Preservation of *Nitrosomonas*

It is well known that the isolation of *Nitrosomonas* in pure culture is rather difficult; another problem is how the pure cultures can be successfully preserved. *Nitrosomonas* seems to be more frail than most other bacteria when in pure culture, and unfortunately the majority of strains isolated during past years have been lost.

There is not much information in the literature regarding the means by which cultures of *Nitrosomonas* have been preserved, but it seems probable that most workers have kept their isolates simply by regularly transferring material into fresh liquid media the composition of which has not deviated essentially from the original ammonium medium of Winogradsky. Kingma Boltjes, however, who made a comprehensive study of nitrification in the 'thirties', kept his isolates of both *Nitrosomonas* and *Nitrobacter* on agar slants with ammonium sulphate and sodium nitrite respectively, and enriched with 0.5 per cent 'Nährstoff Heyden' (this preparation is no longer available). As the bacteria became accustomed to laboratory conditions, this method of preservation worked satisfactorily if subcultures were made every three to four months (personal communication). Unfortunately, Kingma Boltjes lost all his cultures of nitrifying bacteria during the Second World War. Of more recent isolations, all subcultures of the strain of *Nitrosomonas europaea* isolated by Jensen in 1949² have died in spite of approximately monthly subculturing in liquid mineral medium during three years. Even the strain of *N. europaea* isolated by Meiklejohn³ in 1949 has been lost. Three subcultures of this strain were alive as late as in 1952 (although in the meantime contaminated with other bacteria). At this time the cultures were partly freeze-dried, partly inoculated into sterilized soil and kept at 4°C.; after having been left in this state for eighteen months, all the cultures were dead (personal communication).

In this Laboratory an experiment was initiated more than two years ago in order to compare different methods of preservation of *Nitrosomonas*. The methods were:

(1) Monthly transfers in a liquid medium (5 ml. in test-tubes) of the following composition:

(NH ₄) ₂ SO ₄	0.4 per cent (w/v)
KH ₂ PO ₄	0.025 per cent (w/v)
MgSO ₄ ·7H ₂ O	0.0125 per cent (w/v)
NaCl	0.0125 per cent (w/v)
FeSO ₄ ·7H ₂ O	0.0025 per cent (w/v)
MnSO ₄ ·4H ₂ O	0.0025 per cent (w/v)
CaCO ₃	e. 0.05 gm. per 5 ml. of medium
Tap water	

pH in autoclaved medium 8.0. Size of inoculum one drop 0.03–0.05 ml. of a one-month old culture. Incubation at 25–28°C.

(2) Preservation under a layer of sterile paraffin oil. The same medium was used. After inoculation the cultures were covered with 5 ml. of sterilized oil without previous incubation. Storage at room temperature.

(3) Preservation in sterile soil. A sample of garden soil was slightly moistened with the medium described above, dried in the air, and 10-gm. portions distributed in Freudenreich flasks. The media were autoclaved at 120° for 90 min. and finally inoculated with 0.5 ml. of an actively nitrifying culture. Storage at room temperature.

(4) Freeze-drying of six-weeks old cultures. The ampoules were stored at 0°C. (The freeze-drying was

Table 1. NITRITE PRODUCTION BY *Nitrosomonas* AFTER STORAGE FOR VARIOUS TIMES IN DIFFERENT MEDIA

Months	Pure culture				Mixed culture			
	Preservation medium							
	(1)	(2)	(3)	(4)	(1)	(2)	(3)	(4)
1	+++	+++	++	—	+++	+++	++	—
3	+++	+++	—	—	+++	+++	—	—
6	+++	+++	—	—	+++	+++	—	—
12	+++	+++	—	—	+++*	+++	—	—
25	—†	—	—	—	—	—	—	—

Nitrification was not continued in subculture:

* after 11 months.

† after 13 months.

kindly carried out by Dr. Alice Reyn, State Serum Institute, Copenhagen.)

Two strains of *Nitrosomonas* have been used for these experiments: (a) a pure culture of *N. europaea* (Lees strain), and (b) a natural mixed culture of *Nitrosomonas* sp. and three heterotrophic micro-organisms, namely, *Hyphomicrobium vulgare* and two species of *Pseudomonas*⁴.

At intervals material from the stock cultures was transferred to fresh liquid medium, and nitrite production in the latter determined after about two weeks of incubation. If no nitrite was detectable at this time, incubation was continued up to five weeks. Cultures giving no nitrite reaction with Griess reagent at the end of the incubation period were regarded as dead. The results are given in Table 1.

The two strains of *Nitrosomonas* showed almost the same reaction to the four methods of preservation. Freeze-drying proved to be of little value, as subcultures failed to show any nitrification after only one month of storage. Probably the bacteria died during the actual freezing process. Likewise sterile soil gave disappointing results. Substances toxic to *Nitrosomonas* are apparently produced when soil is autoclaved, as unsterilized air-dried soil is known to retain its nitrifying capacity for several years. Preservation by monthly transfers and storage under oil gave essentially the same results. Hence it seems that *Nitrosomonas* might be preserved under paraffin oil. This method has the advantage that the risk of contamination is small compared with the method of regular transfers. Although promising, the paraffin oil method is by no means perfect. It is possible that the number of cells initially present was too small, as the cultures were covered with the oil immediately after inoculation without allowing further growth after the oxygen dissolved in the media had been consumed. Experiments with the paraffin oil preservation method are being continued.

The factors controlling ageing and death of bacteria under otherwise non-toxic conditions are little understood. The frequently observed sudden death of *Nitrosomonas* in liquid media is usually not caused by exhaustion of substrate or oxygen. It has been suspected that accumulation of nitrite might have killed the bacteria; but *Nitrosomonas* is known to tolerate considerable quantities of nitrite although this is toxic to many organisms. A possible explanation might be that lethal mutations occur or that a phage interferes.

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¹ Kingma Boltjes, T. Y., *Arch. Mikrobiol.*, **6**, 79 (1935).

² Jensen, H. L., *Tidsskr. Planteavl.*, **54**, 62 (1950).

³ Meiklejohn, J., *J. Gen. Microbiol.*, **4**, 135 (1950).

⁴ Gundersen, K., *Plant and Soil*, **7**, 26 (1955).