Length of Incubation for Enumerating Nitrifying Bacteria Present in Various Environments

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The effect of incubation time on most-probable-number estimates of autotrophic nitrifying bacteria was investigated by using waters, rooted aquatic plants, sediments, and slimes as inoculum sources. Maximum most probable numbers of the NH$_4^+$-oxidizing group were attained in 20 to 55 days (median, 25). Estimates of NO$_2^-$ oxidizers were highest at termination (103 to 113) days.

Viable autotrophic nitrifying bacteria in environmental materials are usually enumerated by indirect most-probable-number (MPN) techniques. Various lengths of incubation have been used. Most commonly, estimates of nitrifying populations in soils have been based on examinations of the media 21 days after inoculation (1, 11, 13, 16, 17). A 28-day incubation has also been used (5). Ammonium oxidizers in seeded synthetic river water were enumerated by means of a 28-day incubation, but 42 days was allowed to elapse before making the estimates of NO$_3^-$ oxidizers (15). In marine environments 60-day MPN procedures have been used for both groups (18), although an earlier qualitative study (2) used incubations lasting up to 90 days.

Ideally the incubation should last just long enough to account for all of the inoculum cells capable of growth under the conditions provided. The present report concerns the effect of time on MPN estimates of nitrifying bacteria, using standardized incubation conditions. To encounter potentially disparate responses, different types of aquatic materials and sewage treatment plant effluents were used as inoculum sources.

MATERIALS AND METHODS

Media. Both media consisted of: NaCl, 2.0 g; K$_2$HPO$_4$, 0.50 g; MgSO$_4$·7H$_2$O, 0.05 g; CaCl$_2$·H$_2$O, 0.02 g; KHCO$_3$, 0.02 g; NaMoO$_4$·2H$_2$O, 2.4 µg; trace metals mixture no. 44 (3), 1 ml; and distilled deionized water, 1 liter. Either 0.50 g of (NH$_4$)$_2$SO$_4$ or 0.10 g of KNO$_3$ was added as the nitrogen and energy source. The components were autoclaved separately (the metals mixture was filter sterilized) and then combined. Six-milliliter portions were transferred to sterile tubes (16 by 150 cm) containing approximately 0.1 g of CaCO$_3$, which were capped with polypropylene culture tube closures. The pH of the NH$_4^+$ medium was 7.8 and that of the NO$_3^-$ medium was 8.0.

Sampling locations. Aquatic materials were obtained from the Passaic River in northeast New Jersey and from Mine Brook in Somerset County. The Passaic is highly polluted from numerous domestic and industrial effluents, whereas Mine Brook receives the discharge of one domestic wastewater activated sludge treatment plant.

Sample collection and preparation. Water samples were skimmed from the river surface. Sediments were removed from the sediment-water interface (0 to 1 cm) and from a deeper level in the profile (9 to 10 cm) with the aid of a 4.8-cm (inner diameter) glass tube. Slimes scraped from rocks, rooted aquatic plant materials, and effluents were dispersed by blending in the cold with a Sorvall Omnimixer. Further details are available elsewhere (6).

Serial 10-fold dilutions of the inoculum sources were prepared in phosphate buffer solution (pH 7.2), and 1-ml portions were transferred to 10 replicate tubes per dilution for each type of medium. Incubation was at 28 ± 1 C. The tubes were slanted to promote aeration.

MPN estimates. The medium in each tube was examined periodically by aseptically removing a few drops to a spot plate depression containing NO$_3^-$ test reagent (14). If the NH$_4^+$ medium gave a strong reaction (dark red) compared with the uninoculated control, the tube was scored positive for NH$_4^+$ oxidizers. When necessary to guard against a false-negative score, Devarda alloy was added to test for NO$_3^-$. Tubes containing NO$_3^-$ medium were scored positive for NO$_3^-$ oxidizers if the test indicated that this substrate had decreased in concentration or disappeared. Tubes scored negative and those showing weak reactions were reincubated. Control NH$_4^+$ medium developed a light pink reaction to the reagent, which became darker as the experiment progressed. Control NO$_3^-$ medium did not change perceptibly. The MPN values were obtained from a table (10).

RESULTS

At inoculation, each tube contained 7 ml of liquid. This decreased to 2 to 5 ml during the
103- to 113-day incubation period. A total of 1.5 ml at most was removed for the NO₃⁻ tests. Additional losses were by evaporation.

With few exceptions, the appearance of NO₃⁻ in NH₄⁺ medium, and its disappearance from NO₂⁻ medium, were abrupt, indicating a period of rapid growth during the interval since the previous observation. The resultant tube score patterns were as expected of viable bacteria subjected to a dilution-to-extinction procedure (e.g., 10+, 5+, 0+).

In all of the trials (Tables 1 and 2), NH₄⁺ oxidizers appeared to have completed their response within the experimental period. The median length of incubation necessary to attain a stable maximum MPN estimate of this group was 25 days (range, 20 to 55). Completion of the response was not observed for the NO₃⁻ oxidizers.

The use of five activated sludge sewage treatment plant effluents as inoculum sources yielded results similar to those tabulated above.

### DISCUSSION

In practice, the selection of appropriate periods of incubation must balance convenience against the purposes of the study. The disadvantages of an unnecessarily prolonged incubation are many, including the delayed acquisition of data that could aid in framing experimental questions. Too brief an incubation of either nitrifying group results in an underestimation of unknown and variable magnitude. These results, derived from various nonmarine aquatic habitats and effluents, suggest that a 35-day period would generally be sufficient to attain the maximum estimate of NH₄⁺ oxidizers possible under the incubation conditions described. Obviously it is difficult to make a specific recommendation for NO₃⁻ oxidizers.

These results also indicate that the incubation period reported in some investigations of nitrifying bacteria may be too short to yield maximum estimates. It would appear that NO₃⁻ oxidizers have been more seriously underestimated than NH₄⁺ oxidizers. Because a number of different medium formulations have been used, any retrospective evaluation must be tentative.

The time period required for maximum recovery is a function of the physiological state of the inoculum cells as it influences the duration of the lag phase of growth, and of the growth rate once active proliferation commences. In the present study, the NO₃⁻ oxidizers that manifested themselves at high inoculum dilutions did so after a very long period of incubation. The literature provides no opportunity for a comparative evaluation of this finding. It cannot easily be explained on the basis of unusually

### Table 1. Effect of incubation time on estimates of nitrifying bacteria in waters and sediments

| Incubation time (days) | Mine Brook water* | Estimate (MPN/ml of water or sediment) | Passaic River |
|------------------------|-------------------|----------------------------------------|---------------|
|                        | NH₄⁺ oxid | NO₃⁻ oxid | NH₄⁺ oxid | NO₂⁻ oxid | NH₄⁺ oxid | NO₃⁻ oxid | NH₄⁺ oxid | NO₂⁻ oxid | NH₄⁺ oxid | NO₃⁻ oxid | NH₄⁺ oxid | NO₂⁻ oxid |
| 5                      | 2.7 ND*    | 2.3 ND    | 329 ND    | 4.5 ND    | 275 ND    | 23.1 ND   |           |           |           |           |           |           |
| 10                     | 298 ND     | 31.0 ND   | 49,300 ND  | 3,990 ND  | 150 ND    | 27,500 ND | 39.9 ND   | 1,300 ND  |           |           |           |           |
| 15                     | 2,750 ND   | 288 ND    | 130,000 ND | 15,000 ND | 365 ND    | 101,000 ND | 39.9 ND   | 2,750 ND  |           |           |           |           |
| 20                     | 3,290 ND   | 1,710 ND  | 275,000 ND | 62,200 ND | 875 ND    | 197,000 ND | 79.2 ND   | 5,890 ND  |           |           |           |           |
| 25                     | NC 0.9    | 1,960 2.8 | NC 2.8    | NC 101,000 | 2,280 ND  | 275,000 ND | 79.2 ND   | 4,190 ND  |           |           |           |           |
| 30                     | NC 3.3    | NC 7,920 300 | 130,000 | 3,990 ND  | NC 3,990 300 | 39.9 ND   | 101,000 ND | 39.9 ND   |           |           |           |           |
| 35                     | NC 4.0    | NC 24,000 | NC 6,220 400 | 15,000 NC | 10,100 NC 1,710 | 39.9 ND   | 101,000 ND | 39.9 ND   |           |           |           |           |
| 40                     | NC 7.9    | NC 32,900 | NC 27,500 | NC 24,000 | NC 24,000 | NC 27,500 | NC 24,000 | NC 27,500 |           |           |           |           |
| 55                     | NC 27.5   | NC 49,300 | NC 39,900 | NC 116,000 | NC 24,000 | NC 24,000 | NC 24,000 |           |           |           |           |           |
| 70                     | NC 62.2   | NC 62,200 | NC 39,900 | NC 116,000 | NC 24,000 | NC 24,000 |           |           |           |           |           |           |
| 103-113                | NC 298    | NC 11.6   | NC 62,200 | NC 116,000 | NC 24,000 |           |           |           |           |           |           |           |

*Collected approximately 4 miles (about 6.4 km) below the discharge point of the treatment plant. Oxid, Oxidizer.

*Collected at Chatham.

*Collected at Pine Brook where the sediment (Sed) consists of a black organic ooze.

*Collected at Pine Brook where the sediment is sandy.

*ND, None detected.

*NC, No change.
slow growth rates, which, contrary to observation, would have resulted in a gradual disappearance of substrate. It can be inferred, therefore, that the NO$_3^-$ oxidizing cells responsible for late accretions to the MPN estimate passed through a prolonged lag phase. This analysis is consistent with the observation that NH$_4^+$ and NO$_3^-$ oxidizers are capable of similar logarithmic growth rates (4, 8, 9).

It should be noted that the spot test used for scoring the tubes is more sensitive to changes in the NH$_4^+$ medium than in the one formulated with NO$_3^-$. This could bias the results in the direction of an apparently faster response by NH$_4^+$ oxidizers. Because of the length of the intervals between observations, any such bias is probably slight.

Existing data on the optimization of growth conditions of nitrifying bacteria (12) may be only marginally relevant to the problem of enumerating viable cells in the environment. Such information has been obtained with the use of active pregrown cultures or with continuous cultures. Conditions that promote a short generation time by cells in the logarithmic phase of growth are not necessarily those that best initiate active growth by cells recently removed from the environment.

The wide range of incubation times needed for NO$_3^-$-oxidizing bacteria to manifest themselves could reflect, in part, the presence of different strains in a common habitat. This is open to investigation by means of the fluorescent antibody technique, which has been used to distinguish between *Nitrobacter agilis* and *N. winogradskyi* (7).

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**Table 2. Effect of incubation time on estimates of nitrifying bacteria in slimes on rocks and plant surfaces**

| Incubation time (days) | Estimate (MPN/g of slime or plant material) | Mine Brook | Passaic River | Yellow pond lily | Pondweed |
|------------------------|------------------------------------------|------------|---------------|-----------------|----------|
|                        |                                             | Rocks (above outfall) | Rocks (below outfall) |                  |          |
| NH$_4^+$ oxid | NO$_3^-$ oxid | NH$_4^+$ oxid | NO$_3^-$ oxid | NH$_4^+$ oxid | NO$_3^-$ oxid | NH$_4^+$ oxid | NO$_3^-$ oxid |
| 5         | 16,300 | ND* | 2,740,000 | ND | 3,710 | 26.6 | 1,900 | 6.6 |
| 10        | 64,500 | 54.6 | 30,200,000 | ND | 271,000 | 147 | 24,800 | 1,690 |
| 15        | 245,000 | NC* | NC | 121 | 2,220,000 | 3,360 | 363,000 | 3,840 |
| 20        | 639,000 | 4,640 | 36,200,000 | 220 | 3,100,000 | NC | 581,000 | NC |
| 25        | 464,000 | 5,760 | NC | 1,110 | NC | NC | 5,820 | |
| 30        | NC | 13,700 | NC | 3,020 | NC | NC | 6,910 | |
| 35        | NC | 16,300 | NC | 6,840 | NC | NC | 58,000 | |
| 40        | NC | 34,800 | NC | 11,100 | NC | NC | 58,000 | |
| 55        | NC | 113,000 | NC | 54,200 | NC | 11,400 | 668,000 | 189,000 |
| 70        | NC | NC | NC | 111,000 | NC | NC | 248,000 | |
| 103-113   | NC | 198,000 | NC | 143,000 | NC | 14,700 | 350,000 | |

* Rocks collected from 30 m above or 200 m below the treatment plant outfall. oxid. Oxidizer.
* *Nuphar advena* (Alt.) Ait. F.
* *Potamogeton diversifolius* Raf.
* ND, None detected.
* NC, No change.

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