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An improved method for the semicontinuous culture of bacterial populations on Nuclepore Membrane Filters[1])

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Eine verbesserte Methode für die semikontinuierliche Kultur von Bakterienpopulationen auf Nuclepore Membranfiltern (Zusammenfassung): Die vorliegende Arbeit beschreibt eine verbesserte Methode für die semikontinuierliche Kultur von Bakterienpopulationen auf Nuclepore Membranfiltern. Die auf Schwimmringen deponierten Filter werden in einem Fließ-System unter direktem Kontakt mit der Wasseroberfläche bebrütet und fluoreszenzmikroskopisch ausgewertet. Vergleichende Untersuchungen der Teilungsaktivität von Bakterienpopulationen (Reinkulturen und natürliche Populationen) zeigen, daß das Fließ-System herkömmlichen Kulturverfahren (Bebrütung der Filter auf mit Kulturmedium getränkten Kartonscheiben) überlegen ist: Die Anzahl und das Spektrum koloniebildender Keime werden erheblich vergrößert. Die Gründe hierfür werden diskutiert.

(Summary): The present paper describes an improved method for the semicontinuous culture of bacterial populations on Nuclepore membrane filters. The filters, placed on swimming rings, are incubated in direct contact with the water surface of the flow system, and examined by fluorescence microscopy. The growth activity of both, pure cultures and natural bacterial populations, measured in the flow system is compared to the activity measured in a conventional culture system (incubation of membrane filters on the surface of paper disc saturated with incubation medium). The flow system increases the number and the spectrum of colony forming cells. The reasons for the superiority of the flow system are discussed.

Introduction

There are two main problems in determining the growth activity of natural bacterial populations: first, the recognition of microorganisms (especially small cells) and distinguishing them from detritus and their growth upon detritus (for references see Zimmermann and Meyer-Reil, 1974), and second, the cultivation of microorganisms under natural conditions (for references see Brock, 1971).

Lately a paper has been published describing a new method for fluorescence staining of bacteria on Nuclepore membrane filters with acridine orange (Zimmermann and Meyer-Reil, 1974). This method is characterized by a specific, intensive and long lasting orange or green fluorescence of even small bacterial cells (down to about 0.2μ), while the filter backround remains dark homogenous.

Different methods have been used for the cultivation of natural bacterial populations and the assessment of their growth activity. By exposure of glass slides or other objects to the natural environment (Cholodny, 1930; Zobell, 1943; Kriss, 1963; Wood, 1967, Bott and Brock, 1970; Brock, 1971) one has to consider the following factors: suitability of the exposed objects as substrates for bacterial colonization, irreversible attachment of cells (avoided by the technique described by Bott and Brock, 1970), grazing by micro- and macro-predators and the removal of organisms by removing the exposed surfaces.

The incubation of water samples in the laboratory according to different methods; ("slide culture" technique, "surface drop" technique, "spread plate" technique, "poured plate" technique, "membrane filter" technique, Jannasch and Jones, 1959; Hopton et al., 1972; Straskrabova, 1972, Kunicki-Goldfinger, 1974) deviates from

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the natural conditions very much. The quality of agar differs according to the brand, and agar can cause inhibitory effects on the activity of microorganisms (SANDS and BENNETT, 1966; BÖLTER and MEYER-REIL, in press). The water content of the agar and the incubation temperature (SIEBURTH, 1967, 1968, 1971; HOPTON et al., 1972) have to be taken into consideration also. However, the most important factor is the micro climate, which is impaired by increasing microbial growth, due to the competition of the cells, the exhaustion of nutrients and the accumulation of inhibitory and toxic substances (JANNASCH, 1967; KUNICKI-GOLDFINGER, 1974). In most of these methods the water sample is enriched with various additional nutrients, which select only a small part of the versatile natural bacterial population (GODLEWSKA-LIPOWA, 1974). Nutrients even in small amounts can alter the natural bacterial behaviour (JANNASCH, 1967), and bacterial counts "are not acceptable as an adequate measure of bacterial activity in natural populations" (JANNASCH, 1965). The results obtained with the methods mentioned above only show a small part of the natural bacterial population which actually exists and is capable of growth under natural conditions.

Searching for an improved culture system which would be able to simulate the natural conditions as far as possible, KUNICKA-GOLDFINGER and KUNICKI-GOLDFINGER (1972) described an assay device for the semi-continuous culture of bacteria on membrane filters. This flow system renders possible the cultivation of microorganisms under nearly natural conditions (same quality and quantity of nutrients, same physico-chemical parameters as in the natural environment). Since the results of the experiments with this assay device were not satisfactory, this introductory paper describes a modified and improved culture apparatus for the semi-continuous culture of bacteria on Nuclepore membrane filters. The bacterial growth activity measured in this flow system is compared with the activity measured in a conventional system (incubation of membrane filters on the surface of paper discs).

Materials and Methods

Culture apparatus (see Fig. 1): The culture apparatus consists of a round glass vessel (breeding chamber) with the water inlet (silicone stopper with glass tube) in the central part of the lid and with the water outlet the lower edge of the vessel. 4 swimming rings (5.9 cm in diameter, upper part of plastic stoppers) are placed into the breeding chamber, touching each other on one side. The swimming rings set free a central square area for the water inlet. In the central part of the swimming rings are round recessed apertures (2.4 cm in diameter), on which the filters (Nuclepore 0.2 μ, 2.5 cm in diameter) are placed with direct contact to the water surface. A water supply feeds the chamber and after flowing through the system, the water is collected in a flask. A peristaltic metering pump (Desaga, flow rate about 500 ml per day) controls the flow rate, and the water in the water supply and the breeding chamber is mixed by a magnetic stirrer. Floating on the water, the swimming ring system is free to move within the chamber. The entire apparatus is maintained at a constant temperature with both water supply and chamber additionally protected by asbestos pads and styrofoam from warming by the stirrer. The culture apparatus can be sterilized by autoclaving and the swimming rings by flaming with alcohol.

Test organisms: The introductory experiments were carried out with pure cultures and natural bacterial populations from water samples of the Kiel Firth. Pure cultures were taken from cultures growing exponentionally in nutrient broth (Bacto peptone 0,05%, Bacto yeast extract 0,01%, aged sea water diluted with distilled water 1 : 4,

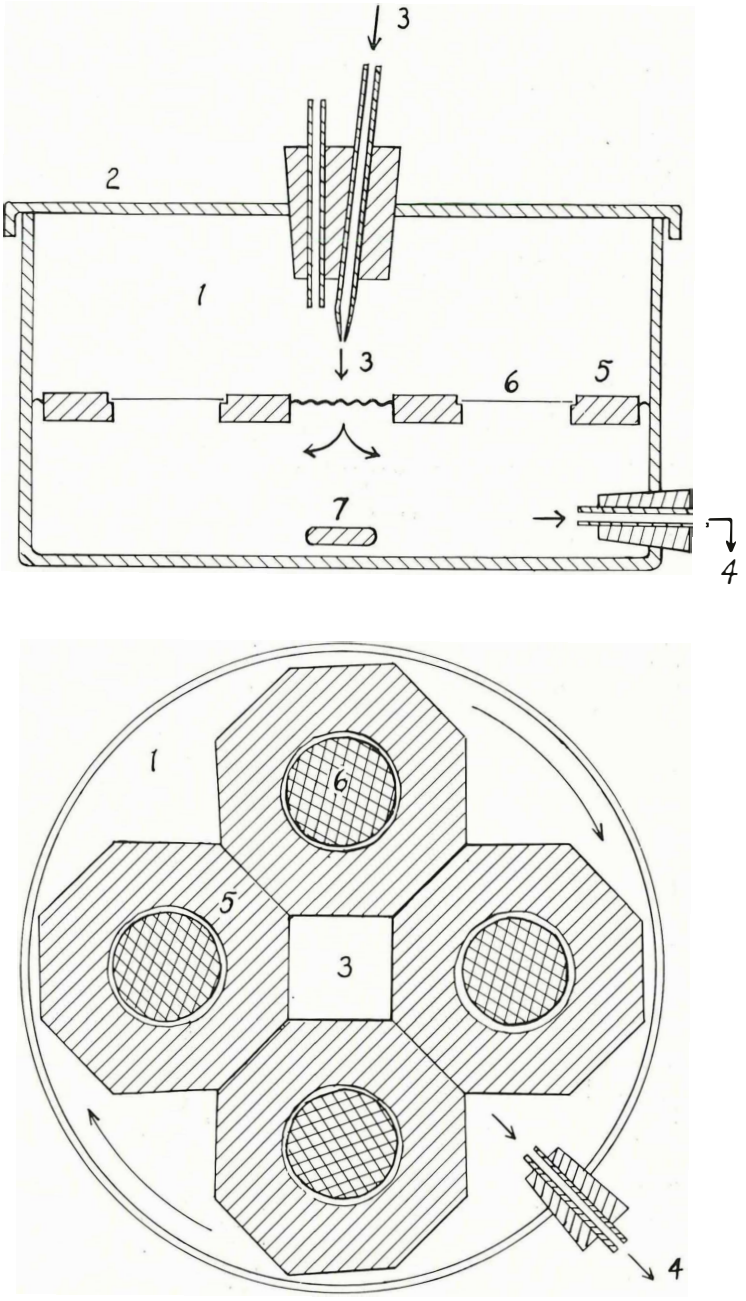

Fig. 1: Culture apparatus (Fig. is shown one half actual size).
Above: cross section. Below: top view.
1 —breeding chamber; 2 — lid of the breeding chamber; 3 — water inlet; 4 — water outlet; 5 — swimming ring; 6 — filter; 7 — magnetic stirring bar. Arrow indicates the floating direction of the swimming ring system.

Tafel 2 (zu L.-A. MEYER-REIL)

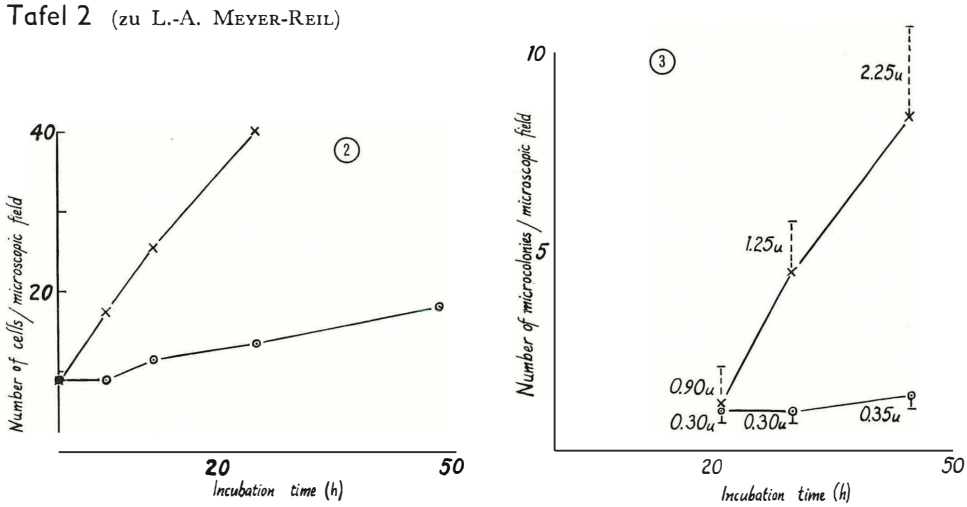

Fig. 2: Strain 1,3. Number of cells per microscopic field as a function of incubation time.
Crosses: incubation of the filters in the flow system.
Circles: incubation of the filters on paper discs.
Medium: see methods, incubation temperature: 20°C.

Fig. 3: Strain 3,60. Number and size of microcolonies per microscopic field as a function of incubation time.
Crosses: incubation of the filters in the flow system.
Circles: incubation of the filters on paper discs.
Broken line: microcolony size in arbitrary microscopic units (u).
Medium: see methods, incubation temperature: 20°C.

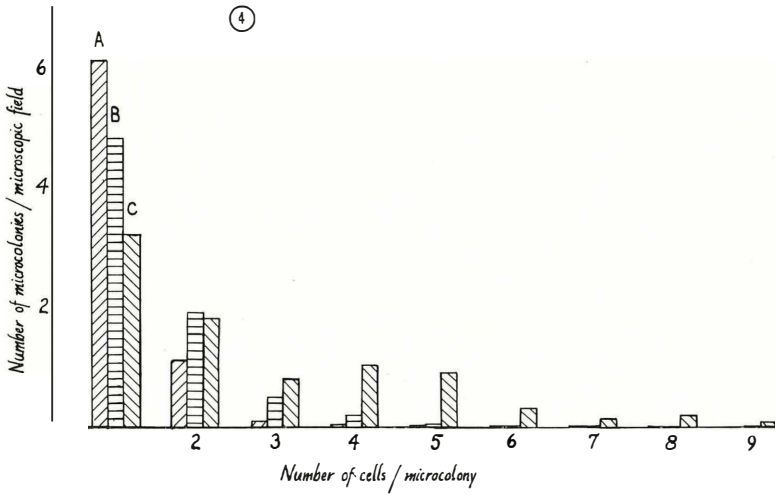

Fig. 4: Strain 1,3. Number of microcolonies per microscopic field as a function of number of cells per microcolony.
A: 0 hours, control. B: 12 hours, incubation of the filters on paper discs. C: 12 hours, incubation of the filters in the flow system.
Medium: see methods, incubation temperature: 20°C.

S=0,8‰). The bacterial suspension was then diluted with the above mentioned sea water mixture and filtered onto Nuclepore membrane filters (filtration apparatus Schleicher and Schüll). For the investigations with natural bacterial populations, 1—2 ml of the water sample was diluted with 10 ml of filter sterilized water from the same station. This mixture was then filtered onto Nuclepore membrane filters. Parallel filters were incubated in the flow system and on chromatographic paper pads in petri dishes. Nutrient broth (for pure cultures) and filter sterilized natural water (for natural bacterial populations) were used as culture medium for the flow system and for saturating the paper pads. It is necessary to boil the Nuclepore filters and the chromatographic paper pads with distilled water before autoclaving. The number of cells per microscopic field should be limited to a maximum of about 15 single cells for pure cultures and 25 cells for natural bacterial populations.

Assay analysis: After different times of incubation single filters were removed, fixed on chromatographic paper pads saturated with 3% formalin for 10 minutes, air dried, and stained with acridine orange (see ZIMMERMANN and MEYER-REIL, 1974). The number of single cells and microcolony forming cells was determined by fluorescence microscopic examination (Zeiss Universal, condensor for epifluorescence, Neofluar 100, 12,5 × oculars). One filter at the beginning and one at the end of filtration was immediately fixed and stained. Each filter was cut into 8 equal wedges with 4 alternate wedges used for counting. Every third field of a wedge was counted, a total of 15 fields per wedge, in general 60 fields per filter.

Results and Discussion

In the culture apparatus originally proposed by KUNICKA-GOLDFINGER and KUNICKI-GOLDFINGER (1972) and copied by us, the use of a sintered-glass disc supporting the membrane filters, is problematic. Separating the incubation and the flow chamber, this disc evolves strong capillary efficacy, and it prevents the free exchange without connection between the two chambers. Consequently, air bubbles often remain under the sintered-glass disc, and the exchange between the two chambers occurs uncontrollably (water rises through the disc and washes off the filters). In model assays with dye solutions, the diffusion of the substances through the disc could be demonstrated to be slow and incomplete. The fixed water inlet and outlet effects an irregular supply of the single filters on the surface of the sintered-glass disc. Therefore, the assay device described by the authors (KUNICKA-GOLDFINGER and KUNICKI-GOLDFINGER, 1972; KUNICKA-GOLDFINGER, 1973; KUNICKI-GOLDFINGER, 1974) was modified and improved to overcome the above mentioned difficulties (see methods).

In the culture apparatus described in this paper, the filters are held by recessed swimming rings with direct contact to the water surface. This system is rendered possible by using Nuclepore filters. In contrast to the relatively thick filters with a cellulose base (Millipore) used by the above mentioned authors, these thin filters are floating on the water surface, not influenced by saturation with water and slight water movement.

The introductory experiments were carried out with pure cultures of two obligate marine halophilic brackish water bacterial strains (1,3 and 3,60), which were isolated from the Baltic during earlier investigations (MEYER-REIL, 1973, 1974). The results of the parallel incubation of the filters in the flow system and on paper pads (incubation medium for both is nutrient broth, see methods) are shown in Fig. 2 and 3. By incubation in the flow system the cell numbers of the strain 1,3 increase linearly with incubation time, and they are duplicated within 6 hours. However, incubation of the filters on

paper pads leads to a slight increase of the cell numbers after a prolonged lag phase, and it takes 40 hours to duplicate the cell numbers (see Fig. 2). Comparable results can be obtained for the microcolony growth of the strain 3,60. In the flow system the number of cells increases 7 times and the size of microcolonies increases 3 times during an incubation interval of 24 hours. Parallel filters on paper pads, however, after a prolonged lag phase show a 1.4 times increase in number of microcolonies, whereas the microcolony size remains nearly unchanged (see Fig. 3). Fig. 4 illustrates for the strain 1,3 the influence of the flow system on the growth of the cells. In contrast to cultivation on paper pads, more single cells are dividing (the number of single cells decreases) and the microcolony growth is promoted (the number of microcolonies consisting of 3, 4 and 5 cells is higher and microcolonies of up to 9 cells are observed).

After these preliminary tests ,experiments were then carried out with natural bacterial populations, which were incubated in the flow system and on paper pads with filter sterilized water from the same station. For the cells incubated in the flow system a generation time (time interval of duplication) of 10 hours was measured, whereas the generation time of the cells incubated on paper pads comes to 75 hours (temperature of the natural water 15°C, incubation temperature 18°C). After an incubation time of 50 hours the number of microcolonies in the flow system was twice that on paper pads. The average size of microcolonies in the flow system, however, was smaller (enlarged spectrum of colony forming cells, greater number of small microcolonies).

The reason for the superiority of incubation in the flow system in contrast to cultivation in a "constant" system (e. g. on paper pads in petri dishes) depends on the properties of the flow system and its influence on the micro climate of the microorganisms. The cells are continually supplied with fresh nutrients through the filter, inhibitory and toxic substances are diluted or washes out, and the growth limiting influence of minimum factors is avoided or at least prolonged. Even bacteria with slow growth activities and limited cell divisions are able to divide. In contrast to common culture system the flow system allows the incubation under nearly natural conditions. It increases the number and the spectrum of colony forming cells.

A probable migration of motile cells over the surface of the filter has not yet been investigated sufficiently. Further investigations have to be carried out about the adsorption of nutrients at the surfaces of the swimming rings and the filters, as such an adsorption of nutrients is well known for glass surfaces (for literature see Moebus, 1972; Kunicki-Goldfinger, 1974) However, it should be considered, that in the natural invironment adsorption of nutrients at different surfaces (detritus, debris of plants and animals) occurs too. Another point that has to be studied are the changes of different chemical parameters in the flow system.

The described flow system is presently used to determine the growth activity of natural bacterial populations and to estimate the bacterial production (increase of the bacterial biomass) in the Baltic. The present results of these investigations show relationships between the initial number of bacterial cells, the generation time and the increase of the bacterial number and biomass after incubation in the flow system. The results in detail will be published in a later paper.

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