| CPRI | 3,600 | 4.14 | – | – |
| I3 | 210 | 1.85 | 510 | 2.45 |
| K3 | 1,700 | 2.81 | – | – |
| L3 | 2,900 | 1.23 | 3,070 | 1.45 |
| M3 | 150,000 | 1.08 | 160,000 | 1.55 |

**The diversity index is based on 55 colonies, except for sample M3, in which 40 colonies were used.*
*†Forty-day counts were determined from 28-day plates that had been refrigerated. Increased counts on plates were due to growth of Hyphomonas.*
*‡Diversity index is based on Hyphomonas colonies. Total colonies counted for I3 were 81; L3 were 58; and M3 were 44.*

such as prosthecate bacteria. Prosthecate bacteria identified included *Caulobacter* and strains recognized as belonging to the *Hyphomonas—Hyphomicrobium* group. In some samples from the Seattle system, *Hyphomonas* constituted from 2.8% to 12.8% of the viable count. Other morphologically distinct isolates included a species of *Vibrio*, three spiral-shaped organisms identified as *Aquaspirillum* strains, and three large, ovoid, motile cells identified as *Azomonas*.

The majority of the isolates (44) were Gram-negative, nonfermentative, oxidase positive, aerobic rods, and 24 of these were pigmented. Of the nonpigmented group, only one isolate was identified as a typical *Pseudomonas*

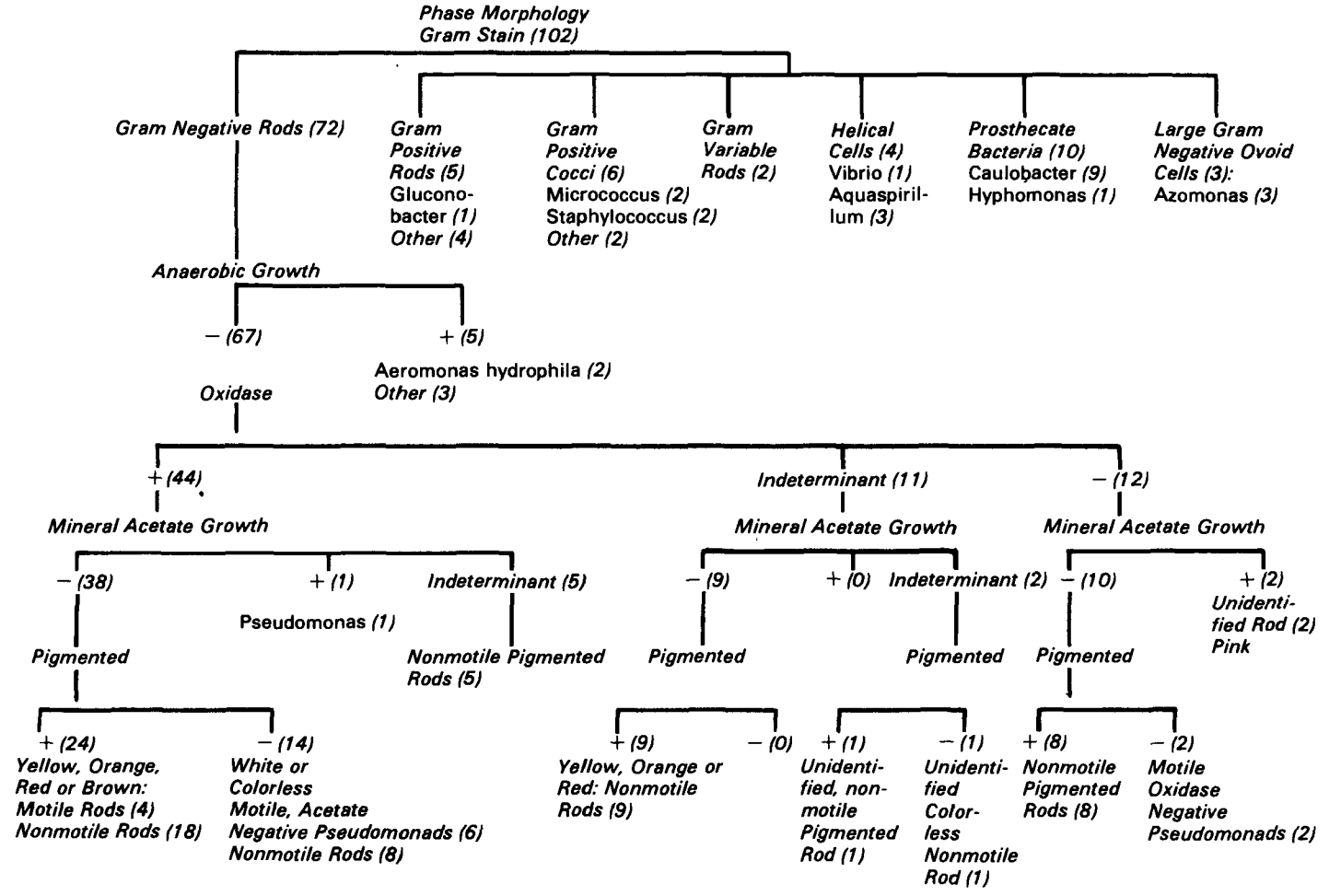


***Figure 1.*** *Flow diagram showing characteristics of 102 bacterial strains from chlorinated drinking water.*

strain, although several isolates resembled *Pseudomonas* species in all respects except for their inability to grow on mineral medium with acetate as the carbon source. About half of the pigmented Gram-negative rods (24) were unable to grow at 37°C and 7 did not grow at 30°C. Many did not grow sufficiently well to permit thorough characterization. All of the pigmented strains were negative for cellulose digestion, and none survived the heat survival tests for sporeformers.

A few Gram-positive and some Gram-variable isolates were obtained. Of the five Gram-positive cocci, three were identified as *Staphylococcus,* one was *Micrococcus luteus,* and one was possibly a strain of *Planococcus.* One of the Gram-positive rods was identifiable to genus; its characteristics were typical of the genus *Gluconobacter.* The remaining four Gram-positive rods and two Gram-variable rods were not identifiable. All but one were pigmented strains.

Because the pigmented Gram-negative rods made up an especially large group of the isolates and were well represented in both distribution systems, much effort was made to identify them. Because of the difficulty in characterizing those isolates, however, identification was not successful. Many of these isolates could possibly be classified as members of the genus *Flavobacterium,* which is a broadly defined genus that encompasses virtually all pigmented Gram-negative rods. So broadly is it defined, however, that it is known as a wastebasket genus. The pigmented isolates from this study were not identified as *Flavobacterium* for several reasons. First, there was no convincing evidence that the pigmented distribution system isolates were related to those that are currently placed in the *Flavobacterium* genus. Second, some nonpigmented strains closely resemble the pigmented strains except for the pigmentation. Thus, until both the pigmented and nonpigmented strains are better characterized and studied, it seemed inappropriate to make an arbitrary assignment on the basis of only one characteristic, pigmentation. The third reason was that the group of pigmented isolates found in this study is a very diverse group, and because of the wide range of phenotypic characteristics, it is doubtful that all pigmented isolates could be placed in a single genus. Even the genus *Flavobacterium* as currently defined does not contain organisms that demonstrate the diversity shown by the pigmented bacterial isolates found in this study.

## Conclusions

A variety of media were tested to determine which provided the highest viable counts of heterotrophic bacteria in chlorinated drinking waters from the City of Seattle water distribution system. The most dilute medium tested consistently provided the highest recoveries of heterotrophic bacteria from the chlorinated water samples examined during this study.

The highest viable counts of heterotrophic bacteria from the chlorinated samples were obtained when (1) plates were incubated at 20°C rather than at 30° or 35°C, and (2) incubation time was longer than 14 days. Use of dual-temperature incubation (20°C for 48 hr followed by 30°C for the remainder of the incubation period) provided higher counts than did incubation at 30°C but lower counts than incubation at 20°C. Twenty-eight days of incubation normally gave maximal recovery at 20°C, but some bacteria did not appear until after about 40 days.

Total direct microscopic count using AO generally decreased following chlorination. Viable count results indicate that only about 1% of the total bacteria can be cultivated on laboratory media.

The diversity of heterotrophic bacteria on each enumeration medium was determined using the Shannon diversity index formula. Chlorination resulted in a diversity index decrease (1.08 to 2.81) compared with values before chlorination (>4.0).

A total of 102 bacterial isolates were obtained from the two treated municipal drinking water systems in Seattle and Cincinnati. Of about 50 isolates from each system, half were isolated during the summer months and the other half during the winter months.

The bacterial isolates were characterized and identified, where possible, to known genera using phenotypic tests. Seventeen of the isolates were morphologically distinctive and included *Caulobacter* (9 strains), *Hyphomonas* (1 strain), *Aquaspirillum* (3 strains), a black pigmented strain of *Vibrio* (1 strain), and *Azomonas* (3 strains). This is apparently the first report of *Caulobacter* and *Hyphomonas* in chlorinated drinking water. Among a group (14 cultures) of Gram-positive and Gram-variable nonfermentative rods, only one was identified, a strain of *Gluconobacter.*

Most of the isolates were Gram-negative, obligately aerobic rods. Only one was clearly identifiable as a strain of *Pseudomonas,* although several other isolates were motile and nonpigmented and shared many features of the genus. Many of the Gram-negative rods could be superficially classified in the genus *Flavobacterium* because of their pigmentation and other features. However, these organisms showed greater diversity than even that poorly defined genus. Further study, including DNA hybridization, is needed to determine whether any of the isolates resemble known strains of the genus *Flavobacterium* closely enough to be classified as such.

Only a few of the Gram-negative rods were facultative anaerobes, some of which were identified as strains of *Aeromonas hydrophila.*

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*The complete report, entitled "Enumeration and Identification of Heterotrophic Bacteria from Drinking Water," (Order No. PB 85-207 496/AS; Cost: $11.50, subject to change) will be available only from:*
*National Technical Information Service*
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